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

Running Head: Subtype Specificity of Stroke Genetic Loci

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Abstract

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

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

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

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

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

In order to minimize the possibility of unintentionally sharing information that can be used to re-identify private information, a subset of the data generated for this study are available at dbGAP and can be accessed at https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000615.v1.p1.

All contributing studies were approved by institutional review committees; subjects gave informed consent.

Full methods are provided in the Data Supplement.

Results

After QC, there were up to 16,664 cases and 32,792 controls remaining for analysis (Table 1). In the merged dataset, a binomial genome-wide analysis of all cases against controls had a genomic inflation lambda=1.09, while the LDSCORE intercept value was 1.04, [10] suggesting that the majority of inflation was due to polygenicity and that any bias introduced by merging the datasets was minimal.

Sixteen loci contained SNPs with log(Bayes factors) of at least 4 in analyses of alternative stroke classification systems: Trial of Org 10172 Acute Stroke Treatment Classification System (TOAST) or Causative Classification of Stroke System (CCS) (causative system). We took these sixteen loci forward for further model selection. Plots for all loci under each classification system are provided in Supplementary Figures 1-16. For each of the sixteen loci, we identified the most likely combination of associated phenotypes at each locus (Figure 1, Table 2) based on model selection. A comparison of odds ratios for analysed loci from MEGASTROKE and the most recent ICH publication with those from our analysis showed high consistency (r^2=0.95, Supplementary Figure 17) despite slightly differing samples. LD values between our lead and previously published SNPs for the 16 loci in this analysis are provided in Supplementary Table 1.

For seven loci, the combination of phenotypes most likely to be influenced by the lead genetic variant at the loci included both ischaemic and haemorrhagic stroke subtypes. Four of these are shown in Figure 2. At these four loci: *EDNRA*, *1q22*, *MMP12*, *SH3PXD2A*, the ischaemic subtype included SVS, highlighting shared mechanisms underlying ICH and SVS, likely through predisposition to cerebral small vessel disease. At the *EDNRA* locus, the direction of association for ICH was opposite to that for LAS and SVS, pointing to contrasting influence on ischaemic and haemorrhagic stroke risk. We explored whether ICH-associated loci were specific to deep or lobar ICH. As in previous reports, [11, 12] associations at 1q22 and *COL4A2*

appear to be specific to deep ICH, with no effect in lobar ICH. For other regions, the evidence for specificity was more equivocal (Supplementary Table 2).

For four loci: *HDAC9*, *PITX2*, *ZFHX3*, *ANK2,* only one phenotype was affected by the lead variant (Figure 1, Supplementary Figures 10, 13, 16, 5) in the most likely configuration across all classification systems. Several other loci: 9p21, 12q24, 16q24, *FOXF2* were associated with only one phenotype under particular classification systems, but did not show consistency across TOAST and CCS (Supplementary Figures 2, 3, 4, 9). For *TSPAN2*, which was previously identified as being associated with LAS, [13] the best-fit model also included CES under CCS, albeit with a much weaker effect than LAS (rs17479660; CES, OR=1.08; LAS, OR=1.19 under CCS). Echoing previous results, the locus showed much stronger significance under CCS classifications than under TOAST (Supplementary Figure 15).

For *COL4A2*, the strongest association found under TOAST was for rs9515201. The most likely model contained ICH (OR=1.14) and SVS (OR=1.13), consistent with findings from previous analyses. [12] However, under CCS an alternate SNP, rs1927349, was the strongest associated. No association with SVS was observed, and a weak association with CES was observed instead. Reasons for this discrepancy between CCS and TOAST are not immediately clear, but non-overlapping samples between the two classification systems are a likely factor.

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

Discussion

We performed a large-scale genetic analysis, characterising the effects of established stroke risk loci with ischaemic and haemorrhagic stroke subtypes in up to 16,664 cases and 32,792 controls. Our main findings are twofold. First, for the vast majority of loci studied, multiple but never all stroke subtypes were affected at the locus. Only one locus (12q24) was assumed to influence all ischaemic stroke subtypes. This indicates that although these loci were identified in analyses of inclusive stroke phenotypes, in the main their effects are specific to particular combinations of stroke subtypes. The mean number of subtypes affected was 1.88 for TOAST and 1.69 for CCS classification systems. Notable exceptions were the *PITX2* and *ZFHX3* loci, which were associated with cardioembolic stroke most likely through atrial fibrillation (for which they are well-established loci [14]), and *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 (all in concordant direction with ischaemic stroke), as well as intracranial aneurysm (in concordant direction with intracerebral haemorrhage). [15-18] The locus has also been associated with migraine in candidate gene studies, [19] but this has not been validated in GWA studies and is likely a false positive. [20] *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, [21] suggesting that higher levels of ET_A are pro-atherogenic: consistent with the observation that higher ET_A levels are observed in atherosclerotic plaques. [22] Based on this, one might expect the *EDNRA* risk variant (C allele of rs17612742 in this study) to lead to increased risk of ischaemic stroke through elevated ET_A levels. Indeed, in GWA studies of intracranial aneurysm the susceptibility variant (in LD with the T allele of rs17612742 in our study) was shown to result in higher transcription factor binding affinity, likely resulting in repression of the transcriptional activity of *EDNRA*. [17] This suggests that carries of the C allele have lower levels of *EDNRA,* which consequently higher *ET-1* levels and greater susceptibility to atherosclerosis. The reason why for carriers of T allele lower levels of ET_A might promote intracranial aneurysm and intracerebral haemorrhage is not immediately obvious, but several mechanisms are possible. Levels of *ET-1* have been linked to vascular remodelling, an important process underlying ICH and IA; [23, 24] subtle changes in this process induced by altered availability of ET_A is one such mechanism. Deep ICH and ischaemic SVS arise due to the same arteriopathy that arises in the deep perforating arteries of the brain. The *EDNRA* variant in this study points to a mechanism that influences whether the resulting pathology is ischaemic or haemorrhagic, and as such warrants further detailed investigation.

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

Our study has several strengths. The dataset was a large stroke population including 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. The implementation of a multinomial regression approach enabled us to systematically assess which stroke subtypes were likely to be affected at each locus, which would not be formally possible under standard binomial regression approaches which analyse each stroke subtype separately. Ultimately, mechanistic studies will be required to determine the influence of associated genetic variants, but analyses such as this have utility in directing the focus and model systems suitable for such follow up studies.

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. One consequence of this is that there remains the potential that some associations are due to random variation rather than true biological differences. It

would therefore be prudent to treat some of the findings here as preliminary until confirmed in larger samples. Due to the challenges of performing these analyses across different ancestry populations, and as we only had a small number of non-European ancestry ICH cases available which could lead to overfitting, we performed analyses in European populations only. The results can therefore not be generalized to all populations. Repeating these analyses once sufficient data from other ancestral groups are available should be highly prioritized to ensure advancements in the field are made for all ancestral groups. 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. The multinomial regression approach used here provided 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.

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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

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

Availability of data and materials

Data from the NINDS-SIGN Stroke study are available to researchers through dbGAP: https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000615.v1.p1.

Trinculo v0.96 is available from: https://sourceforge.net/projects/trinculo/files/.

MEGASTROKE data is available from http://megastroke.org.

Competing interests

Dr. Anderson has consulted for ApoPharma, Inc.

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Tables and Figures

Table 1. Sample Sizes

	TOAST			CCS		
	N	Age (mean(SD))	Male (%)	N	Age (mean(SD))	Male (%)
CES	3847	72(14)	49	2826	75(12)	44
LAS	2803	68(12)	65	2204	67(12)	62
SVS	3976	64(13)	62	3093	63(13)	62
UND	4085	65(16)	54	4013	65(15)	53
ICH	1953	71(13)	53	1953	71(13)	53
Controls	32792	62(17)	46	28052	62(17)	48

CES, cardioembolic Stroke; LAS, large artery atherosclerotic stroke; SVS, small artery occlusion stroke; UND, stroke of undetermined etiology; ICH, intracerebral haemorrhage; TOAST, Trial of Org 10172 Acute Stroke Treatment Classification System; CCS, Causative Classification of Stroke System (causative system). Age available not available for controls from WTCCC2 studies.

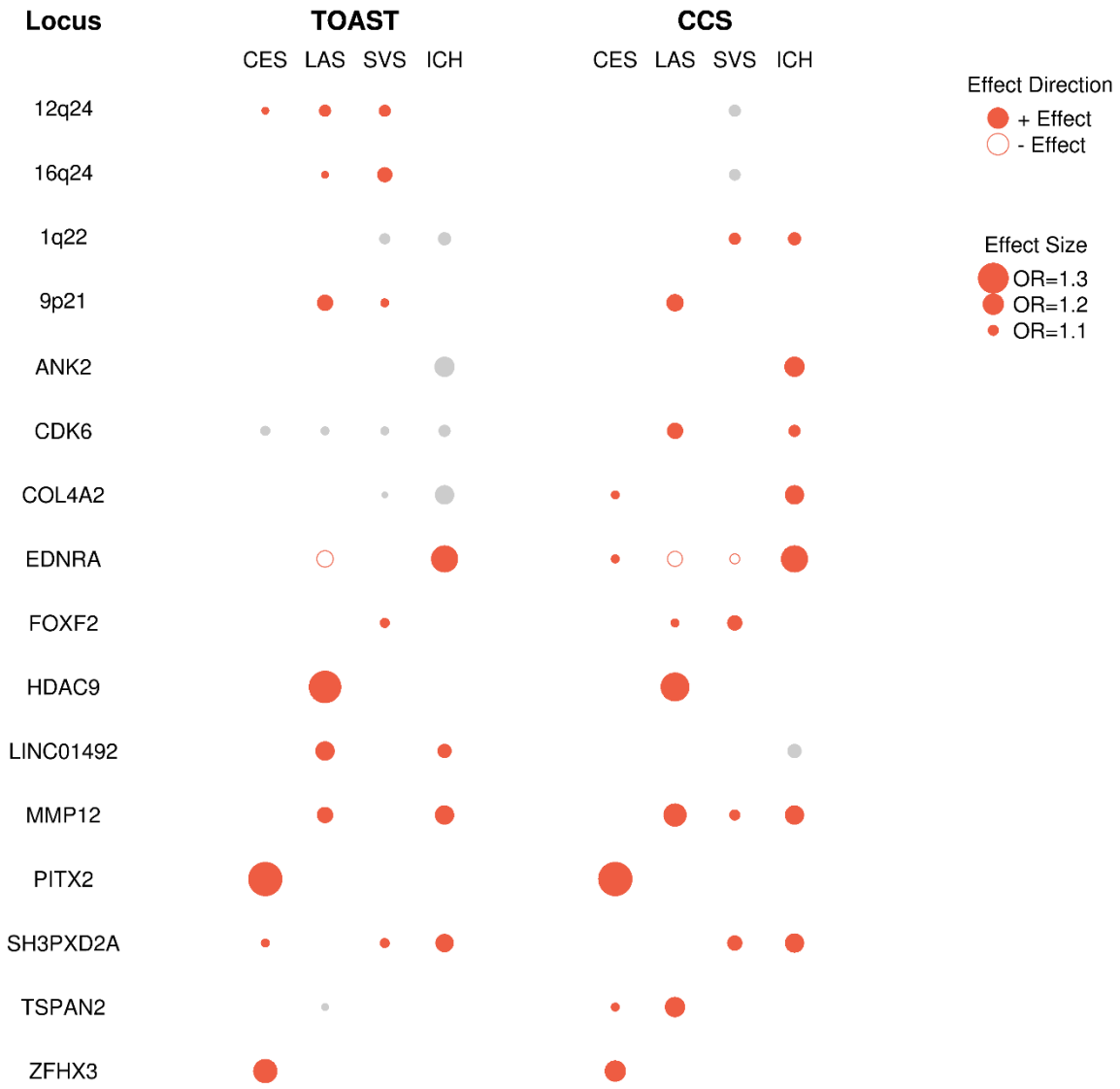
Table 2 – Lead SNPs, Association Statistics, and Affected Stroke Subtypes for Each Locus

Locus	Lead SNP [Best Model]	log OR (SE)	log BF	Subtypes in Best Fitting Model
1q22	rs2758603 [CCS]	0.10 (0.03) SVS 0.11 (0.05) ICH 0.02 (0.03) CES 0.07 (0.03) UNK 0.07 (0.03) LAS	4.0	SVS, ICH
9p21	rs1412830 [TOAST]	0.08 (0.03) SVS 0.07 (0.04) ICH -0.01 (0.03) CES 0.03 (0.03) UNK 0.14 (0.03) LAS	5.7	LAS, SVS
12q24	rs10774624 [TOAST]	0.10 (0.03) SVS -0.03 (0.05) ICH 0.07 (0.03) CES 0.07 (0.03) UNK 0.10 (0.03) LAS	5.8	CE, LAS, SVS
16q24	rs12445022 [TOAST]	0.13 (0.03) SVS 0.05 (0.05) ICH -0.01 (0.03) CES 0.07 (0.03) UNK 0.07 (0.03) LAS	5.8	LAS, SVS
ANK2	rs149538932 [CCS]	0.07 (0.03) SVS 0.18 (0.05) ICH 0.04 (0.03) CES 0.08 (0.03) UNK 0.02 (0.03) LAS	6.4	ICH
CDK6	rs4272 [CCS]	0.05 (0.04) SVS 0.10 (0.05) ICH 0.07 (0.03) CES 0.12 (0.03) UNK 0.14 (0.04) LAS	8.5	LAS, ICH
COL4A2	rs1927349 [CCS]	-0.02 (0.03) SVS 0.16 (0.05) ICH 0.08 (0.03) CES 0.04 (0.03) UNK 0.02 (0.03) LAS	5.0	CES, ICH
EDNRA	rs17612742 [CCS]	0.09 (0.04) SVS -0.23 (0.06) ICH -0.08 (0.04) CES -0.00 (0.04) UNK 0.13 (0.04) LAS	10.5	CES, LAS, SVS, ICH

FOXF2	rs11242678 [CCS]	0.13 (0.03) SVS -0.05 (0.05) ICH 0.07 (0.03) CES 0.09 (0.03) UNK 0.09 (0.04) LAS	7.4	LAS, SVS
HDAC9	rs2107595 [TOAST]	0.04 (0.04) SVS -0.08 (0.06) ICH 0.05 (0.04) CES 0.06 (0.03) UNK 0.27 (0.04) LAS	19.2	LAS
LINC01492	rs10990643 [TOAST]	-0.02 (0.04) SVS 0.12 (0.06) ICH 0.03 (0.04) CES 0.01 (0.03) UNK 0.17 (0.04) LAS	4.1	LAS, ICH
MMP12	rs470234 [CCS]	0.09 (0.04) SVS 0.17 (0.06) ICH 0.04 (0.04) CES 0.03 (0.04) UNK 0.20 (0.04) LAS	8.7	LAS, SVS, ICH
PITX2	rs2723334 [TOAST]	0.0 (0.04) SVS 0.08 (0.06) ICH 0.29 (0.04) CES 0.03 (0.03) UNK -0.03 (0.04) LAS	48.0	CES
SH3PXD2A	rs10883922 [CCS]	0.13 (0.03) SVS 0.16 (0.05) ICH 0.02 (0.03) CES 0.02 (0.03) UNK 0.04 (0.03) LAS	6.0	SVS, ICH
TSPAN2	rs7537796 [CCS]	-0.05 (0.03) SVS -0.06 (0.05) ICH 0.06 (0.03) CES -0.02 (0.03) UNK 0.14 (0.03) LAS	6.8	CES, LAS
ZFHX3	rs67329386 [TOAST]	-0.02 (0.03) SVS -0.05 (0.05) ICH 0.20 (0.03) CES 0.02 (0.03) UNK 0.00 (0.03) LAS	13.8	CES

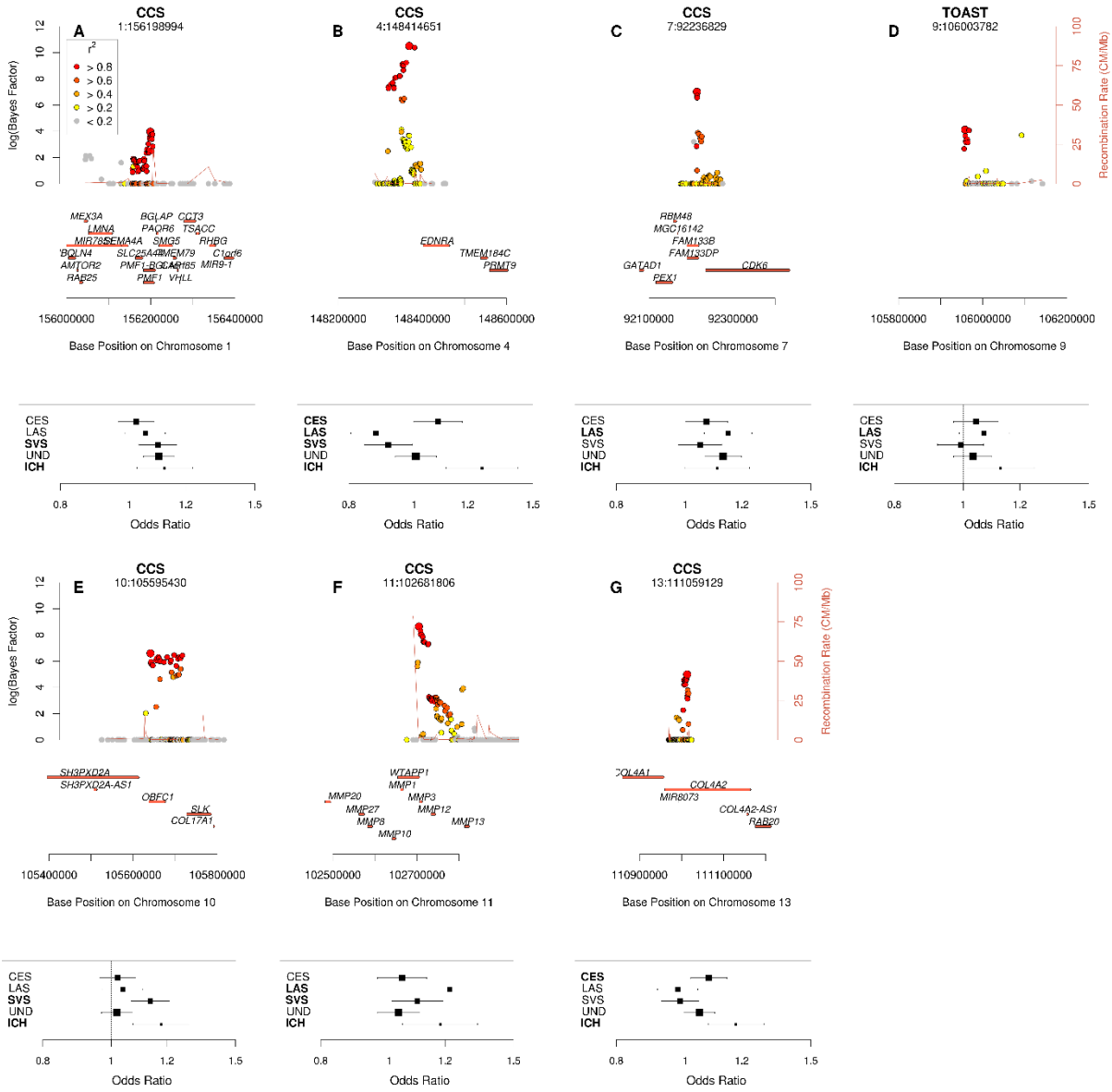
406 CES, Cardioembolic Stroke; LAS, Large artery Stroke; SVS, Small Vessel Stroke; ICH,
407 Intracerebral Haemorrhage; log BF, log transform of Bayes Factor; log OR, log transform of
408 Odds Ratio; SE, standard error; CCS, causative classification system of stroke; TOAST, Trial
409 of Org 10172 Acute Stroke Treatment Classification System

Figure 1. Stroke Subtypes in Best Fitting Model at Each Locus, for CCSc, CCSp, and TOAST classification Systems, with Size Weighted by Association Odds Ratio



CES, Cardioembolic Stroke; LAS, Large artery Stroke; SVS, Small Vessel Stroke; ICH, Intracerebral Haemorrhage. Results are presented for the 16 loci showing log(Bayes Factor)>4 in CCS or TOAST analyses. Classification/Locus combinations in grey indicate that the locus did not reach log(Bayes Factor)>4 in that analysis.

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



A, 1q22 region; B, EDNRA region; C, CDK6 region; D, LINC01492 region; E, SH3PXD2A region; F, MMP12 region; G, COL4A2 region; CE, cardioembolic stroke; LAS, large artery atherosclerotic stroke; SVS, small vessel stroke; ICH, intracerebral haemorrhage. Results are presented for the classification system in which the locus showed strongest significance. Stroke subtypes in bold indicate those included in the best fitting model and therefore predicted to be influenced by the lead genetic variant, based on Bayesian model selection.