# Genetic Variation at 16q24.2 is associated with small vessel stroke.

Running head: 16q24.2 and small vessel stroke

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## Abstract

Objective: Genome-wide association studies (GWAS) have been successful at identifying associations with stroke and stroke subtypes, but have not yet identified any associations solely with small vessel stroke (SVS). SVS comprises a quarter of all ischaemic stroke and is a major manifestation of cerebral small vessel disease, the primary cause of vascular cognitive impairment. Studies across neurological traits have shown younger onset cases have an increased genetic burden. We leveraged this increased genetic burden by performing an age-at-onset informed GWAS meta-analysis, including a large younger onset SVS population, to identify novel associations with stroke.

Methods: We used a three-stage age-at-onset informed GWAS to identify novel genetic variants associated with stroke. On identifying a novel locus associated with SVS, we assessed its influence on other small vessel disease phenotypes, as well as on mRNA expression of nearby genes, and on DNA methylation of nearby CpG sites in whole blood and in the fetal brain.

Results: We identified an association with SVS in 4,203 cases and 50,728 controls on chromosome 16q24.2 (OR(95% CI)=1.16(1.10-1.22); $p=3.2x10^{-9}$). The lead SNP (rs12445022) was also associated with cerebral white matter hyperintensities (OR(95% CI)=1.10(1.05-1.16); $p=5.3x10^{-5}$; N=3,670), but not intracerebral haemorrhage (OR(95% CI)=0.97(0.84-1.12); p=0.71; 1,545 cases, 1,481 controls). rs12445022 is associated with mRNA expression of *ZCCHC14* in arterial tissues ($p=9.4x10^{-7}$), and DNA methylation at probe cg16596957 in whole blood ($p=5.3x10^{-6}$).

Interpretation: 16q24.2 is associated with SVS. Associations of the locus with expression of *ZCCHC14* and DNA methylation suggest the locus acts through changes to regulatory elements.

## Introduction

Genome-wide association studies (GWAS) enable identification of common genetic variants that influence disease risk and have proved successful in elucidating pathophysiological mechanisms underlying diseases with a genetic influence.[1] A number of GWAS associations have recently been identified with ischaemic stroke, almost all of which have been associated with specific stroke subtypes.[2-4] A number of genetic associations have been reported with cardioembolic (CE) and large artery stroke (LAS), but in contrast there have been no robust associations solely with small vessel stroke (SVS). This is despite epidemiological data that suggest genetic factors are particularity important for SVS. For example, there are a number of monogenic stroke disorders associated with SVS,[5] and family history studies have shown a strong association between SVS and a family history of stroke.[6] Similarly, related traits, including white matter hyperintensities, have been shown to have high heritability.[7]

SVS itself comprises a quarter of all ischaemic stroke and is one of the clinically overt manifestations of cerebral small vessel disease (SVD), the major cause of vascular cognitive impairment. Other radiological features of SVD include white matter hyperintensities (WMH) best seen on T2-weighted MRI, cerebral microbleeds - seen on gradient echo MRI, and intracerebral hemorrhages (ICH).[8] Despite its importance, the pathogenesis of SVD remains poorly understood and this limits the development of proven treatments for established disease.

One consistent finding across adult-onset neurological complex diseases including Parkinson's disease,[9] Alzheimer's disease,[10] and stroke,[11] is that younger onset cases have a stronger genetic burden from common disease-associated SNPs. Leveraging this

increased burden, by focussing on younger onset cases in analysis of genetic data, can lead to detection of novel trait-associated variants.[11] This may be particularly relevant for SVS, as epidemiological studies have shown stronger associations with SVS and a family history of stroke in younger stroke cases.[6]

Here, we perform an age-at-onset informed GWAS meta-analysis in stroke, including a large population of younger onset (age<70) small vessel stroke (SVS) cases. We perform analysis for all ischaemic stroke (IS) and its three subtypes: cardioembolic (CE), large artery stroke (LAS) and SVS. Using this approach we identify a novel association with SVS, seek further validation of the locus in other SVD phenotypes, and assess the influence of SNPs at the locus on mRNA expression of nearby genes and DNA methylation at nearby CpG sites.

## Methods

### *Study design*

We employed a three-stage design for the association analysis (Figure 1). In brief, in stage I we performed association analysis of stroke phenotypes in 10,210 cases and 12,285 controls of European ancestry from Europe, United States, and Australia; most of which contributed to the METASTROKE ischaemic stoke GWAS meta-analysis – and all of which have been described previously (Table 1). [2, 12, 13] In all cases, diagnosis of stroke was based on clinical evaluation with radiological confirmation. Subtyping of stroke cases was based on the TOAST criteria; in this analysis we considered the CE, LAS and SVS subtypes.[14] Of note, our SVS analysis included a large sample (1,012 cases, 970 controls) of younger onset (age<70) MRI-confirmed lacunar strokes, meaning that although we investigated all subtypes, we had most power to identify associations with SVS.

In stage II, we took 3 SNPs from the top 25 loci from each phenotype forward for a first in silico replication in the NINDS Stroke Genetics Network (SiGN), [2] which consisted of 7,743 cases and 17,790 controls. We meta-analysed stages I and II together and identified 3 loci with $p<5x10^{-7}$. Finally in stage III, we determine whether these 3 SNPs were associated with the phenotype in which they were identified (CE or SVS) by *in silico* replication in a large Icelandic population (deCODE; 520 SVS cases, 1,100 CE cases, 50,728 controls; stage III).

*Genotyping and Imputation*

Genotyping, quality control and imputation of all studies has been described previously.[2, 3, 13] All studies were genotyped on commercially available arrays from Illumina or Affymetrix and imputed to 1000 Genomes phase 1 reference panels using IMPUTE or MACH.[15] Imputation quality score was assessed by calculating the ratio of the observed to the expected binomial variance of the allele dosage.

*Association analysis*

Association analysis was performed using a covariate-informed approach,[11, 16] which we, and others, have implemented previously.[11, 17] Briefly, the approach uses case/control status and a covariable – in this case age-at-onset – to estimate each individual's stroke liability, which can be interpreted as their underlying propensity to stroke, on a normally-distributed scale. In this analysis cases with an earlier age-at-onset take more extreme positive values than late onset cases as, due to the lower prevalence of stroke at younger ages, they are assumed to have higher stroke liability. Conversely, controls who are older and stroke-free at age-at-

observation take more extreme negative value than younger controls as they have been stroke-free for a longer time and are therefore assumed to have a lower stroke liability.

In this analysis, the approach was implemented in our software, CIAO (provided at https://sites.google.com/site/mtraylor263/software/covariate-informed-gwas-analysis). Specifically, the approach taken is to model phenotype data using a continuous unobserved normally distributed quantitative trait, called the disease liability ($\varphi = \sum_{j=1}^{J} c_j\left(t_j - \overline{t_j}\right) + m + \varepsilon$), where $\varepsilon = \gamma g + N(0,1)$ and $g$ denotes the genetic effects. Then an individual is a case (*z=1*) if and only if $\varphi \geq 0$ and is a control (*z=0*) otherwise. $c_j$ is a parameter estimating the effect of a given covariate *j* on the liability scale. $m$ denotes the disease prevalence *p* at the covariate mean $\overline{t_j}$ under a normal cumulative distribution function ( $\Phi(-m) = p$). This model is used to approximate the effect of a disease covariate – in this case age-at-onset - on the liability scale, based on estimates of risk of ischaemic stroke by age from epidemiological data, thereby estimating $c_j$. For this analysis, the gender-specific risk of ischaemic stroke by age from an index age of 55 was obtained from population-based estimates (1.8%, 5.4%, and 12.1% before 65, 75, and 85, respectively in women; 2.4%, 7.3%, and 12.6% before 65, 75, and 85, respectively in men).[18] We assumed that 20% of ischaemic stroke cases had each of the cardioembolic, small vessel or large vessel stroke subtypes, approximating proportions observed in population-based studies..[19] We developed two models for our analysis; one based on the risk rates for all ischaemic stroke, and secondly for the three stroke subtypes. We used these models to calculate posterior mean liabilities after conditioning on age-at-onset for the four stroke phenotypes separately ($E(\varepsilon|z,t) = \frac{\int_{-c(t-\bar{t})-m}^{\infty} \varepsilon \frac{1}{\sqrt{2\pi}} e^{\left(\frac{-\varepsilon^2}{2}\right)} d\varepsilon}{\int_{-c(t-\bar{t})-m}^{\infty} \frac{1}{\sqrt{2\pi}} e^{\left(\frac{-\varepsilon^2}{2}\right)} d\varepsilon}, if\ z = 1$). Controls were modelled in the same way, but were assumed to take the posterior mean from the lower (unaffected) portion of the distribution in

the liability threshold model ($E(\varepsilon|z,t) = \frac{\int_{\infty}^{-c(t-\bar{t})-m} \varepsilon \frac{1}{\sqrt{2\pi}} e^{\left(\frac{-\varepsilon^2}{2}\right)} d\varepsilon}{\int_{\infty}^{-c(t-\bar{t})-m} \frac{1}{\sqrt{2\pi}} e^{\left(\frac{-\varepsilon^2}{2}\right)} d\varepsilon}, if\ z = 0$). Where age data was missing, individuals were assigned the median age value (<1% of cases). Regression was then performed on posterior liabilities ($E(\varepsilon|z,t)$) by multiplying the number of samples by the squared correlation between the expected genotype dosage and posterior mean liabilities for each of the discovery cohorts in the four ischaemic stroke phenotypes (all IS, CE, LAS, SVS). Ancestry-informative principal components were included where appropriate, using the EIGENSTRAT procedure.[20] Any residual inflation was accounted for by adjusting results by the genomic inflation factor, $\lambda$.[21] In all analyses, SNPs with imputation quality score<0.7 or minor allele frequency<0.01 were excluded and meta-analysis was performed using Stouffer's method in METAL.[22]

*Further analysis of a novel locus associated with small vessel stroke*

For a novel variant associated with SVS, we performed further analysis to elucidate the association for different groups based on age-at-onset. Firstly, for datasets in stage I and II, we divided the cases into quartiles based on age-at-onset and estimated the association of the SNP with each quartile using logistic regression with all controls, meta-analysing using a fixed-effects inverse variance weighted approach (data not available in BRAINS, MGH-GASROS, ISGS/SWISS). Secondly, we interrogated associations at the locus in non-European ancestry populations, comprising 657 small vessel African-American stroke cases and 3,251 matched controls from the NINDS Stroke Genetics Network and African or African-Caribbean ancestry individuals from the South London Ethnicity and Stroke Study (SLESS), [2, 23] and 314 SVS cases and 5,193 controls of Pakistani ancestry from the RACE study.[3] We used logistic regression to evaluate the association within each group, and evaluated the overall transethnic association by meta-analysing using Stouffer's method.

In addition, we explored association of the SNP with other SVD phenotypes. We evaluated association of the SNP with 1) white matter hyperintensity volumes (WMHV) measured on T2-weighted MRI in 3,670 ischaemic stroke patients of European ancestry,[24] 2) in MRI-defined small subcortical brain infarcts (SSBI) brain infarcts in 17,197 trans-ethnic individuals (85.7% European; 8.8% African-American; 3.5% Hispanic; 1.0% Chinese; 1.0% Malay) from community studies recruited within the neuro-CHARGE consortium (mean age 68.90 ± 10.31; 1,986 with infarcts). SSBI were defined as MRI-defined brain infarcts of 3-15 mm or 3-20 mm in size, located in the basal ganglia, the white matter, or the brainstem. Association analysis was performed overall, and for the subset of cases with extensive WMH burden – defined as the top age-specific quartile of WMH volume on a quantitative scale or above the age-specific median by 5-year age-categories for studies using semi-quantitative measurements of WMH burden; N=549; 3) ICH in 1,545 European ancestry cases and 1,481 controls, described previously,[25] and stratified according to lobar or nonlobar location.

*Evaluation of regulatory chromatin states, mRNA expression and DNA methylation*

To investigate a novel locus, we used existing resources and performed some further analyses to characterize its regulatory potential. We interrogated chromatin states and regulatory motifs from ENCODE and Epigenomics Roadmap using Haploreg v4.1.[26] We also evaluated whether the associated SNP influences gene expression using GTEx portal.[27] Upon identifying an association between the SNP and expression of a nearby gene, we evaluated the evidence that the association signal for SVS and gene expression derives from the same causal variant using a Bayesian colocalisation test.[28] Using the R coloc package (http://cran.r-project.org/web/packages/coloc), we compared 5 models for SNPs with 50Kb of our lead SNP using the approach ($H_0$: No association with either trait; $H_1$:

303 Association with SVS, not with expression; $H_2$: Association with expression, not with SVS;
304 $H_3$: Association with SVS and expression, two independent SNPs; $H_4$: Association with SVS
305 and expression, one shared SNP).

306

307 Next, we assessed whether the lead SNP (rs12445022), or 3 SNPs in linkage disequilibrium
308 (LD) (rs4843625, rs12920915, rs12444224), influence DNA methylation levels in whole
309 blood. We evaluated genetic associations of whole blood DNA methylation levels at selected
310 CpG-sites profiled on the Illumina Infinium HumanMethylation450 BeadChip array in a group
311 of 660 monozygotic (MZ) female twins (mean age 59, age range 18 to 79). These
312 individuals were research volunteers from the TwinsUK cohort in the United Kingdom.[29] All
313 were of European ancestry. For each CpG-site of interest we calculated the normalised
314 methylation means for the 330 MZ twin pairs as a phenotype in the genetic analysis, and
315 took into account covariates including smoking, BMI, age, methylation plate, and blood cell
316 count estimates. TwinsUK imputed genotypes were obtained for the 1000 genomes
317 reference set,[30] where we excluded SNPs with Hardy–Weinberg $p<1x10^{-4}$, Minor allele
318 frequency (MAF) <5% and those with IMPUTE info value <0.8. We tested for association
319 with our SNP, or SNPs in close LD ($r^2>0.6$) with DNA methylation at CpG-sites. We used
320 $p<4x10^{-5}$, equivalent to a false discovery rate (FDR) <5%,[31] to identify significant cis-mQTL
321 associations.

322

323 Finally, we explored genetic associations at 16q24.2 (defined as within 50Kb of rs12445022)
324 with DNA methylation profiles in 166 human fetal brain samples (92 male, 74 female)
325 ranging from 56–166 days post-conception initially using publically available data – which
326 holds results for mQTL associations reaching the study-wide significance threshold
327 (http://epigenetics.essex.ac.uk/mQTL/). Methods for this study have been published in detail

elsewhere.[32] Briefly, DNA methylation levels were profiled on the Illumina Infinium HumanMethylation450 BeadChip array and SNP genotypes were obtained from the Illumina HumanOmniExpress BeadChip and imputed to 1000 Genomes phase 3 using SHAPEIT and Minimac3 via the Michigan Imputation Server.[15, 33] SNP-methylation probe pairs were tested using the R package MatrixEQTL,[34] including covariates to control for age, sex and ancestry-informative principal components. Upon identifying a significant association at 16q24.2, we performed additional analyses (not publicly available: we gained access to the data) to test whether any of our 4 SNPs (rs12445022, rs4843625, rs12920915, rs12444224) were associated with methylation at the identified probe. We again used $p<4x10^{-5}$, equivalent to a false discovery rate (FDR) <5%,[31] to identify significant cis-mQTL associations.

## Results

### *Association analysis*

In phase I association analysis we confirmed previous associations between *HDAC9* and LAS (rs2107595, $p=3.0x10^{-8}$) and between *PITX2* and CE (rs192172299, $p=2.0x10^{-9}$).[3, 4] Previous associations between *ZFHX3* and CE and between *MMP12* and LAS did not reach genome-wide significance in this analysis (rs879324, $p=5.0x10^{-7}$ and rs586701, p=0.0014; respectively).[11] A SNP in a region close to *HABP2* previously associated with young onset ischaemic stroke was also significant, albeit not genome-wide, in this analysis (rs11196288; $p=2.4x10^{-4}$).[35] Genomic inflation $\lambda$ and the equivalent values scaled to 1000 cases and 1000 controls ($\lambda_{1000}$),[36] were well controlled across all analyses (IS, $\lambda$ ($\lambda_{1000}$) =1.05 (1.00); CE, $\lambda$ ($\lambda_{1000}$) =1.02 (1.00); LAS, $\lambda$ ($\lambda_{1000}$) =1.02 (1.00); SVS. $\lambda$ ($\lambda_{1000}$) =1.01 (1.00)).

We took 25 independent loci forward (3 SNPs in LD from each locus selected on p-value) from each analysis (IS, CE, LAS, SVS) for *in silico* replication in the NINDS Stroke Genetics Network study (stage II). Information on these SNPs is provided in Supplementary Tables 1-4. Following this analysis , excluding previously reported associations, three loci showed significance at $p<5x10^{-7}$ (two with SVS, one with CE) and one was genome-wide significant (rs12445022, $p=4.4x10^{-8}$, associated with SVS). We followed up all three loci in a second *in silico* replication (stage III) in a large Icelandic population (deCODE). A single SNP, rs12445022, showed evidence of replication (p=0.011). When performing a meta-analysis across all populations, rs12445022 was associated with SVS at genome-wide significance ($p=3.2x10^{-9}$; Figure 2). The SNP was either genotyped or well imputed (info>0.9) in all cohorts and lies in an intergenic region between Junctophilin 3 (*JPH3*) and Zinc Finger, CCHC Domain Containing 14 (*ZCCHC14*). To confirm the association with rs12445022, we repeated the analysis using logistic regression; the approach taken in a conventional GWAS. The association was validated using this method, and associations were consistent across populations (OR(95% CI)=1.16(1.10-1.22); $p=1.3x10^{-8}$; heterogeneity p=0.56; Figure 3).

*Further analysis of a 16q24.2 novel locus associated with small vessel stroke*

We evaluated association of the lead SNP in different quantiles of age at stroke onset, using all controls in each analysis. The strongest associations were observed in younger onset cases, suggesting the influence of the SNP might be greatest in these individuals (Figure 4). However, this was not demonstrated statistically (p>0.05).

We performed further analysis to assess whether the SVS-associated SNP influenced other manifestations of cerebral SVD. The SNP (rs12445022) was also associated with increased T2-WMHV (OR(95% CI)=1.10(1.05-1.16); $p=5.3x10^{-5}$; Figures 2,5), and showed little

heterogeneity across study groups (heterogeneity p=0.58). Conversely, the SNP was not associated with ICH – neither overall, nor in subgroups divided by lobar/non-lobar location. For SSBI, the direction of effect was the same as for SVS, but the effect was weaker and non-significant (OR(95% CI)=1.05(0.97-1.14); p=0.28). For the subgroup with WMH, the effect was stronger – and similar to that observed for SVS, but was again non-significant (OR(95% CI)=1.15(0.99-1.33); p=0.076).

We next evaluated the identified locus in non-European ancestry populations. The SNP had a similar frequency to Europeans in South Asians from RACE (MAF=37%), but was rarer in African ancestry populations, consisting of African-Americans from NINDS Stroke Genetics Network and United Kingdom individuals of African or African-Caribbean ethnicity from SLESS (MAF=14%). Associations with the SNP were in the same direction as in European ancestry populations (Figure 4), but did not reach statistical significance in either ancestry, reflecting the much smaller sample sizes. However, when combining data from all populations, evidence for association at the SNP ($p=1.4x10^{-9}$) was stronger than in European ancestry populations alone, which might suggest a common association across populations. Indeed, there was not evidence of a significant difference in the strength of association between the European and non-European ancestry individuals (p=0.64).

*Regulatory chromatin states, mRNA expression and DNA methylation related to 16q24.2*

We used existing databases to assess the functional consequences of SNPs in the 16q24.2 region. Firstly, we used the Haploreg v4.1 database to interrogate chromatin states and regulatory motifs from ENCODE and NIH Roadmap Epigenomics Mapping Consortium. [26, 37, 38] The database showed that our lead SNP influences chromatin states in multiple tissues.

400 The SNP is classified as a genic promoter in 9 tissues, an enhancer in 13 tissues and
401 overlaps DNAse1 hypersensitivity sites in 21 tissues.

402

403 Secondly, we used publicly available databases to evaluate the evidence that the lead SNP
404 influences expression of nearby genes using the GTEx portal.[27] The implicated A allele of
405 our lead SNP (rs12445022) was associated with decreased expression of *ZCCHC14* in tibial
406 arterial tissue ($p=9.4x10^{-7}$; Figure 2). We used a Bayesian colocalisation technique to assess
407 whether the same variant drives the both the SVS association signal and mRNA expression
408 of *ZCCHC14*.[28] There was overwhelming evidence in support of $H_4$ (Posterior probability =
409 99.7%), strongly indicating that a single variant - most likely to be rs12445022 - influences
410 both SVS and expression of *ZCCHC14*.

411

412 Finally, we performed analyses to assess whether the lead SNP, or the 3 SNPs in LD,
413 influence DNA methylation at CpG probes in whole blood. We found evidence that the lead
414 SNP, and 3 SNPs in close LD ($r^2>0.6$), influence DNA methylation at 4 nearby CpG sites
415 (cg16596957, cg10312981, cg03020503, cg00555085; all $p<4.0x10^{-5}$, Table 2). The
416 implicated A allele of rs12445022 was associated with decreased methylation at the
417 cg16596957 probe (beta(SE)=-0.38(0.082); $p=5.3x10^{-6}$). The SNPs explained between 5-8%
418 of the methylation variance at the given CpG sites. The same 16q24.2 region by CpG probe
419 (cg16596957) association was also recently reported in another study in whole blood.[40] In
420 addition, we looked for an association between SNPs at the 16q24.2 locus and DNA
421 methylation levels in fetal brains, initially using publicly available data
422 (http://epigenetics.essex.ac.uk/mQTL/). There was a strong association with SNPs in distant
423 LD with our lead SNP (rs8047314 ~ cg08031982; $p=7.1x10^{-14}$; $r^2=0.16$ with rs12445022). We
424 then performed additional analyses (not publicly available: we gained access to the data) to

test if our lead SNP, or the SNPs in close LD, were associated with methylation at cg08031982. We could identify no associations that reached our significance threshold ($p<4.0x10^{-5}$). However, there was a near-significant association of rs12920915 and rs4843625 with methylation at the cg08031982 probe (both $p=7.8x10^{-5}$). Our lead SNP, rs12445022 was not associated ($p=9.9x10^{-4}$).

## Discussion

Genome-wide association studies in SVS have largely been disappointing. Some studies have suggested that an association with all IS at the highly pleiotropic 12q24.12 is driven by an association with SVS, [2] but no genome-wide significant associations specifically with SVS have yet been identified. Using an age-of–onset informed analysis approach we identified a novel locus at 16q24.2 associated with SVS. The SNP was also associated using a standard logistic regression approach, but was less significant – a difference of almost an order of magnitude ($p=3.2x10^{-9}$ compared to $p=1.3x10^{-8}$). In addition, the association was stronger with younger onset SVS, suggesting a greater influence in these individuals. We tested whether the 16q24.2 association extends to other cerebral SVD related phenotypes. We showed that the same locus also influences WMH, and may have a similar effect on MRI-defined subcortical brain infarcts from prospective studies, although the association did not reach significance in our analysis. However, the locus does not appear to influence risk of ICH. A SNP in the same 16q24.2 region (rs4081947), in partial LD with our SNP ($r^2=0.28$), was also recently reported to be associated with migraine in a large GWAS meta-analysis. [41] These data provide strong supportive evidence that this 16q24.2 locus harbours variants that influence diseases of the cerebral vasculature.

449 Identifying the mechanisms by which GWAS associations influence disease risk presents
450 additional challenges. In this case, the underlying mechanism and the specific genes
451 implicated remains uncertain. Interrogation of mRNA expression data points to the lead SNP
452 influencing expression of the nearest gene, *ZCCHC14*. This gene is ubiquitously expressed,
453 but is highly expressed in arterial tissues and in the brain. However, its function is not well
454 characterized. Zinc fingers of the CCHC-type contain an 18 digit residue found in the
455 nucleocapsid of retroviruses, and therefore may be important in viral response. Other
456 plausible candidate genes reside nearby. The locus lies around 1Mb away from genes
457 encoding forkhead box proteins including *FOXC2, FOXL1,* and *FOXF1.* These proteins,
458 particularly the closely related *FOXC1* – a paralogue of *FOXC2*, have been implicated in
459 Mendelian forms of SVS.[42] We found no evidence linking our SNP to expression of these
460 genes. However, the function of these proteins changes dramatically between early
461 development and in adult tissues,[43] which might explain the absence of an association. This,
462 coupled with the fact that *FOXF2* variants have also recently been implicated in ischaemic
463 stroke,[44] make forkhead box proteins exciting targets for follow-up experiments.

464

465 Assessing DNA methylation, the process by which methyl groups are added to DNA thereby
466 modifying its function, offers another potential method for mechanistic insight. This
467 epigenetic process influences gene expression and regulation in humans, and may be
468 particularly relevant for diseases such as stroke where gene-environment interactions are
469 likely play an important role in pathogenesis.[45] Substantial inter-individual variation exists
470 with respect to age and tissue type.[46] However, an important emerging mechanism
471 influencing methylation is local sequence content.[47] Notably, recent studies have shown that
472 GWAS findings from stroke-relevant traits such as blood pressure are likely to act by
473 influencing DNA methylation.[48] This may be particularly relevant for SVS, in which
474 environmental and other vascular risk factors such as hypertension are important and have

been shown to interact with disease risk.[49] We evaluated whether our associated SNP (rs12445022), or SNPs in close LD, influence methylation of nearby CpG sites. We found evidence from whole blood that the same genetic variation influences DNA methylation. SNPs in distant LD also influenced DNA methylation at a different probe (cg08031982) in the fetal brain. Further evidence comes from published studies in lung, breast, and kidney tissues,[31] as well as in utero,[50] all of which have shown that the genetic variation at the same 16q24.2 region influences methylation at the cg08031982 probe. Interestingly, the CpG sites influenced by the locus appear to differ by tissue, with different probes affected in whole blood compared to fetal brain. This might imply tissue-specific functional consequences of the locus and therefore highlights the importance of performing follow-up experiments in appropriate tissues. Based on the evidence presented here, we can only speculate on how genetic variation at the locus leads to increased risk of SVS. One hypothesis is that expression of *ZCCHC14*, or other proteins, is mediated through altered methylation of the probes identified. This might occur, in part, in response to environmental stimuli. Evaluating these hypotheses in a relevant tissue type will be an important future analysis to identify the causal mechanisms leading to SVS.

This study has limitations. Our results suggested that the association may be present in other ethnicities, but we had an insufficient number of cases to establish common risk conclusively. Follow-up studies are therefore required in other ethnic groups. In addition, downstream functional experiments will be required to determine the consequences of the identified association. The mRNA expression and methylation analyses presented herein were constrained by available tissue types. Validation of the findings in more disease relevant tissue types such as cerebral small vessels therefore represent important follow-up analyses, although obtaining such tissue in a state to allow mRNA studies is very challenging. We performed mRNA expression and methylation analyses using either the

lead SNP (rs12445022) or 3 LD SNPs. The results should be interpreted with the limitation that we cannot be certain that any of these SNPs is the causal variant. Radiological confirmation of SVS in this study was performed using either CT or MRI. Evidence shows that MRI is considerably more reliable at identifying SVS. Replication of the association in an MRI-confirmed population may therefore provide a more accurate estimate of the effect of the locus on SVS risk. Similarly, interrogation of causative classification system (CCS) definitions of SVS may provide further insights. [51] Another method of interrogating the combined influence of age and genotype is by testing for an interaction. In this analysis, we were unable to do this as age was not available in some sets of controls (e.g WTCCC2).

In this large genome-wide meta-analysis using an age-at-onset informed approach, we have identified the first genome-wide significant locus that is associated solely with SVS. Our findings, which point to subtle changes in gene expression and DNA methylation influencing disease risk, show that strategies that account for different liability across disease related covariates such as age can identify novel associations with disease.

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537

538 **Author Contributions**

539 M.T, R.M, C.M.L, B.B.W, H.S.M conceived and designed the study. M.T, B.B.W, H.S.M
540 drafted the manuscript. M.T, K.B.H drew the figures. M.T, R.M, M.A.N, I.C, F.R, P.S, D.S,
541 M.A.H-B, C.L.M.S, P.M.R, G.B, V.T, R.L, C.L, J.F.M, J.R, M.D, B.B.W, H.S.M contributed

acquisition and analysis of METASTROKE datasets. H.X, L.H, M.F, C.J, J.F.M, B.D.M, S.J.K, M.D, B.B.W J.J-C, J.W.C, R.S, A.S, R.L, A.L, O.M, R.P.G, R.L.S, T.R, K.R, D.K.A, J.A.J, O.R.B, S.W-S, J-M.L, M.T, S.S.R, P.D.B, S.L.P, Q.W, P.F.M, D.W, C.D.A, J.R contributed acquisition and analysis of the NINDS-SIGN data. J.R, D.W, C.L, C.D.A contributed acquisition and analysis of ICH data. G.C, S.D, L.J.L, S.S, J.C.B, W.T.L Jr, contributed acquisition and analysis of neuro-CHARGE data. I.Y, T.D.S, J.T.B, E.H., J.M contributed acquisition and analysis of DNA methylation data. M.T, H.S.M, N.S.R contributed acquisition and analysis of WMH data. G.T, K.S, U.T, S.G contributed acquisition and analysis of deCODE data.

**Conflicts of Interest**

Authors whose affiliations are listed as deCODE/Amgen are employees of deCODE/ Amgen. The remaining authors declare no conflicts of interest.

558 Table 1 – Ischaemic Stroke Study participants

| Population | IS | CE | LAS | SVS | Controls | % cases with MRI | Age of cases (mean(s.d)) |
|---|---|---|---|---|---|---|---|
| Stage I Populations | | | | | | | |
| ASGC | 1,162 | 240 | 421 | 310 | 1,244 | 43.0% | 72.9 (13.2) |
| WTCCC2-Germany | 1,174 | 330 | 346 | 106 | 797 | 83.0% | 66.7 (12.9) |
| WTCCC2-UK | 2,374 | 474 | 498 | 460 | 5,175 | 37.2% | 72.2 (12.5) |
| Milano | 366 | 64 | 73 | 25 | 407 | 86.7% | 57.4 (15.6) |
| DNA-lacunar / GENESIS | 1,287 | 80 | 64 | 1,012 | 970 | 100.0% | 59.6 (12.0) |
| LSS | 455 | 157 | 70 | 55 | 455 | 89.0% | 67.7 (14.5) |
| ISGS / SWISS | 1,014 | 235 | 217 | 187 | 1,370 | 83.0% | 66.5 (13.6) |
| BRAINS | 361 | 29 | 120 | 97 | 444 | 30.8% | 74.4 (14.2) |
| MGH-GASROS | 294 | 106 | 68 | 23 | 376 | 60.0% | 66.7 (14.5) |
| VISP | 1,723 | - | - | - | 1,047 | 47.0% | 68.0 (10.7) |
| Total (discovery) | 10,210 | 1,715 | 1,877 | 2,275 | 12,285 | | |
| Stage II populations | | | | | | | |
| NINDS Stroke Genetics Network | 7,743 | 2,001 | 1,130 | 1,408 | 17,970 | 62.0% | 66.3 (14.8) |
| Stage III populations | | | | | | | |
| deCODE | - | 1,100 | - | 520 | 20,473 | NA | 72.7 (11.6) |
| TOTAL | 17,953 | 4,816 | 3,007 | 4,203 | 50,728 | | |

559

560 IS, all ischaemic stroke; CE, cardioembolic stroke; LAS, large artery stroke; SVS, small
561 vessel stroke; ASGC, Australian Stroke Genetics Collaborative; WTCCC2, Wellcome Trust
562 Case Control Consortium 2; LSS, Leuven Stroke Study; BRAINS, Bio-repository of DNA in
563 stroke; MGH-GASROS, The MGH Genes Affecting Stroke Risk and Outcome Study; VISP,
564 The Vitamin Intervention for Stroke Prevention Trial; NA, information not available.

565 Table 2 – Significant associations between rs12445022 and LD SNPs

566 ($r^2$>0.6) with cis-methylation probes in whole blood

567

| SNP Variant | SNP BP | CpG Probe | Probe BP | RA | beta (SE) | $R^2$ | P-v |
|---|---|---|---|---|---|---|---|
| rs12445022 | 87,575,332 | cg16596957 | 87,575,151 | A | -0.38 (0.082) | 0.058 | 5.3x |
| rs4843625 | 87,576,996 | cg16596957 | 87,575,151 | C | -0.33 (0.075) | 0.053 | 1.3x |
| rs4843625 | 87,576,996 | cg10312981 | 87,577,304 | C | 0.39 (0.074) | 0.077 | 1.9x |
| rs4843625 | 87,576,996 | cg03020503 | 87,577,656 | C | 0.35 (0.075) | 0.059 | 5.0x |
| rs4843625 | 87,576,996 | cg00555085 | 87,616,248 | C | 0.34 (0.075) | 0.057 | 6.6x |
| rs12920915 | 87,577,521 | cg16596957 | 87,575,151 | T | -0.38 (0.075) | 0.069 | 7.3x |
| rs12920915 | 87,577,521 | cg10312981 | 87,577,304 | T | 0.38 (0.075) | 0.068 | 1.0x |
| rs12920915 | 87,577,521 | cg03020503 | 87,577,656 | T | 0.34 (0.076) | 0.055 | 1.1x |
| rs12920915 | 87,577,521 | cg00555085 | 87,616,248 | T | 0.33 (0.076) | 0.051 | 2.2x |
| rs12444224 | 87,580,855 | cg16596957 | 87,575,151 | T | -0.38 (0.075) | 0.068 | 8.0x |
| rs12444224 | 87,580,855 | cg10312981 | 87,577,304 | T | 0.38 (0.075) | 0.069 | 8.7x |
| rs12444224 | 87,580,855 | cg03020503 | 87,577,656 | T | 0.35 (0.076) | 0.054 | 1.1x |
| rs12444224 | 87,580,855 | cg00555085 | 87,616,248 | T | 0.32 (0.076) | 0.050 | 2.6x |

568 BP, base position; RA, reference allele; $R^2$, proportion of methylation

569 variance explained by respective genotype.

570 Figure Legends

571 Figure 1 – Flow chart of analyses performed

572 Figure 2 – Associations at 16q24.2 with A) small vessel stroke, B) cerebral
573 white matter hyperintensities, C) mRNA expression of ZCCHC14; and D)
574 Gene Locations and associations of the locus with DNA methylation
575 SVS, small vessel stroke; WMH, white matter hyperintensities; ZCCHC14, Zinc Finger,
576 CCHC Domain Containing 14; JPH3, Junctophilin 3; meQTL, methylation quantitative trait
577 locus.

578 Figure 3 – Forest Plot of Associations with rs12445022 under a logistic
579 regression model
580

581 Figure 4 – Association of rs12445022 with small vessel stroke by quartiles of
582 age-at-stroke onset in Europeans
583

584 Figure 5 – Associations with rs12445022 for stroke and cerebral small vessel
585 disease phenotypes
586

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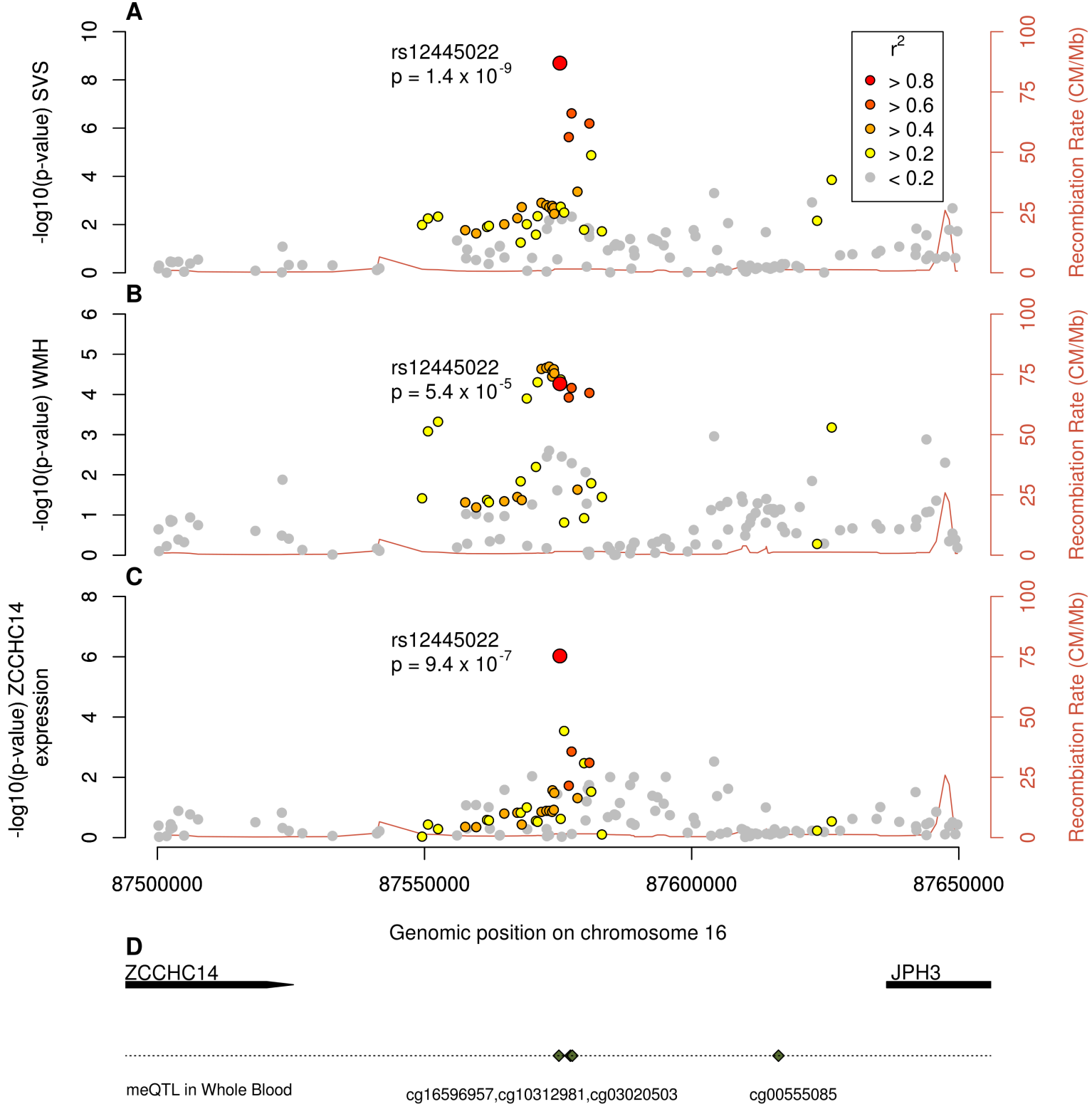
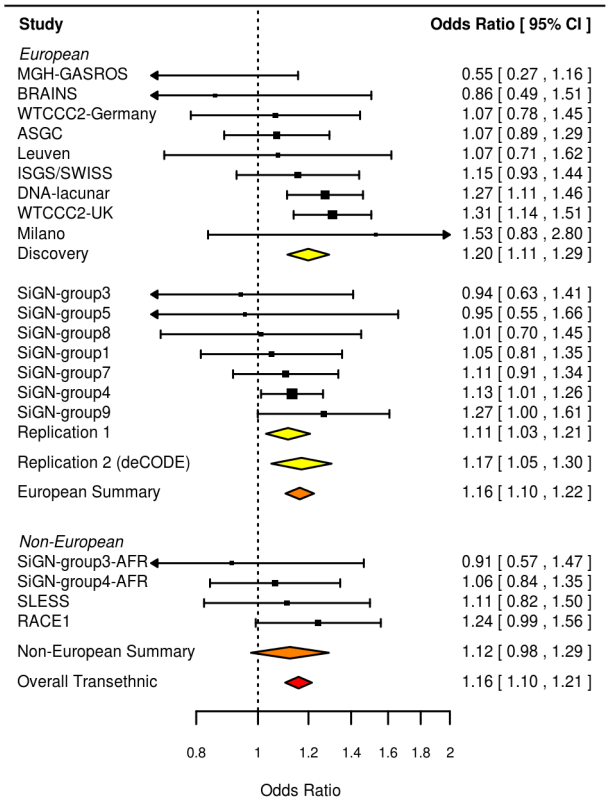
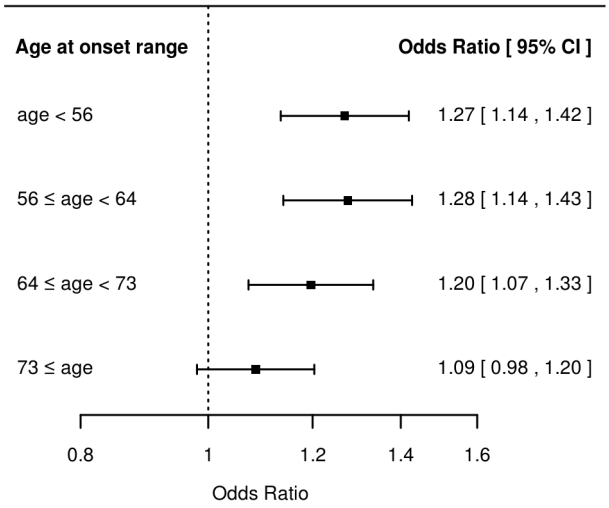
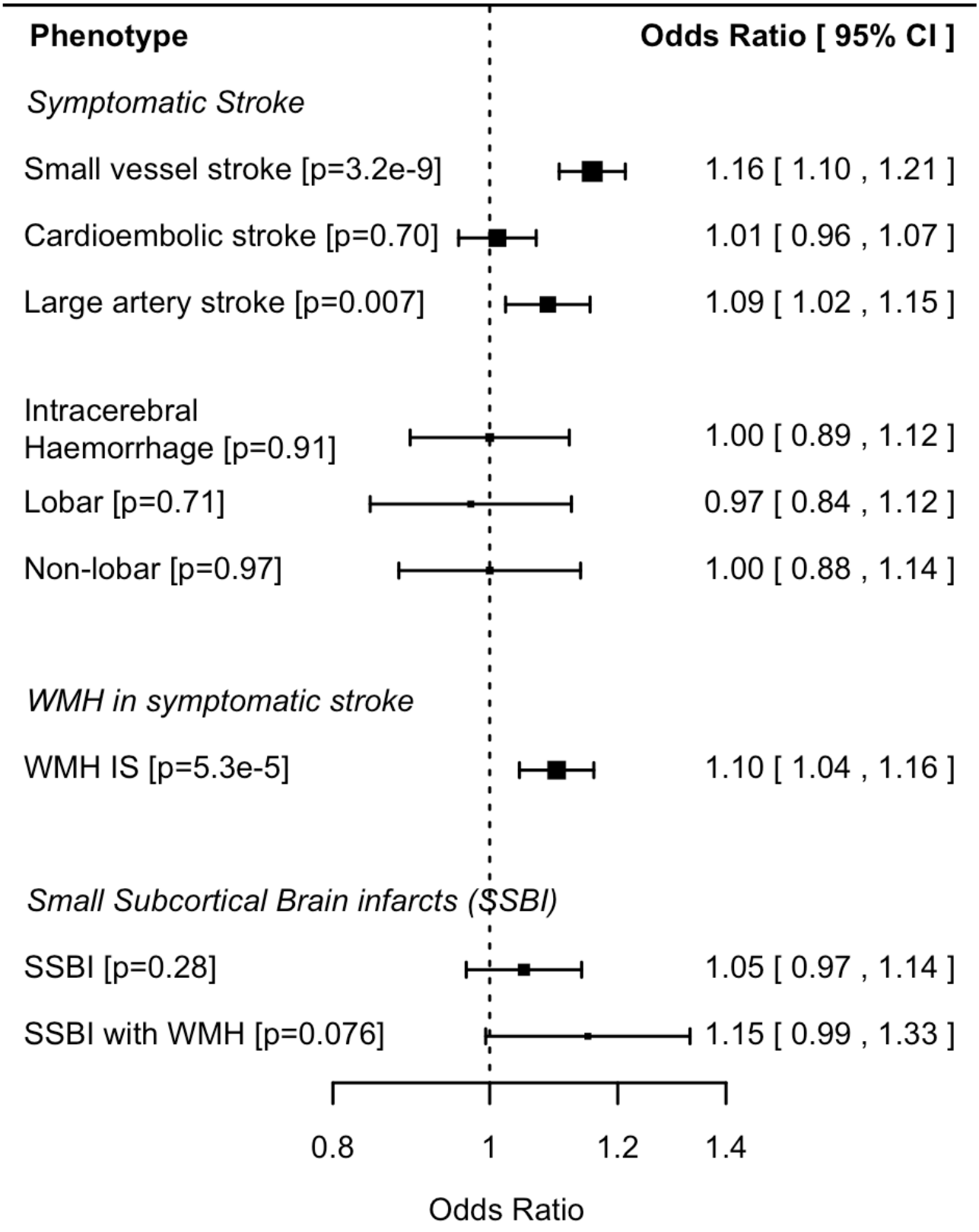
710


METASTROKE (stage I)
10,210 cases:
1,715 cardioembolic; 1,877 large vessel;
2,275 small vessel
12,285 controls
NINDS-SiGN (stage II)
7,743 cases:
2,001 cardioembolic; 1,130 large vessel; 1,408 small vessel
17,790 controls
deCODE (stage III)
1,100 cardioembolic cases
520 small vessel cases
20,473 controls
Identifies SNP association at 16q24.2 with SVS
Test SNP for age-at-onset effects
Perform transethnic analyses for associated SNP
Test SNP in other small vessel disease phenotypes
Test for association with mRNA expression in GTEx
Test for association with DNA methylation in whole blood (N=660) and fetal brain (N=166)


A
rs12445022
p = 1.4 x 10-9
r2
> 0.8
> 0.6
> 0.4
> 0.2
< 0.2
-log10(p-value) SVS
Recombiation Rate (CM/Mb)
B
rs12445022
p = 5.4 x 10-5
-log10(p-value) WMH
Recombiation Rate (CM/Mb)
C
rs12445022
p = 9.4 x 10-7
-log10(p-value) ZCCHC14 expression
Recombiation Rate (CM/Mb)
87500000
87550000
87600000
87650000
Genomic position on chromosome 16
D
ZCCHC14
JPH3
meQTL in Whole Blood
cg16596957,cg10312981,cg03020503
cg00555085


Study
Odds Ratio [ 95% CI ]
European
MGH-GASROS
0.55 [ 0.27 , 1.16 ]
BRAINS
0.86 [ 0.49 , 1.51 ]
WTCCC2-Germany
1.07 [ 0.78 , 1.45 ]
ASGC
1.07 [ 0.89 , 1.29 ]
Leuven
1.07 [ 0.71 , 1.62 ]
ISGS/SWISS
1.15 [ 0.93 , 1.44 ]
DNA-lacunar
1.27 [ 1.11 , 1.46 ]
WTCCC2-UK
1.31 [ 1.14 , 1.51 ]
Milano
1.53 [ 0.83 , 2.80 ]
Discovery
1.20 [ 1.11 , 1.29 ]
SiGN-group3
0.94 [ 0.63 , 1.41 ]
SiGN-group5
0.95 [ 0.55 , 1.66 ]
SiGN-group8
1.01 [ 0.70 , 1.45 ]
SiGN-group1
1.05 [ 0.81 , 1.35 ]
SiGN-group7
1.11 [ 0.91 , 1.34 ]
SiGN-group4
1.13 [ 1.01 , 1.26 ]
SiGN-group9
1.27 [ 1.00 , 1.61 ]
Replication 1
1.11 [ 1.03 , 1.21 ]
Replication 2 (deCODE)
1.17 [ 1.05 , 1.30 ]
European Summary
1.16 [ 1.10 , 1.22 ]
Non-European
SiGN-group3-AFR
0.91 [ 0.57 , 1.47 ]
SiGN-group4-AFR
1.06 [ 0.84 , 1.35 ]
SLESS
1.11 [ 0.82 , 1.50 ]
RACE1
1.24 [ 0.99 , 1.56 ]
Non-European Summary
1.12 [ 0.98 , 1.29 ]
Overall Transethnic
1.16 [ 1.10 , 1.21 ]
0.8
1
1.2
1.4
1.6
1.8
2
Odds Ratio


Age at onset range
Odds Ratio [ 95% CI ]
age < 56
1.27 [ 1.14 , 1.42 ]
56 ≤ age < 64
1.28 [ 1.14 , 1.43 ]
64 ≤ age < 73
1.20 [ 1.07 , 1.33 ]
73 ≤ age
1.09 [ 0.98 , 1.20 ]
0.8
1
1.2
1.4
1.6
Odds Ratio


Phenotype
Odds Ratio [ 95% CI ]
Symptomatic Stroke
Small vessel stroke [p=3.2e-9]
1.16 [ 1.10 , 1.21 ]
Cardioembolic stroke [p=0.70]
1.01 [ 0.96 , 1.07 ]
Large artery stroke [p=0.007]
1.09 [ 1.02 , 1.15 ]
Intracerebral Haemorrhage [p=0.91]
1.00 [ 0.89 , 1.12 ]
Lobar [p=0.71]
0.97 [ 0.84 , 1.12 ]
Non-lobar [p=0.97]
1.00 [ 0.88 , 1.14 ]
WMH in symptomatic stroke
WMH IS [p=5.3e-5]
1.10 [ 1.04 , 1.16 ]
Small Subcortical Brain infarcts (SSBI)
SSBI [p=0.28]
1.05 [ 0.97 , 1.14 ]
SSBI with WMH [p=0.076]
1.15 [ 0.99 , 1.33 ]
0.8
1
1.2
1.4
Odds Ratio