The Subtype Specificity of Genetic Loci Associated with Stroke in 16,664 cases and 32,792 controls

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68 **Abstract**

69 Background: Genome-wide association studies have identified multiple loci associated with
70 stroke. However, the specific stroke subtypes affected, and whether loci influence both
71 ischaemic and haemorrhagic stroke, remains unknown. For loci associated with stroke, we
72 aimed to infer the combination of stroke subtypes likely to be affected, and in doing so assess
73 the extent to which such loci have homogeneous effects across stroke subtypes.

74 Methods: We performed Bayesian multinomial regression in 16,664 stroke cases and 32,792
75 controls of European ancestry to determine the most likely combination of stroke subtypes
76 affected for loci with published genome-wide stroke associations, using model selection.
77 Cases were subtyped under two commonly used stroke classification systems, Trial of Org
78 10172 Acute Stroke Treatment (TOAST) and Causative Classification of Stroke (CCS). All
79 individuals had genotypes imputed to the Haplotype Reference Consortium 1.1 Panel.

80 Results: Sixteen loci were considered for analysis. Seven loci influenced both haemorrhagic
81 and ischaemic stroke, three of which influenced ischaemic and haemorrhagic subtypes under
82 both TOAST and CCS. Under CCS, 4 loci influenced both small vessel stroke and
83 intracerebral haemorrhage. An *EDNRA* locus demonstrated opposing effects on ischaemic
84 and haemorrhagic stroke. No loci were predicted to influence all stroke subtypes in the same
85 direction and only one locus (12q24) was predicted to influence all ischaemic stroke subtypes.

86 Conclusions: Heterogeneity in the influence of stroke-associated loci on stroke subtypes is
87 pervasive, reflecting differing causal pathways. However, overlap exists between
88 haemorrhagic and ischaemic stroke, which may reflect shared pathobiology predisposing to
89 small vessel arteriopathy. Stroke is a complex, heterogeneous disorder requiring tailored
90 analytic strategies to decipher genetic mechanisms.

91 Keywords: Stroke, Multinomial, EDNRA, Genetics, intracerebral haemorrhage

Introduction

The burden of stroke on global healthcare and society is substantial; it is consistently one of the leading causes of death and disability worldwide, [1] and a major cause of cognitive impairment and dementia. However, there exist significant gaps in our understanding of the pathological processes that underlie the disease. In recent years genome-wide association studies (GWAS) have made considerable advances in identifying genetic components underlying complex traits, in many cases identifying novel disease pathways and treatments.[2]

Characterizing the genetic component to stroke has been challenging, in part due to clinical heterogeneity, with at least three distinct major pathological processes (cardioembolism, large artery atherosclerosis, small vessel disease) underlying the majority of ischaemic strokes; and two processes underlying the majority of intracerebral haemorrhagic stroke (small vessel disease and cerebral amyloid angiopathy). [3, 4] However, recent GWAS have made considerable advances; 32 independent genome-wide significant loci were identified in the MEGASTROKE project. [5] The majority of these loci were identified as being associated with inclusive ‘all stroke’ or ‘ischaemic stroke’ categories, rather than specific stroke subtypes. This is in part due to study design, with much larger samples for these broader categories and only a fraction of stroke cases having detailed phenotyping. Indeed, this finding is in contrast to earlier studies that identified loci such as *HDAC9, PITX2* as being associated with specific subtypes. [6, 7] In order to interpret genetic risk associations in the context of biological mechanisms, a pertinent question is whether the newly identified stroke-associated loci truly confer risk across all stroke subtypes, or whether isolated or combinations of subtypes are affected. At least one of the novel variants (on chromosome 1q22) shows association with both ischaemic and haemorrhagic stroke, which might point to some shared mechanisms underlying these clinically distinct entities, which have thus far been separated in genetic studies.

Conventional approaches to GWAS, which employ within study analysis and subsequent meta-analysis across groups, do not enable detailed model comparison across different subgroups. In this analysis, we used multinomial logistic regression on well-characterized subjects with individual-level data to investigate the association of all identified genetic GWAS loci to date with all stroke subtypes (cardioembolic (CES), large artery stroke (LAS), small vessel stroke (SVS) and intracerebral haemorrhage (ICH)), determining the most likely combination of stroke subtypes affected at each locus. We performed our analysis using two established subtyping approaches: the Trial of Org 10172 in Acute Stroke Treatment (TOAST), [8] and Causative Classification of Stroke (CCS) system,[9] to provide a comprehensive account of these loci across available classification systems. Our overall aim was to evaluate genetic loci identified in previous studies using stroke datasets with well-defined phenotyping to determine if subtype specificity or cross-subtype associations could be identified.

Methods

Cohort Characteristics

The data used in this analysis were derived from several sources: the NINDS-SIGN Stroke Genetics study, [10] the Wellcome Trust Case Control Consortium 2 Stroke and Immunochip studies, [6, 11] the UK Young Lacunar Stroke Study, [12] Genetics of Cerebral Hemorrhage with Anticoagulation (GOCHA), [13] Genetic and Environmental Risk Factors for Hemorrhagic Stroke (GERFHS), [13] Cambridge ICH Genetics Study. Almost all samples (>95%) were included in the previous MEGASTROKE genome-wide association study of stroke. [5]

Stroke Phenotyping

Stroke was defined according to the World Health Organization (WHO), i.e. rapidly developing signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death with no apparent cause other than that of vascular origin. Strokes were defined as ischaemic stroke (IS) or intracerebral haemorrhage (ICH) based on clinical and imaging criteria. ICH stroke events were divided into lobar or deep, which have different presumed etiology, [3] based on location of the primary event. Ischaemic stroke cases were classified under the TOAST or CCS stroke classification systems (causative and phenotypic), or both. [8, 9] TOAST and CCS both include an 'undetermined ischaemic stroke' group (UND) denoting individuals for which it is not possible to determine the ischaemic stroke subtype. Full details are provided in Additional Files 1-2.

Genotyping and Imputation

Genotyping of datasets has been described in detail elsewhere. [6, 10-13] In this analysis, we imputed all datasets to the Haplotype Reference Consortium 1.1 panel, using the Michigan Imputation Server. [14] For each separately imputed dataset, we extracted SNPs with MAF>1% and imputation INFO values>0.8. All datasets were subsequently merged using bcftools and SNPs with a MAF>5% in the combined dataset and present in 66% of samples were included in further analyses.[15] We removed any duplicate or related (3^{rd} degree or closer) samples at this stage and calculated ancestry informative principal components on a linkage-disequilibrium pruned subset of SNPs on the remaining individuals using the --pca approx function in plink 2.0.[16]

Locus and SNP Selection

For each locus associated with stroke or stroke subtypes at genome-wide significance in MEGASTROKE,[5] we identified all SNPs in LD (r^2>0.2) with the lead reported SNP based on the five European populations from 1000 Genomes.[17] These SNPs were then extracted from

the merged dataset for analysis. We did not analyse two regions from MEGASTROKE: *RGS7* and *TMFSF1-TMFSF4*, as the previously associated variants in these regions were low frequency variants that were filtered out in our analysis. We additionally considered the *COL4A2* locus as it been robustly associated with stroke phenotypes in other large-scale studies. [18]

Multinomial Logistic Regression

We used a Bayesian multinomial logistic regression approach, implemented in *Trinculo*, [19] to evaluate the association of SNPs at each locus. Multinomial logistic regression is a natural extension of logistic regression that enables modelling of multiple phenotypic categories simultaneously against a common set of controls. The benefit of this approach, which is leveraged in this analysis, is that it enables comparison of models that include different combinations of phenotypes. In the context of genetic studies, this enables determination of the combination of phenotypes that are mostly likely to be associated with the genetic variant of interest.

We used the default prior, which assumes effect sizes are independent with variances of 0.04. All analyses included eight ancestry-informative principal components, and batch covariates for each study.

Based on their association at genome-wide significance in previous analyses, we assumed *a priori* that each region was associated with stroke. However, to avoid overfitting for weakly associated loci in our data, we performed model selection only for loci that had a Bayes Factor of at least 4 in either TOAST or CCS analyses.

No prior genome-wide association study of stroke has identified a significant association with strokes of undetermined or cryptogenic cause. Given that this study was intended to evaluate potential shared mechanisms between subtypes, we excluded strokes of undetermined cause in model fitting.

196

197 *Statistical Analysis*

198 For each locus we performed the following steps:

199 1. Use multinomial logistic regression to model the association between each genetic variant
200 and stroke subtypes under TOAST and CCS classifications, in each case including ICH
201 as an additional outcome. We therefore tested a common set of Controls against CES,
202 LAS, SVS, UND, and ICH cases.
203 2. Identify the most significant SNP in the locus under any classification system
204 3. For this SNP, calculate marginal likelihoods for all combinations of phenotypes
205 4. Identify the combination of phenotypes with the largest marginal likelihood (discarding any
206 groups containing UND) and infer that this indicates the most likely combination of
207 phenotypes for which the SNP confers risk

208

209

210 **Results**

211 After QC, there were up to 16,664 cases and 32,792 controls remaining for analysis (Table 1).
212 In the merged dataset, a binomial genome-wide analysis of all cases against controls had a
213 genomic inflation lambda=1.09, while the LDSCORE intercept value was 1.04, [20] suggesting
214 that the majority of inflation was due to polygenicity and that any bias introduced by merging
215 the datasets was minimal. A comparison of odds ratios for analysed loci from MEGASTROKE
216 and the most recent ICH publication with those from our analysis showed high consistency
217 (r^2=0.95, Additional File 3) despite slightly differing samples.

218

219 Sixteen loci contained SNPs with Bayes factors of at least 4 in either TOAST or CCS analyses.
220 We took these sixteen loci forward for further model selection. Plots for all loci under each

221 classification system are provided in Additional Files 4-19. For each of the sixteen loci, we
222 identified the most likely combination of associated phenotypes at each locus (Figure 1) based
223 on model selection. Apart from one locus (*FOXF2*), we found identical results between the two
224 CCS systems, so for simplicity of presentation results for CCS causative are presented only.

225

226 For seven loci, the combination of phenotypes most likely to be influenced by the lead genetic
227 variant at the loci included both ischaemic and haemorrhagic stroke subtypes. Four of these
228 are shown in Figure 2. At these four loci: *EDNRA*, *1q22*, *MMP12*, *SH3PXD2A*, the ischaemic
229 subtype included SVS, highlighting shared mechanisms underlying ICH and SVS, likely
230 through predisposition to cerebral small vessel disease. At the *EDNRA* locus, the direction of
231 association for ICH was opposite to that for LAS and SVS, pointing to contrasting influence on
232 ischaemic and haemorrhagic stroke risk. We explored whether ICH-associated loci were
233 specific to deep or lobar ICH. As in previous reports, [13, 18] associations at 1q22 and
234 COL4A2 appear to be specific to deep ICH, with no effect in lobar ICH. For other regions, the
235 evidence for specificity was more equivocal (Additional File 20).

236

237 For four loci: *HDAC9*, *PITX2*, *ZFHX3*, *ANK2,* only one phenotype was affected by the lead
238 variant (Figure 1, Additional Files 13, 16, 19, 8) in the most likely configuration across all
239 classification systems. Several other loci: 9p21, 12q24, 16q24, *FOXF2* were associated with
240 only one phenotype under particular classification systems, but did not show consistency
241 across TOAST and CCS (Additional Files 5, 6, 7, 12). For *TSPAN2*, which was previously
242 identified as being associated with LAS, [10] the best-fit model also included CES under CCS,
243 albeit with a much weaker effect than LAS (rs17479660; CES, OR=1.08; LAS, OR=1.19 under
244 CCS). Echoing previous results, the locus showed much stronger significance under CCS
245 classifications than under TOAST (Additional File 18).

246

247 For *COL4A2*, the strongest association found under TOAST was for rs9515201. The most
248 likely model contained ICH (OR=1.14) and SVS (OR=1.13), consistent with findings from
249 previous analyses. [18] However, under CCS an alternate SNP, rs1927349, was the strongest
250 associated. No association with SVS was observed, and a weak association with CES was
251 observed instead. Reasons for this discrepancy between CCS and TOAST are not
252 immediately clear, but non-overlapping samples between the two classification systems are a
253 likely factor.

254

255 The mean (SD) number of stroke subtypes affected at each locus were 1.88 (0.89) under
256 TOAST and 1.69 (0.87) under CCS. Under CCS, the most common combination of affected
257 subtypes was SVS and ICH (4 loci).

258

259 **Discussion**

260 We performed a large-scale genetic analysis, characterising the effects of established stroke
261 risk loci with ischaemic and haemorrhagic stroke subtypes in up to 16,664 cases and 32,792
262 controls.

263

264 Our main findings are twofold. First, for the vast majority of loci studied, multiple but never all
265 stroke subtypes were affected at the locus. Only one locus (12q24) was assumed to influence
266 all ischaemic stroke subtypes. This indicates that although these loci were identified in
267 analyses of inclusive stroke phenotypes, in the main their effects are specific to particular
268 combinations of stroke subtypes. The mean number of subtypes affected was 1.88 for TOAST
269 and 1.69 for CCS classification systems. Notable exceptions were the *PITX2* and *ZFHX3* loci,
270 which were associated with cardioembolic stroke most likely through atrial fibrillation, and
271 *HDAC9* which is associated with large vessel stroke. Under TOAST, the *FOXF2* locus was

associated solely with SVS. However, under CCS, LAS was also implicated. For CCS, the 9p21 locus was predicted to influence only LAS. However, under TOAST, SVS was also implicated. Our analyses suggest that *ANK2* confers risk of stroke predominantly through its influence on *ICH.* We were unable to identify any loci for which the most likely model included all stroke phenotypes in the same direction and only one (12q24) which for which the most likely model included all ischaemic stroke subtypes.

Secondly, we find evidence that several loci influence both haemorrhagic and ischaemic stroke. This was evident for seven loci in total (1q22, *COL4A2*, *EDNRA*, *LINC01492*, *MMP12*, *SH3PXD2A*, *CDK6*). Under CCS, 4 loci (*SH3PXD2A*, *MMP12*, *EDNRA*, 1q22) influenced both SVS and ICH, highlighting shared mechanisms underlying small vessel disease. Previous GWAS analyses have tended to separate ischaemic and haemorrhagic stroke on the basis of presumed differing etiologies. Our results suggest that including haemorrhagic alongside ischaemic stroke in multiphenotype analyses will provide further insights.

For one locus: Endothelin Receptor Type A (*EDNRA*), the association with ICH was in the opposite direction to the ischaemic stroke subtypes, suggesting opposing risk mechanisms. This locus has previously been associated with a variety of vascular phenotypes, including coronary artery disease, carotid plaques, and peripheral arterial disease (in concordant direction with ischaemic stroke), as well as intracranial aneurysm (in concordant direction with intracerebral haemorrhage). [21-24] The locus has also been associated with migraine in candidate gene studies, [25] but this has not been validated in GWA studies. [26] *EDNRA* encodes the type A receptor (ET_A) for Endothelin-1 (*ET-1*), a potent vasoconstrictor with pro-inflammatory effects. ET_A -specific antagonists increase Nitric Oxide (NO)-mediated endothelium-dependent relaxation, reduce *ET-1* levels and inhibit atherosclerosis in mice, [27] suggesting that higher levels of ET_A are pro-atherogenic: consistent with the observation that

298 higher ET_A levels are observed in atherosclerotic plaques. [28] This is also consistent with the
299 C allele of rs17612742 in our study leading to increased risk of ischaemic stroke through
300 elevated ET_A levels. Indeed, in GWA studies of intracranial aneurysm the susceptibility variant
301 (in LD with the T allele of rs17612742 in our study) was shown to result in higher transcription
302 factor binding affinity, likely resulting in repression of the transcriptional activity of *EDNRA*.
303 [23] The reason why lower levels of ET_A might promote intracranial aneurysm and
304 intracerebral haemorrhage is not immediately obvious, but several mechanisms are possible.
305 Levels of *ET-1* have been linked to vascular remodelling, an important process underlying ICH
306 and IA; [29, 30] subtle changes in this process induced by altered availability of ET_A is one
307 such mechanism. Deep ICH and ischaemic SVS arise due to the same arteriopathy that arises
308 in the deep perforating arteries of the brain. The *EDNRA* variant in this study points to a
309 mechanism that influences whether the resulting pathology is ischaemic or haemorrhagic, and
310 as such warrants further detailed investigation.

311

312 Some loci were notably more significant when phenotyped using CCS; *SH3XPD2A, MMP12*,
313 *TSPAN2, FOXF2, EDNRA,* which might point to CCS having greater accuracy and therefore
314 utility in stroke GWA studies. However, the opposite was also true for others: *16q24, HDAC9*.
315 We note that some differences may be due to the fact that not all individuals were subtyped
316 under both CCS and TOAST; the TOAST cohort was a least 20% larger. A detailed discussion
317 of the relative merits of TOAST and CCS is beyond the scope of this article, but our results
318 highlight that the importance of collecting individual phenotypic qualities that make up the
319 etiologic subtypes in genetic studies of stroke so that associated loci can be more
320 systematically examined.

321

322 Our study has several strengths. The dataset was a large stroke population including
323 intracerebral haemorrhage and ischaemic stroke cases, the majority of which were subtyped

under both TOAST and CCS. We had full access to genotype-level data enabling us full control over all analyses. Similarly there are limitations. We present results for the most likely combination of stroke phenotypes affected at each locus: the 'best-fitting' model. We had limited statistical power to determine with statistical certainty that this was the correct model; significantly larger samples would be required to achieve this. Due to the challenges of performing these analyses across different ancestry populations, we performed analyses in European populations only. The results can therefore not be generalized to all populations. In all analyses we assume there is a single causal variant at the locus, which may not be true in all cases. Our analyses are based on use of a default prior, which has been used in many genetic studies. An alternative is to derive an empirical prior from associated genetic loci. As more loci are identified as being associated with stroke, this will become a more realistic possibility and should be explored in future analyses.

Conclusions

Our findings suggest that although large scale genome-wide studies of broad 'all stroke' or 'all ischaemic stroke' phenotypes are able to identify multiple associations, it should not be assumed that such associations confer risk equally across stroke subtypes. Heterogeneity in the influence of genetic variants on different stroke subtypes is the norm, not the exception. Analyses such as the current one provide insights into the etiological stroke subtypes most prominently influenced by genetic variants at these loci – a prerequisite to decide on the most appropriate model systems to choose for further mechanistic studies. Stroke is a complex, heterogeneous disorder: our findings highlight the ongoing need for large, well phenotyped case collections and tailored analytic strategies to decipher the underlying genetic mechanisms.

Abbreviations

CES, cardioembolic stroke; LAS large artery stroke; SVS, small vessel stroke; ICH, intracerebral haemorrhage; SNP, single nucleotide polymorphism.

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Author's Contributions

MT and RM designed the experiments. MT and MC performed the imputations. MT performed the statistical analyses. MT, CDA, LCARJ, HSM, DW, and RM wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

373 All research participants contributing clinical and genetic samples for analysis in this study
374 provided written informed consent.

375

376 **Availability of data and materials**

377 Data from the NINDS-SIGN Stroke study are available to researchers through dbGAP:
378 https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000615.v1.p1.
379 Trinculo v0.96 is available from: https://sourceforge.net/projects/trinculo/files/

380

381 **Competing interests**

382 Dr. Anderson has consulted for ApoPharma, Inc.

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474

475

476 **Tables and Figures**

477 **Table 1.** Sample Sizes

Classification System	CES	LAS	SVS	UND	ICH	Controls
TOAST	3847	2803	3976	4085	1953	32,792
CCS	2826	2204	3093	4013	1953	28,052

478 CES, cardioembolic Stroke; LAS, large artery atherosclerotic stroke; SVS, small artery
479 occlusion stroke; UND, stroke of undetermined etiology; ICH, intracerebral haemorrhage;
480 TOAST, Trial of Org 10172 Acute Stroke Treatment Classification System; CCS, Causative
481 Classification of Stroke System (causative system).

482

483

484 **Figure 1.** Stroke Subtypes in Best Fitting Model at Each Locus, for CCSc, CCSp, and TOAST

485 classification Systems, with Size Weighted by Association Odds Ratio

486

487 CES, Cardioembolic Stroke; LAS, Large artery Stroke; SVS, Small Vessel Stroke; ICH,

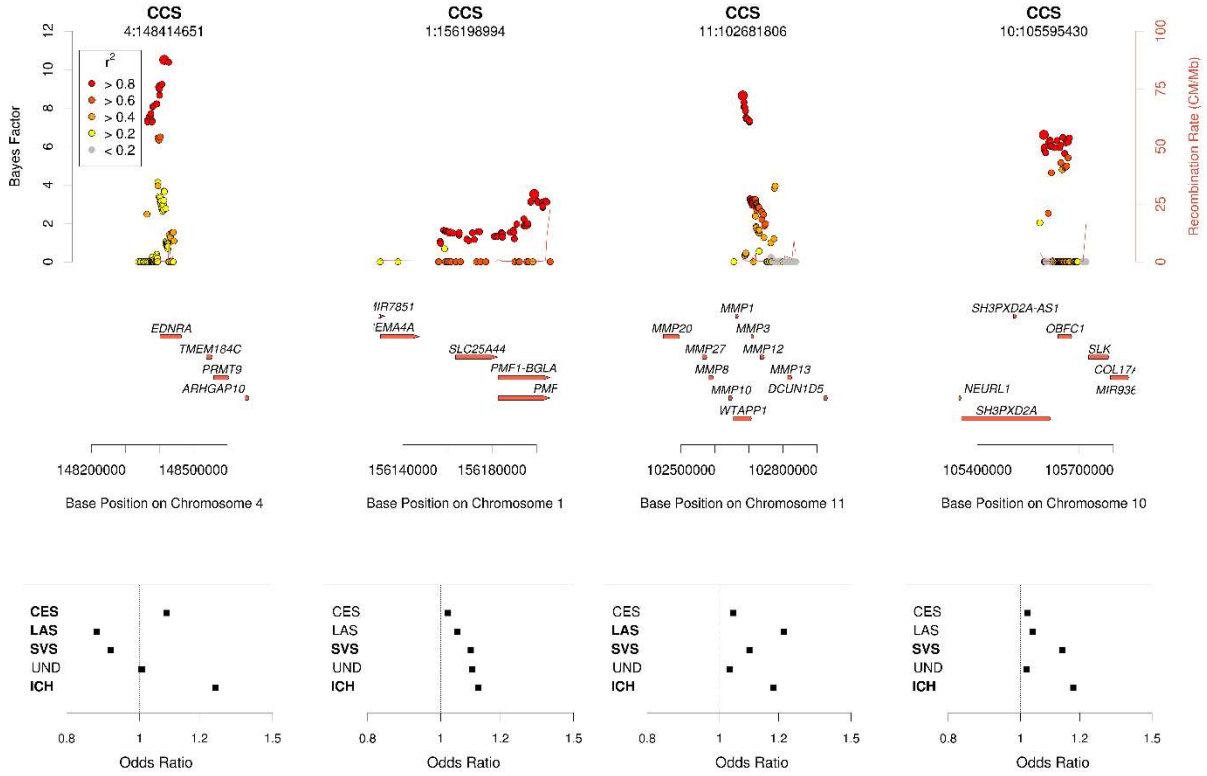
488 Intracerebral Haemorrhage. Results are presented for the 16 loci showing BF>4 in CCS or

489 TOAST analyses. Classification/Locus combinations in grey indicate that the locus did not

490 reach BF>4 in that analysis.

491

492 **Figure 2.** Local Plots showing Associations with 4 Regions Conferring Risk of Ischaemic and
493 Haemorrhagic Stroke and Odds Ratios for all stroke Subtypes



494

495 CE, cardioembolic stroke; LAS, large artery atherosclerotic stroke; SVS, small vessel stroke;
496 ICH, intracerebral haemorrhage. Results are presented for the classification system in which
497 the locus showed strongest significance. Stroke subtypes in bold indicate those included in
498 the best fitting model and therefore predicted to be influenced by the lead genetic variant,
499 based on Bayesian model selection.

500

Additional Files

Additional File 1. Stroke Phenotyping

Additional File 2. Cohort Descriptions

Additional File 3. Comparison of log(odds ratio) from most recent publication with those from this analysis for 16 SNPs tested in this analysis

ICH, Intracerebral haemorrhage; CES, cardioembolic stroke; LAS, large artery stroke; SVS, small vessel stroke. Where the lead SNP from previous publication was not available, [5, 13] we used the nearest proxy (r^2>0.6 in all cases). No SNPs in the 12q24 region passed QC in the most recent ICH publication so are not included here.

Additional File 4. 1q22 Region

Additional File 5. 9p21 Region

Additional File 6. 12q24 Region

Additional File 7. 16q24 Region

Additional File 8. ANK2 Region

Additional File 9. CDK6 Region

Additional File 10. COL4A2 Region

Additional File 11. EDNRA Region

Additional File 12. FOXF2 Region

Additional File 13. HDAC9 Region

Additional File 14. LINC01492 Region

Additional File 15. MMP12 Region

Additional File 16. PITX2 Region

Additional File 17. SH3PXD2A Region

Additional File 18. TSPAN2 Region

Additional File 19. ZFHX3 Region

544

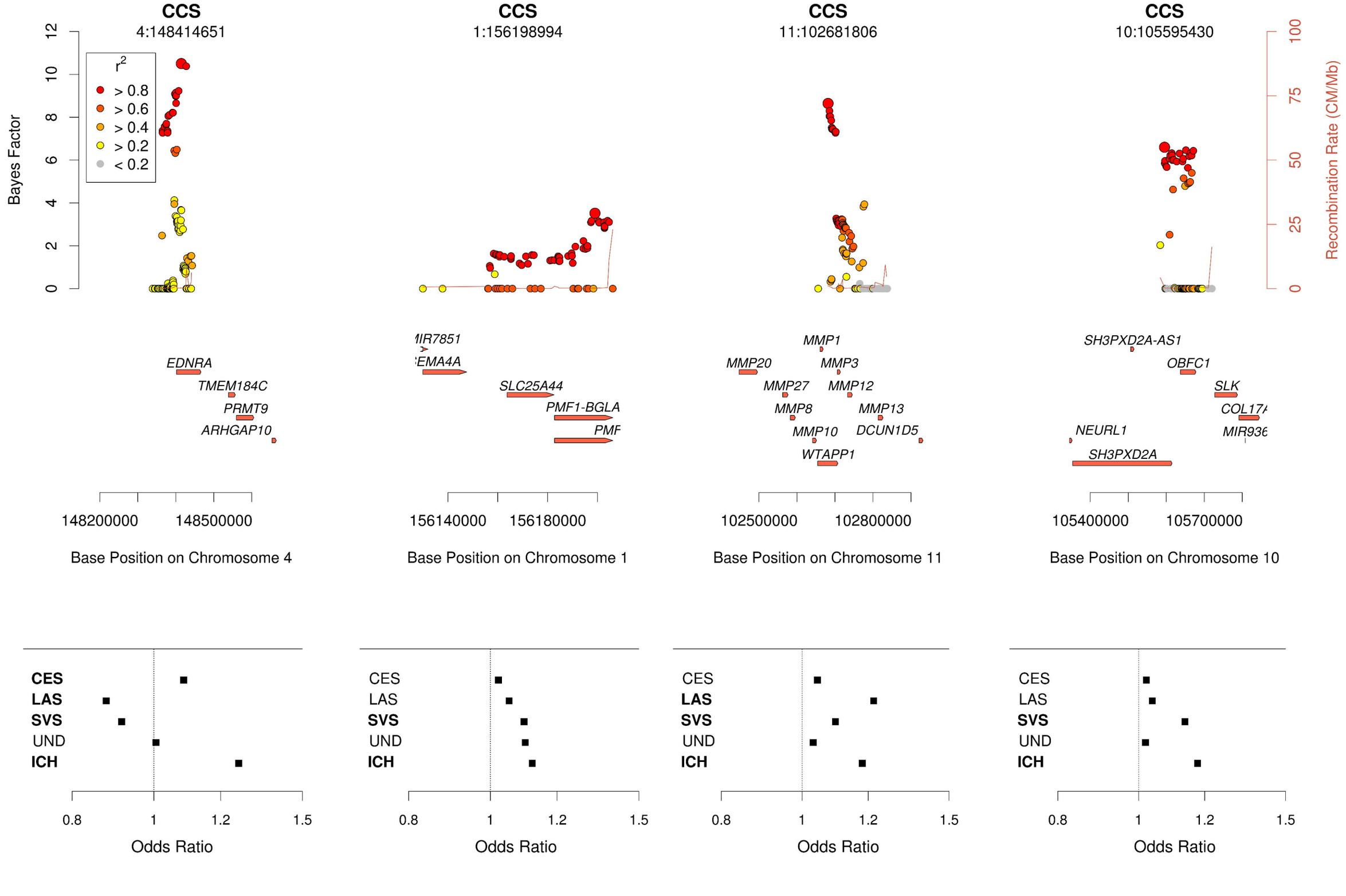
545 **Additional File 20.** Odds ratios for association of ICH-associated loci with ICH subtypes, and

546 evidence for ICH subtype-specific effects

547 OR, odds ratio; BF, Bayes Factor

548


Locus
TOAST
CCS
CES LAS SVS ICH
CES LAS SVS ICH
12q24
16q24
1q22
9p21
ANK2
CDK6
COL4A2
EDNRA
FOXF2
HDAC9
LINC01492
MMP12
PITX2
SH3PXD2A
TSPAN2
ZFHX3
+ Effect
- Effect
OR=1.3
OR=1.2
OR=1.1
BF≥4
BF<4


CCS
4:148414651
CCS
1:156198994
CCS
11:102681806
CCS
10:105595430
Bayes Factor
Recombination Rate (CM/Mb)
r2
> 0.8
> 0.6
> 0.4
> 0.2
< 0.2
EDNRA
TMEM184C
PRMT9
ARHGAP10
SLC25A44
PMF1-BGLA
MMP1
MMP20
MMP3
MMP27
MMP12
MMP8
MMP13
MMP10
DCUN1D5
WTAPP1
SH3PXD2A-AS1
OBFC1
SLK
NEURL1
SH3PXD2A
148200000
148500000
156140000
156180000
102500000
102800000
105400000
105700000
Base Position on Chromosome 4
Base Position on Chromosome 1
Base Position on Chromosome 11
Base Position on Chromosome 10
CES
LAS
SVS
UND
ICH
0.8
1
1.2
1.5
Odds Ratio