Low-frequency and common genetic variation in ischemic stroke
The METASTROKE collaboration

Rainer Malik, PhD*
Matthew Traylor, PhD*
Sara L. Pulit, BA*
Steve Bevan, PhD
Jemma C. Hopewell, PhD
Elizabeth G. Holliday, PhD
Wei Zhao, MS
Patricia Abrantes, PhD
Philippe Amouyel, MD, PhD
John R. Attia, MD, PhD
Thomas W.K. Battey, BS
Klaus Berger, MD
Giorgio B. Boncoraglio, MD
Ganesh Chauhan, PhD
Yu-Ching Cheng, PhD
Wei-Min Chen, PhD
Robert Clarke, FRCP
Ioana Cotlarciuc, PhD
Stephanie Debette, MD, PhD
Guido J. Falcone, MD, ScD, MPH
Jose M. Ferro, MD, PhD
Dale M. Gamble, BS
Andreea Ilinca, MD
Steven J. Kittner, MD, MPH
Christina E. Kourkoulis, BS
Robin Lemmens, MD, PhD
Christopher R. Levi, MBBS, PhD
Peter Lichtner, PhD
Arne Lindgren, MD, PhD
Jingmin Liu, MS
James F. Meschia, MD
Braxton D. Mitchell, PhD, MPH

ABSTRACT

Objective: To investigate the influence of common and low-frequency genetic variants on the risk of ischemic stroke (all IS) and etiologic stroke subtypes.

Methods: We meta-analyzed 12 individual genome-wide association studies comprising 10,307 cases and 19,326 controls imputed to the 1000 Genomes (1 KG) phase I reference panel. We selected variants showing the highest degree of association ($p < 1E-5$) in the discovery phase for replication in Caucasian (13,435 cases and 29,269 controls) and South Asian (2,385 cases and 5,193 controls) samples followed by a transethnic meta-analysis. We further investigated the p value distribution for different bins of allele frequencies for all IS and stroke subtypes.

Results: We showed genome-wide significance for 4 loci: *ABO* for all IS, *HDAC9* for large vessel disease (LVD), and both *PITX2* and *ZFHX3* for cardioembolic stroke (CE). We further refined the association peaks for *ABO* and *PITX2*. Analyzing different allele frequency bins, we showed significant enrichment in low-frequency variants (allele frequency <5%) for both LVD and small vessel disease, and an enrichment of higher frequency variants (allele frequency 10% and 30%) for CE (all $p < 1E-5$).

Conclusions: Our findings suggest that the missing heritability in IS subtypes can in part be attributed to low-frequency and rare variants. Larger sample sizes are needed to identify the variants associated with all IS and stroke subtypes. *Neurology® 2016;86:1217–1226*

GLOSSARY

AF = atrial fibrillation; **CADISP** = Cervical Artery Dissection and Ischemic Stroke Patients; **CE** = cardioembolic stroke; **FDR** = false discovery rate; **GWAS** = genome-wide association studies; **IS** = ischemic stroke; **LD** = linkage disequilibrium; **LVD** = large vessel disease; **MAF** = minor allele frequency; **MAGENTA** = Meta-Analysis Gene-set Enrichment of Variant Associations; **NINDS-SiGN** = National Institute of Neurological Disorders and Stroke–Stroke Genetics Network; **NK** = natural killer; **NO** = nitric oxide; **RACE** = Risk Assessment of Cardiovascular Events; **SNP** = single nucleotide polymorphism; **SVD** = small vessel disease; **TOAST** = Trial of Org 10172 in Acute Stroke Treatment.

Stroke is a leading cause of disability in Western countries and among the most common causes of premature death worldwide.[1,2] Ischemic stroke (IS) accounts for up to 85% of all stroke cases. Evidence for a substantial genetic contribution to IS risk comes from twin and family history studies and the discovery of risk loci for IS through genome-wide association studies (GWAS).[3–7] Most previously identified associations have been confined to etiologic stroke subtypes, which include large vessel disease (LVD), cardioembolic stroke (CE), and small vessel disease (SVD). Despite these discoveries, a significant proportion of heritability remains unexplained.[3,6–8]

Prior GWAS in IS have been based on genetic data imputed to versions of the HapMap panel[9] with training sets of up to 2.5 million single nucleotide polymorphisms (SNPs) of which 85% are common variants (minor allele frequency [MAF] > 5%). Since then, the 1000 Genomes (1 KG) Project[10] has considerably expanded the coverage of human genetic variation especially for low-frequency (MAF 1%–5%) variants. We thus performed an extended meta-analysis informed by 1 KG including low-frequency variants in the human genome not assessed in the previous METASTROKE collaboration to determine whether these variants mediate risk for ischemic stroke.

Sofia A. Oliveira, PhD
Joana Pera, MD, PhD
Alex P. Reiner, MD
Peter M. Rothwell, MD,
    PhD, FMedSci
Pankaj Sharma, MD, PhD
Agnieszka Slowik, MD, PhD
Cathie L.M. Sudlow, MD,
    PhD
Turgut Tatlisumak, MD,
    PhD
Vincent Thijs, MD, PhD
Astrid M. Vicente, PhD
Daniel Woo, MD
Sudha Seshadri, MD
Danish Saleheen, PhD
Jonathan Rosand, MD, MSc
Hugh S. Markus, DM
Bradford B. Worrall, MD,
    MSc
Martin Dichgans, MD
For the ISGC Analysis
    Group
For the METASTROKE
    collaboration
The Wellcome Trust Case
    Control Consortium 2
    (WTCCC2)
The NINDS Stroke
    Genetics Network
    (SiGN)

Correspondence to
Dr. Dichgans:
martin.dichgans@med.uni-muenchen.de

We assembled 10,307 Caucasian cases and 19,326 Caucasian controls from 12 studies for a GWAS meta-analysis of IS based on the 1 KG phase I imputation training set. After quality control, 8.3 million SNPs and 1 million indels were available for analysis. Promising signals were replicated both in Caucasian and non-Caucasian populations.

METHODS **Overall study design.** The discovery stage consisted of a meta-analysis of 12 case-control studies of IS with previously genotyped data (table e-1 on the *Neurology*® Web site at Neurology.org). For each set of cases, population-matched controls were recruited from studies with existing genotyping data (details of study cohorts and controls are given in the supplementary material). For both sample sets, raw autosomal data were imputed to approximately 9 million SNPs using 1 KG phase I data as a reference panel. Genome-wide logistic regression analysis was performed independently in all samples, summary statistics were shared, and meta-analysis was performed centrally for all datasets. Covariates were not considered as they were not equally available over all study sets. Subsequently, the top SNPs ($p < 1E-5$) from the discovery meta-analysis were tested for replication in 3 independent samples (figure 1 and table e-2): (1) 5,137 de novo genotyped stroke samples from Europe and the United States and 2,040 controls; (2) genome-wide data from 8,298 Caucasian stroke patients and 27,229 controls recruited through the Cervical Artery Dissection and Ischemic Stroke Patients (CADISP) and National Institute of Neurological Disorders and Stroke–Stroke Genetics Network (NINDS-SiGN) networks[11,12]; and (3) genome-wide data from South Asian patients recruited through the Risk Assessment of Cardiovascular Events (RACE) study phase 1 and 2.[13]

Standard protocol approvals, registrations, and patient consents. Written or oral informed consent was obtained from all participants, and the study was approved by the respective research ethics committees.

Discovery-stage genotyping. Genotyping was performed individually for all sites and quality control was performed as described previously.[14]

Replication-stage genotyping. The first part of de novo genotyping was done at the Helmholtz Center Munich using iPlex Gold (Sequenom, San Diego, CA) methodology. Amplification reactions and parameters were based on the manufacturer's instructions. Spectrocaller software supplied by the manufacturer was used for automatic genotype calling. Clusters were checked manually, and all doubtful calls were evaluated. Sex was checked to remove any sample misidentifications.

The second part of de novo genotyping was performed at the Psychiatric & Neurodevelopmental Genetics Unit, Boston, Massachusetts, using the Sequenom iPLEX Gold chemistry and the MassARRAY system. Genotypes were called using Spectro-CHIP array and matrix-assisted laser desorption/ionization–time of flight mass spectrometry. Genotype clusters were checked manually, and all doubtful calls were evaluated.

Imputation. We performed imputation separately for each study (table e-1) using the algorithms IMPUTEv2[15] and MACH[16] with standard parameters. We removed SNPs with an imputation quality (info) score <0.3, leaving approximately 8 million variants per individual study.

GWAS and meta-analysis. We performed GWAS on the combined phenotype (all ischemic stroke), as well as for etiologic stroke subtypes classified according to Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria.[17] TOAST subtyping was available for all but 2 studies (Heart Protection Study and Vitamin Intervention for Stroke Prevention; see table e-1). To ensure high quality of the resulting GWAS data, we calculated λ for the overall discovery sample (figure e-1) and for each study individually (figure e-2). We further calculated lambda on sets of SNPs stratified by frequency and imputation quality to determine whether particular bins of SNPs were susceptible to genomic inflation (figure 2). SNPs with frequency <1%, introduced during imputation, showed high levels of genomic inflation and were thus excluded from the trans-ethnic meta-analysis. 1 KG phase I samples were used to calculate reference frequencies for European samples. SNPs with a frequency difference >30% from 1 KG in controls or a difference >30% with any other study in the meta-analysis were removed from the data, as were SNPs with missing *p* values and SNPs that only produced *p* values in <50% of the studies.
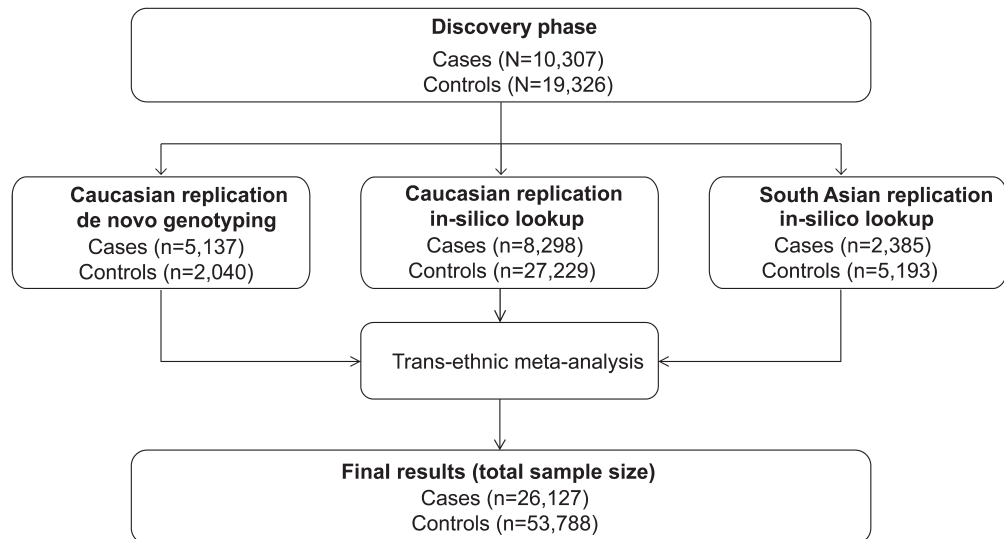
Finally, λ was calculated across the cleaned set of association results, and the results were combined to perform fixed-effects inverse variance weighted meta-analysis using METAL.[18] During meta-analysis, SNPs were analyzed across cohorts to ensure that effect alleles were consistent and that alleles matched those reported in the 1 KG phase I. Outliers in these analyses were excluded from further processing. We used genomic control to correct for incidental inflation of test statistics. Upon completion of the meta-analyses, we again confirmed that the genomic inflation was well behaved (all λ <1.03).

All SNPs with $p < 1E-5$ in any of the performed GWAS (all IS and subtypes) and a median imputation quality >0.7 were selected for downstream replication. This resulted in sets of SNPs analyzed for the following traits: all IS, LVD, SVD, and CE. The replication strategy consisted of 3 parts (figure 1). We first performed replication in the de novo genotyped wet-laboratory studies using Sequenom technology.

Summary statistics for each replication sample were produced using logistic regression with the phenotype of interest as outcome. Model 1 was calculated without covariates; model 2 included sex as a covariate. Since the results did not differ between the 2 models and to ensure consistency between the discovery and replication phase, results are reported only for model 1. Cases from Leuven were folded into the German sample using the German controls. Results were summarized using fixed-effects inverse-variance meta-analysis. We next performed in silico replication in the CADISP[11] and NINDS-SiGN[12] sample. Overlapping cases and controls between the NINDS-SiGN sample and METASTROKE were identified and removed and summary statistics from NINDS-SiGN were recalculated for the replication SNPs.

For both meta-analyses, fixed-effects inverse variance models were used. The first and second replication steps were combined to form the Caucasian replication set. Third, multiethnic meta-analysis was performed by integrating in silico lookup data from RACE1 and RACE2 (forming the South Asian replication set) using METASOFT. We used Han and Eskin's[19] random effects model to maximize power under heterogeneity. Combination of the discovery, the Caucasian replication set, and the South Asian replication set using multiethnic analysis formed the final results (figure 1). Any SNPs with $p < 5E-8$ were considered to be genome-wide significant. Any SNPs with $5E-8 < p < 1E-5$ were considered to have suggestive evidence for association.

Pathway analysis. Pathway analysis was performed using Meta-Analysis Gene-set Enrichment of Variant Associations (MAGENTA).[20] We used all available databases and 10,000

Figure 1   Study profile

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                    │         Discovery phase          │
                    │      Cases (N=10,307)            │
                    │      Controls (N=19,326)         │
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│ Caucasian         │   │ Caucasian         │   │ South Asian       │
│ replication       │   │ replication       │   │ replication       │
│ de novo genotyping│   │ in-silico lookup  │   │ in-silico lookup  │
│ Cases (n=5,137)   │   │ Cases (n=8,298)   │   │ Cases (n=2,385)   │
│ Controls (n=2,040)│   │ Controls (n=27,229)│  │ Controls (n=5,193)│
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                    │   Trans-ethnic meta-analysis     │
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                    │ Final results (total sample size)│
                    │      Cases (n=26,127)            │
                    │      Controls (n=53,788)         │
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Study profile summarizing the study samples and analytical strategy.

permutations to select statistically significant pathways and processes. We deemed a false discovery rate (FDR) q value of $q < 0.05$ or a Bonferroni corrected p value of $p < 0.05$ as significant. Bonferroni correction was performed on the number of gene sets in a pathway.

RESULTS A total of 10,307 cases and 19,326 controls from 12 studies were investigated in the discovery analysis. Data on etiologic stroke subtypes were available for 10 of the 12 studies (table e-1). A set of 15,820 IS cases (3,808 LVD, 3,697 CE, and 2,206 SVD) and 34,462 controls was available for replication (see Methods and table e-2). The overall genomic inflation factors (λ) for the meta-analyses of IS, LVD, CE, and SVD were 1.015, 1.028, 1.029, and 1.029, respectively, indicating minimal inflation due to population stratification or due to cases and controls who had undergone separate genotyping (figure 2). Manhattan plots for the discovery sample are shown in figures e3-e6. QQ plots for all IS and subtypes are depicted in figure e-1 and for individual studies in figure e-2.

Established risk loci for ischemic stroke. We first examined the lead SNPs of established risk loci for IS and subtypes derived from GWAS in our discovery meta-analysis (table 1). The previously identified lead signal for *HDAC9* (LVD)[5] was associated on a genome-wide level in the discovery analysis. We also observed p values $< 1E-3$ for association at chr12q24[21] (all IS, $p = 1.13E-5$), *ABO*[22] (all IS, $p = 5.40E-4$), chr6p21[6] (LVD, $p = 5.83E-5$), chr9p21[23] (LVD, $p = 1.09E-4$), *PITX2*[24] (CE, $p = 2.51E-6$), and *ZFHX3*[25] (CE, $p = 6.73E-5$). In contrast, we found no association of *NINJ2*[26] (all IS, $p = 0.4196$). For full results, see table 1 and figure e-7.

Novel risk loci for ischemic stroke. Our discovery analysis yielded 4 new and independent loci that exceeded the threshold for genome-wide significance of $5E-8$ (table 2) as well as 25 additional loci with association p values $< 1E-5$ (table e-3). This list included 7 loci (13 variants) for all IS, 8 loci (13 variants) for LVD, 7 loci (11 variants) for CE, and 7 loci (13 variants) for SVD, all of which were selected for replication (figure 1). In the Caucasian replication, 3 SNPs for 3 loci (*ABO*, *PITX2*, and *ZFHX3*) were nominally associated after Bonferroni correction ($p < 6.3E-3$, table 2). The results of a transethnic meta-analysis yielded 4 loci genome-wide significant, all of which have been reported previously. The lead SNPs for these loci were rs532436 for *ABO* (all IS, overall $p = 4.28E-8$), rs2107595 for *HDAC9* (LVD, $p = 2.99E-11$), rs2723334 for *PITX2* (CE, $p = 8.37E-24$), and rs12932445 for *ZFHX3* (CE, $p = 1.20E-8$). Association signals for the indels did not achieve genome-wide or near genome-wide significance.

Fine mapping of risk loci for ischemic stroke. In order to refine the association signals at confirmed, previously published loci, we took advantage of the denser imputation panel provided by the 1 KG consortium to produce association signals for an enlarged set of low-frequency variants (figure e-7). We discovered a new peak association for *PITX2* (rs2723334), which is only in moderate linkage disequilibrium (LD) with the previously published lead signal for CE (rs2200733, $r^2 = 0.45$, figure e-7C) and with the previously published lead signal for atrial fibrillation (AF)[27] (rs6817105, $r^2 = 0.46$). Furthermore, our novel lead SNP (rs532436) for *ABO* is only in

Figure 2   Quantile-quantile plots for different allele frequency bins

A

Large vessel disease
(λ=1.028)

- Expected (null)
- Observed
- MAF > 0.30 (2092704)
- 0.1 < MAF < 0.3 (2890435)
- 0.05 < MAF < 0.1 (1290554)
- MAF < 0.05 (3276900)

B

Cardioembolic stroke
(λ=1.029)

- Expected (null)
- Observed
- MAF > 0.30 (2093921)
- 0.1 < MAF < 0.3 (2893088)
- 0.05 < MAF < 0.1 (1290553)
- MAF < 0.05 (3265067)

C

Small vessel disease
(λ=1.029)

- Expected (null)
- Observed
- MAF > 0.30 (2091669)
- 0.1 < MAF < 0.3 (2887145)
- 0.05 < MAF < 0.1 (1286855)
- MAF < 0.05 (3275872)
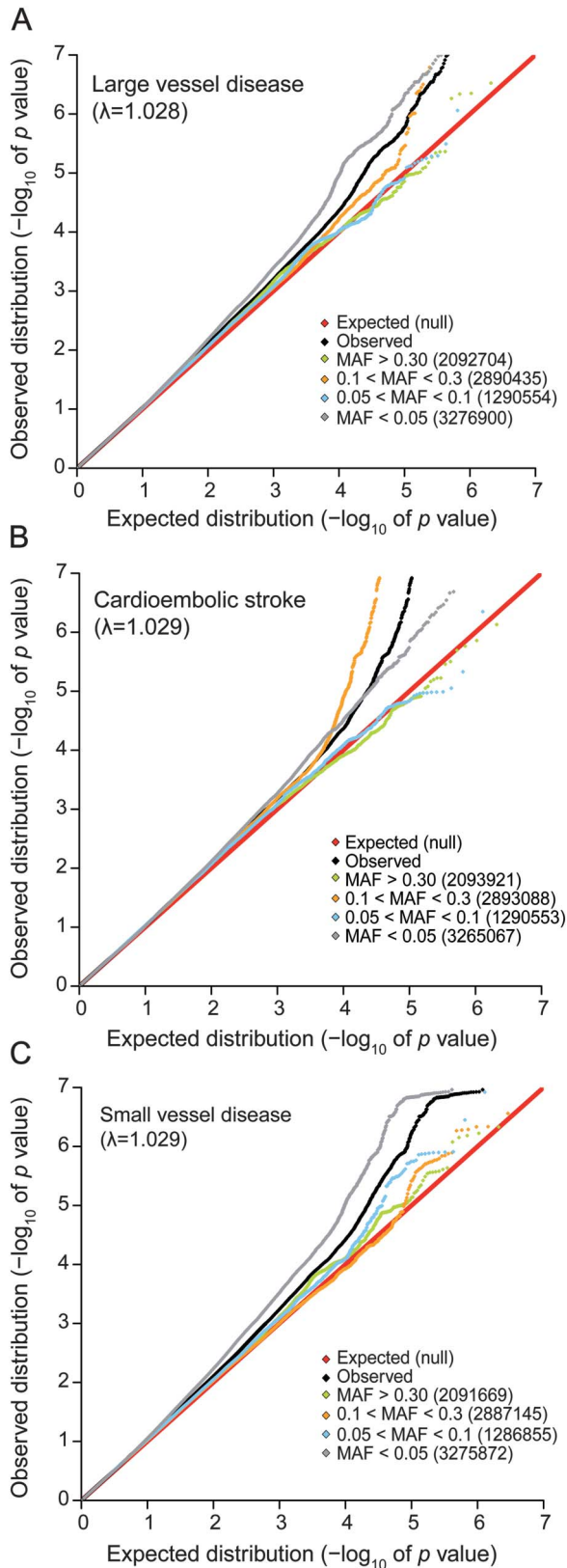
Shown is the distribution of p values for different allele frequency bins: (A) large vessel disease, (B) cardioembolic stroke, (C) small vessel disease. The red line displays the expected (null) distribution of the statistic. The black line shows the observed distribution of all variants studied. Frequency bins are depicted in different colors: green (>30% minor allele frequency [MAF]), orange (10%–30% MAF), blue (5%–10% MAF), and gray (1%–5% MAF). The number in parentheses shows the number of SNPs that were included in the respective bins. Statistical significance was tested using a 2-sample Kolmogorov-Smirnov test.[28]

moderate LD with the previously published variant (rs505922, $r^2 = 0.53$, figure e-7A). In contrast, we found the lead SNPs for *HDAC9* and *ZHFX* to be identical or in high LD ($r^2 > 0.9$) with the lead SNPs reported by prior studies (rs2107595 for *HDAC9*, and rs879324 for *ZFHX3*, figure e-7, B and D; table 1). Functional annotation of all lead SNPs is presented in table e-4.

Role of allele frequency bins. To study the contribution of low-frequency alleles and common alleles to individual stroke subtypes, we further investigated the *p* value distribution across bins of variants categorized according to their minor allele frequencies (>30%; 10–30%; 5–10%; <5%) for all IS and stroke subtypes (figure 2). There was no enrichment of specific bins of allele frequencies for all IS. However, we found an enrichment in low-frequency variants (<5%) for both LVD and SVD. In contrast, CE showed an enrichment of variants between 10% and 30%. The enrichment of these specific variant bins was significant when compared to all SNPs or any other frequency bin using a 2-sample Kolmogorov-Smirnov test (all $p < 1E-5$).[28] Of note, the distribution of observed vs expected *p* values was well-behaved in all analyses (figure 2) and we did not observe a systematic bias towards low-frequency variants that could have been introduced through imputation artifacts.

Pathway analysis. Applying a Bonferroni corrected threshold of $p < 0.05$ we found several pathways for all IS and IS subtypes (table e-5). In total, there were 136 nominally associated pathways for CE, 84 for all IS, 86 for LVD, and 55 for SVD. The following terms showed the highest degree of association: germ cell development (CE), microtubule (IS), mitochondrial envelope (LVD), and SH3 domain binding (SVD). When using a predefined FDR cutoff of q < 0.05, we observed a single association of natural killer (NK) cell signaling with all IS.

DISCUSSION Adopting a classical GWAS approach based on 1 KG imputed data with replication in both de novo and in silico genotype data, we found no novel locus reaching genome-wide significance for ischemic stroke or its subtypes. However, for the first time, we report genome-wide significance for association of the ABO locus with all IS and were able to fine-map 2 known stroke loci (PITX2 and ABO) by making use of the expanded 1 KG panel. We further found enrichment of association of

Table 1 Results for previously established risk loci for IS

Lead SNP	Locus	Phenotype	Discovery p value	Discovery odds ratio (95% CI)
rs17696736[21]	12q24	IS	1.13E-5	1.09 (1.05–1.13)
rs1242579[26]	NINJ2	IS	0.4196	0.98 (0.93–1.03)
rs2107595[14]	HDAC9	LVD	2.99E-11	1.39 (1.26–1.53)
rs556621[6]	6p21	LVD	5.83E-5	1.18 (1.09–1.27)
rs2383207[23]	9p21	LVD	1.09E-4	0.87 (0.80–0.93)
rs2200733[24]	PITX2	CE	2.51E-6	1.30 (1.17–1.46)
rs879324[25]	ZFHX3	CE	1.96E-7	1.29 (1.17–1.42)
rs505922[22]	ABO	IS	5.4E-4	1.08 (1.03–1.12)
		LVD	0.0011	1.14 (1.05–1.23)
		CE	0.0013	1.13 (1.05–1.22)

Abbreviations: CE = cardioembolic stroke; CI = confidence interval; IS = ischemic stroke; LVD = large vessel disease; SNP = single nucleotide polymorphism.
Results for the METASTROKE discovery meta-analysis are shown for the lead SNPs reported by previous studies. The effect direction of the odds ratio is given in the direction of the minor allele.

low-frequency alleles with both LVD and SVD, and of higher frequency variants (10%–30%) with CE. This finding has important implications for future studies, as design and analysis strategies may differ for low-frequency and common variants.

ABO has previously been shown to be genome-wide associated with circulating levels of von Willebrand factor and factor VIII.[22] An assessment of these signals in IS cohorts showed a nominal replication for the lead SNP rs505922 in LVD and CE, but not for SVD.[22] Our findings extend this observation by demonstrating that a different variant in the *ABO* gene (rs532436, $p = 4.30E-8$) is genome-wide associated with all IS and that the association with ABO is strictly confined to LVD ($p = 0.0029$) and CE ($p = 0.0011$) with no signal with SVD ($p = 0.53$). Together, these findings emphasize a role of ABO in thrombosis and associated stroke phenotypes.

Although not reaching genome-wide significance, there are 3 novel loci that deserve attention. *GUCY1A3*, which showed suggestive association with LVD ($p = 8.25E-6$) in the current study, has recently been reported as a risk gene for early-onset myocardial infarction in a family-based study.[29] The allele frequency of the lead SNP in our study was 1.5%. Thus, the low-frequency nature of this variant together with the lower power for association detection in LVD might have hindered our ability to detect a genetic association.

The second locus is *TNFSF11* (*RANKL*), which showed suggestive evidence for association with CE ($p = 1.03E-7$). The allele frequency of the lead SNP in our study was 24%. *TNFSF11*, a major player in bone remodeling and part of the *RANK/RANKL/ OPG* pathway, has repeatedly been reported in the pathogenesis of AF and as a predictor of IS in patients with nonvalvular AF.[30–32] Of note, however, variants in or near this gene have not emerged from prior GWAS of AF, thus highlighting the need for sample expansion in future GWAS.

The third locus is *GCH1*, which showed a p value of 3.31E-5 for association with SVD. The allele frequency of the lead SNP in our study was 1.5%. *GCH1* encodes for GTP cyclohydrolase 1, a rate-limiting factor in the tetrahydrobiopterin (BH4) biosynthesis.[33] BH4 is an essential cofactor for nitric oxide (NO) synthases in endothelial cells and has been shown to enhance NO bioavailability.[34] Supplementation with a synthetic BH4 analog has previously been tested in a trial in monogenic SVD.[35]

Aside from *GUCY1A3* and *GCH1*, we found other low-frequency variants with p values $< 1E-4$ for association and consistent effect directions across all samples. These include *NACC2* as well as an intergenic locus near *KCNN2* (both LVD), *TMEM108* (SVD), and *CBFA2T3* (CE). More work is needed to determine the potential role of these low-frequency variants in stroke subtypes. Our findings on low-frequency variants together with the observed enrichment of association of low-frequency alleles with both LVD and SVD supports the notion that parts of the missing heritability in IS are explained by rare and low-frequency variation. Next-generation sequencing studies and targeted resequencing efforts of known risk loci together with larger sample sizes for stroke subtypes are needed to capture this missing heritability and to depict the heritability of ischemic stroke more precisely.

Previous GWAS have revealed that associations with ischemic stroke are largely confined to etiologic

Table 2 Meta-analysis results for all IS and subtypes: Listed are all SNPs reaching genome-wide significance in the transethnic meta-analysis

SNP	Locus	Phenotype	Effect allele	Effect allele frequency in the discovery sample	Discovery p value	Discovery odds ratio (95% CI)	Caucasian replication p value	Caucasian replication odds ratio (95% CI)	RACE replication p value	RACE replication odds ratio (95% CI)	Overall meta-analysis p value	Overall meta-analysis odds ratio (95% CI)
rs532436	ABO	IS	A	0.1936	3.6E-6	1.12 (1.07–1.18)	0.0004399	1.08 (1.03–1.13)	0.9887	1.00 (0.89–1.11)	4.3E-8	1.09 (1.05–1.12)
rs2107595	HDAC9	LVD	A	0.1642	3E-11	1.39 (1.26–1.53)	NA	NA	0.3248	1.09 (0.92–1.31)	2.5E-10	1.31 (1.21–1.43)
rs2723334	PITX2	CE	T	0.2141	1.6E-8	1.30 (1.18–1.42)	3.4E-18	1.33 (1.25–1.42)	0.3549	1.08 (0.92–1.26)	8.4E-24	1.29 (1.23–1.36)
rs12932445	ZFHX3	CE	T	0.8291	1.3E-7	1.29 (1.18–1.42)	0.001805	0.88 (0.82–0.95)	NA	NA	1.2E-8	0.84 (0.79–0.89)

Abbreviations: CE = cardioembolic stroke; CI = confidence interval; IS = ischemic stroke; LVD = large vessel disease; RACE = Risk Assessment of Cardiovascular Events; SNP = single nucleotide polymorphism. Results are shown for the discovery sample, the Caucasian and South Asian replication samples, and the final transethnic meta-analysis. Results are sorted by stroke phenotype and meta-analysis p value. NA signifies that no information on that SNP was available for quality control reasons.

stroke subtypes. We found the ABO locus to be associated with all IS on a genome-wide level, which is primarily due to its association with LVD and CE. Another major locus that has been reported to be associated with multiple stroke subtypes is the chr12q24 region, which has recently been shown to be implicated in LVD, CE, and SVD.[21] New results, however, show association restricted to SVD without evidence for association in any other subtype.[12] Conceivably, shared associations in conjunction with subtype-specific signals may provide insights into stroke mechanisms.

Our pathway analysis revealed several novel pathways for all IS and etiologic stroke subtypes. The strongest association was seen for all IS and NK cell signaling. It was recently shown that NK cells promote neuronal death in experimental stroke.[36] However, additional work is needed to fully explore the role of this and other candidate pathways in IS. Combining pathway analysis with more detailed phenotyping may provide further insight into specific stroke subtypes. It is well-known that genes do not act in isolation, but rather in complex molecular networks that are often involved in disease susceptibility and progression. Pathway analysis has promise in other diseases like coronary artery disease[37] where canonical pathways like inflammation and lipid metabolism had been identified as key players in disease development. This information is highly valuable in a context of mechanistic and functional studies to elucidate the biological processes in disease development. Further, it also provides potential mechanisms in gene–environment interactions, which are mostly unexplored in the cardiovascular disease context. An additional point to consider is the potential use of such pathways in the discovery of biomarkers. Finally, it may provide researchers with therapeutic targets that could ultimately prove to be of high relevance.

A methodologic strength of our approach is the replication of signals in a wet laboratory environment with de novo genotyping in addition to in silico replication. Prior results have shown that signals confirmed in an in silico setting may not necessarily replicate in a de novo genotyping environment.[38,39] Hence, we have minimized the risk of false-positive reporting by our study design. By integrating genome-wide data from non-Caucasian populations and performing a transethnic meta-analysis, we maximized the chance of detecting association signals across different ethnicities while preserving the power in our dataset. Thus for example, we saw nominally significant replication *p* values for *GUCY1A3* in LVD in the South Asian samples (*p* = 0.012), pointing towards shared risk in Caucasian and South Asian populations. Discoveries of both shared and ethnicity-specific genetic risk factors will be further

facilitated by recently completed GWAS studies in non-Caucasian populations.[40] In the pathway analysis, we made use of the MAGENTA software, which is tailored towards elucidating pathways in a GWAS setting. MAGENTA has been shown to be superior to other pathway analysis tools; for one, MAGENTA accounts for inherent difficulties in the assignment of SNP data to gene/gene products. It ensures that the results are comparable and that there is no inherent bias in the final outcome. Second, it accounts for important confounders on the association scores of genes and gene sets, which cannot be performed by other pathway analysis tools. Our study was limited by the relatively low power for detecting associations in stroke subtypes, especially for low-frequency variants and the heterogeneity of the imputation accuracy, particularly for lower frequency variants, which may have been introduced by decentralized imputation.

Aside from providing new insights into the genetic architecture of IS, this large meta-analysis of 1 KG imputed data provides a valuable resource for even larger meta-analyses with recently published GWAS[12] and provides additional insight into the genetic architecture of ischemic stroke. The complete summary statistics of this analysis are available upon request through the METASTROKE Web site (www.strokegenetics.org).

AUTHOR AFFILIATIONS

From the Institute for Stroke and Dementia Research, Klinikum der Universität München (R.M., M.D.), Ludwig-Maximilians University, Munich, Germany; Department of Clinical Neurosciences (M.T., S.B., H.S.M.), University of Cambridge, UK; Department of Medical Genetics (S.L.P.), Center for Molecular Medicine, University Medical Center Utrecht, the Netherlands; CTSU (J.C.H., R.C.), Nuffield Department of Population Health, University of Oxford, UK; Hunter Medical Research Institute (E.G.H., J.R.A.), Public Health Research Program, Newcastle, Australia; Department of Genetics (W.Z., D.S.), Perelman School of Medicine, University of Pennsylvania, PA; Instituto de Medicina Molecular (P. Abrantes, S.A.O.), Faculdade de Medicina, Universidade de Lisboa (J.M.F.); Instituto Gulbenkian de Ciência (IGC) (P. Abrantes, S.A.O., A.M.V.), Oeiras, Portugal; Inserm U1167 (P. Amouyel), Pasteur Institute Lille, France; Department of Public Health (P. Amouyel), Lille University Hospital, University of Lille Nord de France; Division of Neurocritical Care and Emergency Neurology (T.W.K.B., C.E.K., G.J.F., J.R.), Department of Neurology (T.W.K.B., G.J.F., C.E.K., J.R.), Center for Human Genetic Research (T.W.K.B., G.J.F., C.E.K., J.R.), and J. Philip Kistler Stroke Research Center (G.J.F.), Massachusetts General Hospital, Boston; Institute of Epidemiology and Social Medicine (K.B.), University of Münster, Germany; Department of Cerebrovascular Diseases (G.B.B.), Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy; Team Neuroepidemiology (G.C., S.D.), Inserm Research Center for Epidemiology and Biostatistics (U897), Bordeaux, France; University of Bordeaux (G.C., S.D.), France; Department of Medicine (Y.-C.C., B.D.M.), University of Maryland School of Medicine (S.J.K.), Baltimore; Center for Public Health Genomics and Cardiovascular Research Center (W.-M.C., B.B.W.), University of Virginia, Charlottesville; Institute of Cardiovascular Research Royal Holloway (I.C., P.S.), University of London (ICR2UL), UK; Program in Medical and Population Genetics (G.J.F., C.E.K., J.R.), Broad Institute, Cambridge, MA; Department of Neurosciences (J.M.F.), Neurology, Hospital Santa Maria, Lisbon, Portugal; Department of Neurology (D.M.G., J.F.M.),

Mayo Clinic, Jacksonville, FL; Department of Clinical Sciences Lund (A.I., A.L.), Neurology, Lund University, Sweden; Department of Neurology (S.J.K.), Veterans Administration Medical Center, Baltimore, MD; Department of Neurosciences, Experimental Neurology, and Leuven Research Institute for Neuroscience and Disease (LIND) (R.L., V.T.), KU Leuven, University of Leuven; Laboratory of Neurobiology (R.L., V.T.), VIB, Vesalius Research Center, Leuven, Belgium; Brain and Mental Health Program (C.R.L.), Hunter Medical Research Institute, Newcastle, Australia; Helmholtz Zentrum München and Technische Universität München (P.L.), Institut für Humangenetik, Munich, Germany; Fred Hutchinson Cancer Research Center (J.L., A.P.R.), Seattle, WA; Department of Neurology (J.P., A.S.), Jagiellonian University Medical College, Krakow, Poland; Stroke Prevention Research Unit (P.M.R.), Nuffield Department of Clinical Neurosciences, University of Oxford, UK; Ashford & St Peters NHS Foundation Trust (P.S.), Surrey; Division of Clinical Neurosciences (C.L.M.S.), University of Edinburgh, UK; Department of Neurology (V.T.), Austin Health, Heidelberg, Australia; Florey Institute for Neuroscience and Mental Health (V.T.), University of Melbourne, Heidelberg, Australia; Institute of Neuroscience and Physiology (T.T.), Sahlgrenska Academy at University of Gothenburg; Department of Neurology (T.T.), Sahlgrenska University Hospital, Gothenburg, Sweden; Departamento Promoção da Saúde e Doenças Crónicas (DPSDC) (A.M.V.), Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA), Lisbon; Center for Biodiversity, Functional & Integrative Genomics (BIOFIG) (A.M.V.), Lisbon, Portugal; Department of Neurology and Rehabilitation (D.W.), University of Cincinnati College of Medicine, OH; Department of Neurology (S.S.), Boston University School of Medicine, MA; Departments of Neurology and Public Health Sciences (B.B.W.), University of Virginia Health System, Charlottesville; and Munich Cluster for Systems Neurology (SyNergy) (M.D.), Munich, Germany.

AUTHOR CONTRIBUTIONS

analysis, accepts responsibility for conduct of research and final approval. Robert Clarke: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Ioana Cotlarciuc: drafting/revising the manuscript for content, analysis or interpretation of data, statistical analysis, accepts responsibility for conduct of research and final approval. Stephanie Debette: drafting/revising the manuscript for content, acquisition of data, accepts responsibility for conduct of research and final approval. Guido J. Falcone: drafting/revising the manuscript for content, analysis or interpretation of data, acquisition of data, statistical analysis, accepts responsibility for conduct of research and final approval. Jose M. Ferro: acquisition of data, accepts responsibility for conduct of research and final approval. Dale M. Gamble: analysis or interpretation of data, statistical analysis, accepts responsibility for conduct of research and final approval. Andreea Ilinca: acquisition of data, accepts responsibility for conduct of research and final approval. Steven J. Kittner: drafting/revising the manuscript for content, acquisition of data, accepts responsibility for conduct of research and final approval. Christina E. Kourkoulis: acquisition of data, accepts responsibility for conduct of research and final approval. Robin Lemmens: acquisition of data, accepts responsibility for conduct of research and final approval. Christopher R. Levi: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Peter Lichtner: acquisition of data, statistical analysis, accepts responsibility for conduct of research and final approval. Arne Lindgren: drafting/revising the manuscript for content, acquisition of data, accepts responsibility for conduct of research and final approval. Jingmin Liu: analysis or interpretation of data, acquisition of data, statistical analysis, accepts responsibility for conduct of research and final approval. James F. Meschia: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Braxton D. Mitchell: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Sofia A. Oliveira: acquisition of data, accepts responsibility for conduct of research and final approval. Joana Pera: drafting/revising the manuscript for content, acquisition of data, statistical analysis, accepts responsibility for conduct of research and final approval. Alex P. Reiner: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Peter M. Rothwell: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Pankaj Sharma: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Agnieszka Slowik: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Cathie L.M. Sudlow: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Turgut Tatlisumak: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Vincent Thijs: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Astrid M. Vicente: acquisition of data, statistical analysis, accepts responsibility for conduct of research and final approval. Daniel Woo: acquisition of data, accepts responsibility for conduct of research and final approval. Sudha Seshadri: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Danish Saleheen: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Jonathan Rosand: drafting/revising the manuscript for content, study concept or design, acquisition of data, study supervision or coordination, accepts responsibility for conduct of research and final approval. Hugh S. Markus: drafting/revising the manuscript for content, study concept or design, acquisition of data, study supervision or coordination, accepts responsibility for conduct of research and final approval. Bradford B. Worrall: drafting/revising the manuscript for content, study concept or design, acquisition of data, accepts responsibility for conduct of research and final approval. Martin Dichgans: drafting/revising the

manuscript for content, study concept or design, acquisition of data, study supervision or coordination, accepts responsibility for conduct of research and final approval.

DISCLOSURE

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