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Genetic variants influencing elevated myeloperoxidase levels increase risk of stroke

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Primary intracerebral haemorrhage and lacunar ischaemic stroke are acute manifestations of progressive cerebral microvascular disease. Current paradigms suggest atherosclerosis is a chronic, dynamic, inflammatory condition precipitated in response to endothelial injury from various environmental challenges. Myeloperoxidase plays a central role in initiation and progression of vascular inflammation, but prior studies linking myeloperoxidase with stroke risk have been inconclusive. We hypothesized that genetic determinants of myeloperoxidase levels influence the development of vascular instability, leading to increased primary intracerebral haemorrhage and lacunar stroke risk. We used a discovery cohort of 1409 primary intracerebral haemorrhage cases and 1624 controls from three studies, an extension cohort of 12 577 ischaemic stroke cases and 25 643 controls from NINDS-SiGN, and a validation cohort of 10 307 ischaemic stroke cases and 29 326 controls from METASTROKE Consortium with genome-wide genotyping to test this hypothesis. A genetic risk score reflecting elevated myeloperoxidase levels was constructed from 15 common single nucleotide polymorphisms identified from prior genome-wide studies of circulating myeloperoxidase levels ($P < 5 \times 10^{-6}$). This genetic risk score was used as the independent variable in multivariable regression models for association with primary intracerebral haemorrhage and ischaemic stroke subtypes. We used fixed effects meta-analyses to pool estimates across studies. We also used Cox regression models in a prospective cohort of 174 primary intracerebral haemorrhage survivors for association with intracerebral haemorrhage recurrence. We present effects of myeloperoxidase elevating single nucleotide polymorphisms on stroke risk per risk allele, corresponding to a one allele increase in the myeloperoxidase increasing genetic risk score. Genetic determinants of elevated circulating myeloperoxidase levels were associated with both primary intracerebral haemorrhage risk (odds ratio, 1.07, $P = 0.04$) and recurrent intracerebral haemorrhage risk (hazards ratio, 1.45, $P = 0.006$). In analysis of ischaemic stroke subtypes, the myeloperoxidase increasing genetic risk score was strongly associated with lacunar subtype only (odds ratio, 1.05, $P = 0.0012$). These results, demonstrating that common genetic variants that increase myeloperoxidase levels increase risk of primary intracerebral haemorrhage and lacunar stroke, directly implicate the myeloperoxidase pathway in the pathogenesis of cerebral small vessel disease. Because genetic variants are not influenced by environmental exposures, these results provide new support for a causal rather than bystander role for myeloperoxidase in the progression of cerebrovascular disease. Furthermore, these results support a rationale for chronic inflammation as a potential modifiable stroke risk mechanism, and suggest that immune-targeted therapies could be useful for treatment and prevention of cerebrovascular disease.

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Abbreviations: GRS = genetic risk score; SNP = single nucleotide polymorphism

Introduction

Primary intracerebral haemorrhage and lacunar stroke result from vascular disease involving small cerebral perforating arteries and arterioles, and constitute acute clinical manifestations of the highly prevalent pathological entity of cerebral small vessel disease (Pantoni, 2010). Primary intracerebral haemorrhage and lacunar stroke, collectively representing small vessel-related strokes, comprise approximately one-third of stroke cases (Sacco *et al.*, 2006; Qureshi *et al.*, 2009; Tsai *et al.*, 2013), but account disproportionately towards higher stroke-related mortality and morbidity (Qureshi *et al.*, 2009; Tsai *et al.*, 2013). Preventative strategies that involve controlling vascular risk factors have limited effects on risk reduction for these small vessel-related strokes (ten Dam *et al.*, 2005; The SPS3 Investigators, 2012). Indeed, small vessel strokes seem to demonstrate discrete differences in risk factor profiles (Khan *et al.*, 2007), suggesting a pathophysiology distinct from large vessel atherosclerosis (Wardlaw *et al.*, 2013; Pogessi *et al.*, 2016). Consistent with this, genome-wide association (GWAS) and candidate gene studies have identified genetic variants associated specifically with small vessel-related stroke risk (Biffi *et al.*, 2010; Traylor *et al.*, 2016, 2017). Growing evidence supports a prominent role for inflammation (Kamel and Iadecola, 2012; Ma *et al.*, 2014), as a driver towards endothelial failure and resultant neurovascular unit dysfunction, and suggests that susceptibility to inflammation-mediated injury in conjunction with atherosclerotic risk factors may underlie the vascular changes leading to primary intracerebral haemorrhage and lacunar stroke (Thompson and Hakim, 2009; Pogessi *et al.*, 2016).

Myeloperoxidase (MPO) is an oxidizing lysosomal enzyme that can disrupt major endothelial functions necessary for maintaining vascular homeostasis (Pogessi *et al.*, 2016) including regulation of vasomotor tone (Rudolph

et al., 2012), fibrinolysis and coagulation pathways (Pawlus *et al.*, 2010), modulation of inflammatory reactions (Lau and Baldus, 2006), and angiogenesis, vessel repair and remodelling (Kim and Byzova, 2014). However, evidence from prospective population-based studies examining circulating MPO levels has been inconclusive in demonstrating a pathogenic role of MPO in cerebral small vessel disease (Kaneski *et al.*, 2006; Wright *et al.*, 2009; Shoamanesh *et al.*, 2015), which parallels the lack of consistent outcomes seen in association studies of other circulating inflammatory biomarkers (Pogessi *et al.*, 2016). Measurements of these inflammatory markers in peripheral blood are inconsistent, fluctuate over time, and can be influenced by a multitude of environmental factors (Zoccali *et al.*, 2006; Ebrahim and Davey Smith, 2008). In contrast, genetic variants that influence inflammatory biomarkers remain constant throughout life, are less subject to environmental and medical confounding, and are likely to influence long-term exposures to inflammation (Ebrahim and Davey Smith, 2008).

Well-powered genetic studies have made substantial progress in identifying genetic determinants of circulating MPO levels (Reiner *et al.*, 2013). We used these identified genetic variants collectively in a genetic risk score (GRS) analysis to provide new support for observed relationship between circulating MPO levels and small vessel-related stroke (primary intracerebral haemorrhage and lacunar stroke). We applied the GRS generated from previous GWAS of MPO levels to individual level genome-wide genotype data and summary statistics from the International Stroke Genetics Consortium (ISGC), Stroke Genetics Network (NINDS-SiGN), and METASTROKE Consortium to determine whether genetic predisposition towards high MPO levels was associated with stroke risk.

Materials and methods

Study design and participating studies

We performed a multi-phase study using a case-control methodology to determine whether genetic variants affecting circulating MPO levels were associated with risk of primary intracerebral haemorrhage and lacunar stroke, and a case-only time-to-event design to test for association between MPO-associated single nucleotide polymorphisms (SNPs) and recurrent intracerebral haemorrhage risk.

All primary intracerebral haemorrhage study populations have been previously described in detail (Anderson *et al.*, 2016). In the discovery phase, genotype and phenotype data for primary intracerebral haemorrhage were drawn from three GWAS of intracerebral haemorrhage: the North American (USA) multi-centre Genetics of Cerebral Haemorrhage on Anticoagulation (GOCHA) study, Genetic and Environmental Risk Factors for Haemorrhagic Stroke (GERFHS), and the European member sites contributing to the International Stroke Genetics Consortium (ISGC) Genome-Wide Association Study of Intracerebral Haemorrhage (Woo *et al.*, 2014). Cases and controls were enrolled by previously described methods (Anderson *et al.*, 2016). Primary intracerebral haemorrhage was defined as new, acute (<24 h) neurological deficits with presence of intraparenchymal haemorrhage on brain imaging. Exclusions include brain tumour, haemorrhagic transformation of cerebral infarction, trauma, vascular malformation, and other causes of secondary intracerebral haemorrhage. Briefly, enrolled cases were patients >18 years old presenting to the emergency department of participating institutions with neuroimaging-confirmed acute primary intracerebral haemorrhage. Stroke neurologists or neuroradiologists at individual participating sites performed the neuroimaging assessments. Primary intracerebral haemorrhage was classified by location based on known differences in underlying biology; lobar intracerebral haemorrhage was defined as intracerebral haemorrhage involving cortical-subcortical regions, while non-lobar intracerebral haemorrhage was restricted to haemorrhages involving thalamus, internal capsule, basal ganglia, brainstem or cerebellum. Controls were age-matched, intracerebral haemorrhage-free individuals confirmed by interview and medical record review, who were recruited by random selection at ambulatory clinics from the same population as the cases. An exception was the GERFHS study, which used random digit dialling for control sampling (Woo *et al.*, 2014).

For the extension study in lacunar stroke, we used data from NINDS-SiGN consisting of ischaemic stroke cases and controls derived from 10 previously published populations (Table 2) (Meschia *et al.*, 2013). We then performed replication of the association in lacunar stroke using summary statistics derived from the METASTROKE study, which consists of combined data from 15 GWAS of ischaemic stroke (Traylor *et al.*, 2012). All ischaemic stroke cases were subtyped using the TOAST classification criteria (Adams *et al.*, 1993), including large artery stroke (LAS), cardioembolic stroke (CE), and small vessel-related stroke (SV). We restricted our analysis to individuals of European ancestry from North America, Europe, and Australia. To the best of our knowledge, we removed overlap between individuals in the extension and replication datasets.

For the time-to-recurrent intracerebral haemorrhage study, we used patients who were enrolled in an ongoing longitudinal cohort study of primary intracerebral haemorrhage at Massachusetts General Hospital (Biffi *et al.*, 2015). Individuals selected for the present study were consecutive patients presenting to Massachusetts General Hospital from 1 January 1995 to 31 December 2010 with (i) age >18 years old; (ii) imaging-confirmed diagnosis of primary intracerebral haemorrhage; and (iii) available GWAS data. Longitudinal follow-up involved telephone-based interviews of either the patients and/or their caregivers at 6-monthly intervals after index intracerebral haemorrhage by trained study staff for data collection regarding intracerebral haemorrhage recurrence, death and medication use. Manual review of hospital electronic medical records was also performed where available, to obtain/confirm interval medical history and medication use.

The local institutional review board or ethics committee at each participating institution approved all studies. Informed consent was obtained from all subjects, their legally authorized representatives, or waived via protocol-specific allowance.

Exposure: myeloperoxidase increasing genetic risk score

We used a 'top-hits' strategy to construct our MPO genetic risk score. Eighteen SNPs highly associated with circulating MPO levels were identified from a recent, comprehensive meta-analysis of prior population-based GWAS and gene-centric data (Reiner *et al.*, 2013). We used SNP Annotation and Proxy Search (SNAP) (Johnson *et al.*, 2008) to calculate linkage disequilibrium between SNPs (with reference to 1000 Genomes) and to search for proxy SNPs where index SNPs were absent. Within the identified SNPs, we selected SNPs that were independent ($r^2 < 0.2$), common (minor allele frequency > 0.01), and highly associated with circulating serum or plasma MPO levels ($P < 5 \times 10^{-6}$) (rs2814778, rs800292, rs12049351, rs2144300, rs9332739, rs3134931, rs1390943, rs12940923, rs2680701, rs9911753, rs8081967, rs6503905, rs28730837, rs35897051, rs6042507). Three SNPs, which did not meet the above criteria, were excluded. These selected SNPs were ascertained in the present study by genome-wide genotyping followed by imputation. Genotyping, quality control and imputation processes for all the study populations were previously described (Traylor *et al.*, 2012; Meschia *et al.*, 2013; Woo *et al.*, 2014). Post-imputation, SNPs with low quality imputation scores (confidence score < 0.9), minor allele frequency < 0.01, and $r^2 < 0.5$ were removed. Detailed information of SNPs included in the final MPO genetic risk score are provided in Supplementary Table 1.

We constructed an unweighted, allele-count based MPO-increasing GRS (Smith *et al.*, 2015). The unweighted GRS uses equal weighting of each risk allele, functioning as a simple risk allele count of MPO-increasing alleles under the conservative assumption that all alleles have the same effect. We selected for the other (major) risk allele if the SNPs were reported to have minor alleles associated with lower circulating MPO levels in order to establish a concordant effect direction (increasing serum or plasma MPO levels). Accordingly, this MPO GRS approximating the aggregate burden of genetic variants associated with increased circulating MPO levels was calculated for each patient using an additive genetic model, and represented the summation of each SNP included in the GRS calculation weighted by 0, 1, or 2, corresponding to the number of risk alleles present (Smith *et al.*, 2015). In addition, we also attempted to quantify the magnitude of effect of our MPO genetic risk score on peripheral MPO levels by estimating the change in circulating MPO levels per allele using the average calculated from individual allele-associated serum measurements obtained from population-level data (Reiner *et al.*, 2013).

Statistical analyses

Genetic risk score association analysis

We tested the resulting MPO genetic risk scores for association with primary intracerebral haemorrhage and lacunar stroke risk. To account for population structure, we analysed only individuals of European ancestry and performed principal component analysis using genome-wide data (Price *et al.*, 2006). Population outliers were excluded by visual inspection of these principal component plots, and principal components were recomputed after outlier removal. The MPO GRS associated with elevated circulating MPO levels was modelled as the independent variable in multivariable logistic regression models for both primary intracerebral haemorrhage and ischaemic stroke subtypes, adjusting for age, sex, and all available principal components (maximum of five) in the study cohorts. Separate analyses were performed for lobar and non-lobar primary intracerebral haemorrhage, and for ischaemic stroke subtype according to TOAST criteria (Adams *et al.*, 1993). In all models, the MPO GRS was used as a continuous variable where the effect estimate (beta coefficient) could be interpreted as the increase in risk of outcome (either primary intracerebral haemorrhage or lacunar stroke) per presence of each additional MPO-increasing risk allele. Finally, we performed fixed-effects, inverse-variance weighted meta-analysis for each phenotype of interest to pool effect estimates across the relevant studies (Evangelou and Ioannidis, 2013). Heterogeneity was assessed using Cochrane's Q (with corresponding *P)* and I^2 (as a percentage of the effect size attributable to heterogeneity). Statistical significance was set at $P < 0.05$ for genetic score association testing in the discovery, extension and replication datasets, all of which represented two-tailed, independent tests. Genotype quality control and genetic score calculations were performed using PLINK v1.07 (Purcell *et al.*, 2007), imputation of ungenotyped SNPs was performed using IMPUTE2 (Howie *et al.*, 2011), and all other analyses were performed with R software version 3.1 (The R Foundation for Statistical Computing).

Genetic risk score association with recurrent intracerebral haemorrhage

We obtained longitudinal follow-up on 1051 primary intracerebral haemorrhage patients, of which 181 individuals with genome-wide data available were eligible for our study based on aforementioned selection criteria. We considered primary outcome as recurrent intracerebral haemorrhage. Patients' data were censored at (i) date of first neuroimaging-confirmed recurrent intracerebral haemorrhage; (ii) death; and (iii) date of last recorded follow-up. Age at index intracerebral haemorrhage was reported as a continuous variable, while patients' sex, ethnicity, pre-intracerebral haemorrhage prior medical history, medication exposure, and intracerebral haemorrhage locations were analysed as categorical variables. We performed comparisons of categorical variables using Fisher's exact test and continuous variables using unpaired *t*-test, as appropriate. We modelled the calculated MPO GRS as a continuous variable and as tertiles (representing low, intermediate, and high circulating MPO levels) according to the absolute number of MPO-increasing risk alleles concomitantly carried by a given individual (<5, 5–9, and >9). Univariate predictors of intracerebral haemorrhage recurrence were determined by significance testing using log-rank test. Multivariable Cox regression analysis was used to determine association of increased MPO levels with recurrent intracerebral haemorrhage risk following index intracerebral haemorrhage (Raffeld *et al.*, 2015). We included all covariates that trended in association with recurrent intracerebral haemorrhage in univariate analysis ($P < 0.20$), followed by backward elimination of non-significant variables ($P > 0.05$) to generate the final minimal model. Survival function was adjusted for age at index intracerebral haemorrhage, and lobar location of

haemorrhage. All analyses were performed using STATA v10.0 (StataCorp LP, USA).

Results

The primary intracerebral haemorrhage dataset consisted of 3033 individuals: 1543 intracerebral haemorrhage cases and 1490 intracerebral haemorrhage-free controls. Demographic and clinical characteristics of the intracerebral haemorrhage study populations are presented in Table 1. The MPO GRS frequency was approximately normal in distribution (Fig. 1). Estimates from population-level data suggest that each MPO increasing allele in our score is associated with a mean increase in peripheral MPO levels of 1.12 ± 0.10 pmol/l. The number of risk alleles per individual (associated with increased MPO levels) ranged between two to 12, while the average number of risk alleles per individual was 6.34 ± 2.06 in intracerebral haemorrhage-free controls and 6.35 ± 2.07 in intracerebral haemorrhage participants. We extended our genetic score analyses to all-cause ischaemic stroke and small vessel subtype among 12 577 ischaemic stroke cases and 25 643 stroke-free controls of European ancestry from 10 case-control group strata in NINDS-SiGN (Table 2). A separate dataset from the METASTROKE Consortium, comprising 10 307 ischaemic stroke cases and 19 326 stroke-free controls of European ancestry served as a replication sample.

Table 1 Characteristics of primary intracerebral haemorrhage cohort by study population

	GOCHA (*n* = 774)	GERFHS (*n* = 1201)	ISGC-ICH (*n* = 1058)
Number of cases	387	628	528
Females, *n* (%)	179 (46.3)	314 (50.0)	240 (45.5)
Mean age, years	73.8	68.9	71.5
Lobar ICH, *n* (%)	210 (54.3)	258 (41.1)	182 (34.5)
Non-lobar ICH, *n* (%)	167 (43.1)	370 (58.9)	313 (59.3)
Number of controls	387	573	530
Females, *n* (%)	194 (50.1)	285 (49.7)	260 (49.1)
Mean age, years	72.4	67.5	66

GOCHA = Genetics of Cerebral Haemorrhage on Anticoagulation Study; GERFHS = Genetic and Environmental Risk Factors for Haemorrhagic Stroke Study; ISGC-ICH = International Stroke Genetics Consortium Intracerebral Haemorrhage Study; ICH = intracerebral haemorrhage.

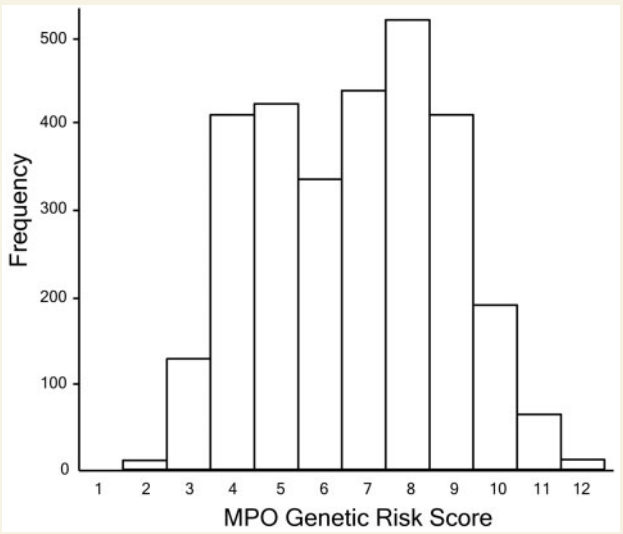

Figure 1 Distribution of MPO GRS in primary intracerebral haemorrhage cohorts.

Genetic determinants of elevated myeloperoxidase levels increase intracerebral haemorrhage risk

Genetic score-based analysis demonstrated an association between MPO-increasing GRS and primary intracerebral haemorrhage, where presence of each additional risk allele increased all intracerebral haemorrhage risk by 7% [meta-analysis odds ratio (OR) 1.07, 95% confidence interval (CI) 1.00–1.14, $P = 0.04$] (Fig. 2). There was no heterogeneity in effect estimates observed across studies ($I^2 = 0\%$, Q-$P = 0.55$). On stratification by location, restriction to lobar intracerebral haemorrhage demonstrated an 11% increase in risk of intracerebral haemorrhage with each additional MPO-increasing risk allele (meta-analysis OR 1.11, 95% CI 1.02–1.18, $P = 0.01$) while no association was found between MPO-increasing GRS and non-lobar location of intracerebral haemorrhage (meta-analysis OR 1.03, 95% CI 0.95–1.10, $P = 0.47$).

Genetic determinants of elevated myeloperoxidase levels increase recurrent intracerebral haemorrhage risk

One hundred and eighty-one patients with primary intracerebral haemorrhage were eligible for the longitudinal intracerebral haemorrhage cohort study. Seven individuals were excluded from analysis due to discrepancies between telephone-collected and electronic medical record-collected data. There was no significant difference in baseline characteristics for the excluded individuals (data not shown). Baseline demographic and clinical characteristics of the final analysis cohort are summarized in Table 3. Among the 174 intracerebral haemorrhage survivors, the overall incidence of new fatal and non-fatal intracerebral haemorrhage was 16.6% (29 events) during a median follow-up period of 52.9 months [interquartile range (IQR) 22.2–83.1] corresponding to an incidence rate of 1.6% per year. Only lobar location of index intracerebral haemorrhage and MPO-increasing GRS were associated with intracerebral haemorrhage recurrence in univariate analysis.

Cox proportional hazards analyses were performed to assess the relative risk for intracerebral haemorrhage recurrence according to the MPO-increasing GRS. Initial multivariable analysis included univariate predictors of intracerebral haemorrhage recurrence (Table 4). After correcting for age and lobar location of index intracerebral haemorrhage, individuals who possess a greater number of MPO risk alleles had increased risk of future intracerebral haemorrhage events (Fig. 3). Each additional MPO-increasing risk allele was associated with increased risk of intracerebral haemorrhage recurrence [hazard ratio (HR) 1.49, 95% CI 1.12–1.98, $P = 0.006$].

Genetic determinants of elevated myeloperoxidase levels increase small vessel ischaemic stroke risk

Genetic score-based analysis demonstrated a weak association between ischaemic stroke and MPO-increasing GRS (meta-analysis OR 1.01, 95% CI 1.00–1.02, $P = 0.02$) within the NINDS-SiGN cohort. Among ischaemic stroke subtypes, only small vessel ischaemic stroke subtype was associated with MPO-increasing GRS (meta-analysis OR 1.02, 95% CI 1.00–1.03, $P = 0.03$) with no heterogeneity observed across studies ($I^2 = 0\%$, Q-$P = 0.69$). There were no associations observed with either large artery atherosclerosis (LAA) or cardioembolic (CE) subtypes ($P = 0.86$ and $P = 0.39$, respectively) (Fig. 4).

Replication of the MPO-increasing GRS association with increased lacunar/small vessel ischaemic stroke risk was performed in a separate dataset using summary statistics from the METASTROKE Consortium. MPO-increasing GRS was similarly also strongly associated with increased risk of lacunar stroke (meta-analysis OR 1.05, 95% CI 1.02–1.08, $P = 0.0012$). There was no association observed with large artery stroke (meta-analysis OR 0.98, 95% CI 0.95–1.00, $P = 0.08$) and weak association with cardioembolic stroke subtypes (meta-analysis OR 1.03, 95% CI 1.01–1.06, $P = 0.01$).

Discussion

In this pathway-based genetic association study, we used a GRS approach to investigate the role of inflammatory pathways promoting MPO release in pathogenesis of small vessel-related stroke represented by primary intracerebral haemorrhage and lacunar stroke. Our results provide

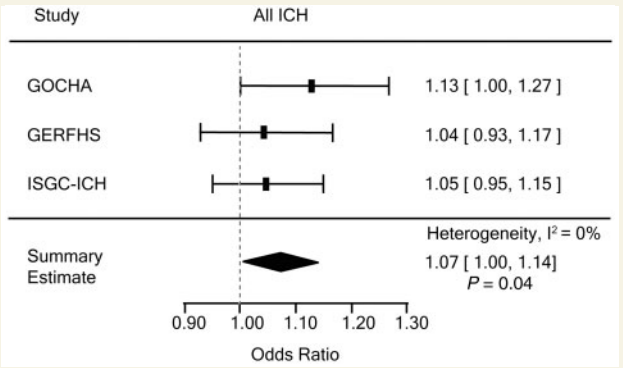

Figure 2 MPO GRS and risk of intracerebral haemorrhage. MPO GRS meta-analysis results for each additional trait-associated allele. GOCHA = Genetics of Cerebral Haemorrhage on Anticoagulation Study; GERFHS = Genetic and Environmental Risk Factors for Haemorrhagic Stroke Study; ISGC-ICH = International Stroke Genetics Consortium Intracerebral Haemorrhage Study.

Table 2 Case and control cohorts, and by ischaemic stroke subtype in NINDS-SiGN

Study stratum	Cohorts	Controls	Cases	LAA	SV	CE
EUR 1	BRAINS, ISGS, GASROS, SWISS, HABC	1586	754	174	87	174
EUR 2	ESS, MUNICH, OXVASC, STGEORGE, WTCCC, KORA	5954	2,572	431	320	755
EUR 3	GEOS	519	460	68	58	31
EUR 4	CIDR[a], HRS, OAI	11 820	3291	728	866	868
EUR 5	KRAKOW	716	878	77	97	381
EUR 6	LSGS	453	459	104	34	127
EUR 7	BASICMAR, ADHD, INMA	1218	868	236	266	385
EUR 8	GRAZ	815	607	142	72	225
EUR 9	SAHLSIS, LSR, MDC	1362	1579	215	200	374
EUR 10	ASGC	1200	1109	54	29	80

[a]CIDR = BRAINS, GASROS, GCNKSS, ISGS, MCISS, MIAMISR, NHS, NOMAS, REGARDS, SPS3, SWISS, WHI, WUSTL.
ADHD = attention deficit hyperactivity disorder; ASGC = Australian Stroke Genetics Collaborative; BASICMAR = Base de Datos de Ictus del Hospital del Mar; BRAINS = Biorepository of DNA in Stroke; CE = cardioembolic; ESS = Edinburgh Stroke Study; GASROS = Genes Affecting Stroke Risk and Outcome Study; GCNKSS = Greater Cincinnati/Northern Kentucky Stroke Study; GEOS = Genetics of Early Onset Stroke; HABC = Health ABC; HRS = Health and Retirement Study; INMA = Infancia y medio ambiente; ISGS = Ischaemic Stroke Genetics Study; KORA = MONICA/KORA Ausburg Study; LAA = large artery atherosclerosis; LSGS = Leuven Stroke Genetics Study; LSR = Lund Stroke Registry; MCISS = Middlesex County Ischaemic Stroke Study; MDC = Malmo Diet and Cancer Study; MIAMISR = Miami Stroke Registry and Biorepository; NHS = Nurses' Health Study; NINDS-SiGN = National Institute of Neurological Disorders and Stroke Genetics Network; NOMAS = Northern Manhattan Study; OAI = Osteoarthritis Initiative; OXVASC = Oxford Vascular Study; REGARDS = Reasons for Geographic and Racial Differences in Stroke; SAHLSIS = Sahlgrenska Academy Study of Ischaemic Stroke; SPS3 = Secondary Prevention of Small Subcortical Strokes; STGEORGE = St George's Hospital; SV = small vessel; SWISS = Siblings with Ischaemic Stroke Study; WHI = Women's Health Initiative; WTCCC = Wellcome Trust Case Control Consortium; WUSTL = Washington University St Louis.

Table 3 Longitudinal primary intracerebral haemorrhage cohort characteristics and univariate analysis

Variable	ICH recurrence	No ICH recurrence	*P*-value
n	29	145	NA
Demographics			
Age, mean ± SD	73.9 ± 9.8	70.8 ± 10.9	0.1595
Sex, *n* (%) male	13 (44.8)	74 (51.0)	0.685
Race, *n* (%) white	29 (100.0)	145 (100.0)	NA
Location, *n* (%) lobar	22 (75.9)	78 (53.8)	0.039*
Prior ICH	3 (10.3)	9 (6.2)	0.424
Pre-ICH medical history			
Hypertension	16 (55.2)	104 (71.7)	0.122
CAD	3 (10.3)	20 (13.8)	0.77
Diabetes mellitus	1 (3.5)	25 (17.2)	0.083
Hypercholesterolaemia	8 (27.6)	57 (39.3)	0.295
Medications			
Warfarin	0 (0.00)	11 (7.6)	0.215
Aspirin	14 (48.3)	67 (46.2)	0.842
Statin	6 (20.7)	46 (32.2)	0.271
Genotype			
Mean MPO genetic score per individual	7.45	6.84	0.03*

CAD = coronary artery disease; ICH = intracerebral haemorrhage.
*Statistical significance at $P < 0.05$.

Table 4 Multivariable analysis of MPO GRS and intracerebral haemorrhage recurrence

Variable	HR	95% CI	*P*-value
Lobar ICH	2.43	1.02–5.80	0.04*
Hypertension	0.66	0.30–1.42	0.29
Diabetes mellitus	0.38	0.05–2.94	0.35
Age	1.04	1.00–1.09	0.03*
Statin exposure	0.56	0.21–1.48	0.24
MPO GRS	1.44	1.05–1.97	0.03*

HR = hazard ratio; ICH = intracerebral haemorrhage; Variables with $P > 0.20$ are not shown.
*Statistical significance at $P < 0.05$.

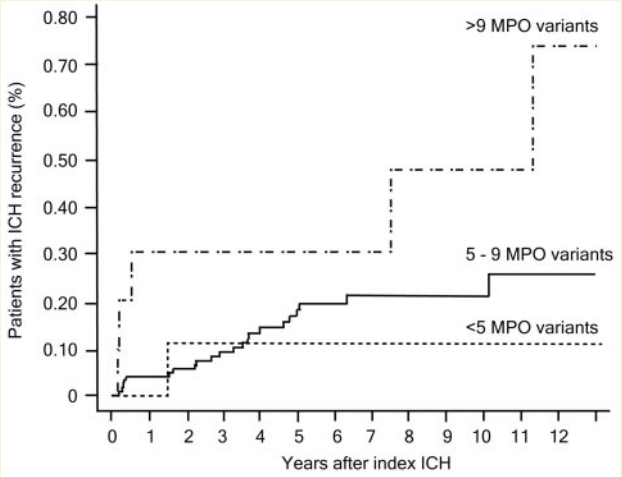

Figure 3 MPO GRS and risk of intracerebral haemorrhage recurrence. Kaplan-Meier plot of intracerebral haemorrhage recurrence rates stratified by number of MPO-elevating risk alleles.

evidence for an association between MPO-mediated inflammation and risk of small vessel-related ischaemic and haemorrhagic stroke across multiple study populations. Furthermore, we also observed that the presence of additional MPO risk alleles conferred increased risk of incident recurrent intracerebral haemorrhage amongst a longitudinal cohort of intracerebral haemorrhage survivors. Collectively, these data suggest that life-long exposures to genetically-mediated differences in circulating MPO levels could lead to development of small vessel disease-related stroke.

Our chosen methodology illustrates some of the advantages of using genetics in surmounting environmental confounding frequently encountered in observational studies using biomarker levels, manifest by conflicting results of serum MPO associations with clinical and neuroimaging surrogates of cerebral small vessel disease (Kaneski *et al.*, 2006; Wright *et al.*, 2009; Shoamanesh *et al.*, 2015). Elevated serum MPO levels had been shown to be (i) predictive of increased risk of lacunar stroke events in patients with Fabry disease (Kaneski *et al.*, 2006); and (ii) associated with increased white matter hyperintensity volumes (WMHV), an imaging surrogate of cerebral small vessel disease, in a population-based study of stroke-free individuals (Wright *et al.*, 2009). However, contrasting results were demonstrated in the Framingham cohort; lower serum MPO levels were associated with increased risk of WMHV and silent lacunar infarcts (Shoamanesh *et al.*, 2015). Our analyses used genetic variants that associate with circulating MPO levels to provide additional support for a potentially unconfounded role of MPO and its reaction products in the underlying pathophysiology of cerebral microvascular disease. Genetic risk factors exert a lifetime influence on MPO levels, are inherited independent of other risk factors for cardiovascular and cerebrovascular disease, and are less subject to confounding from subsequent environmental or medical exposures. Taking advantage of these technical benefits, these results support the hypothesis that MPO-related inflammation may have a causal role in cerebrovascular disease genesis and progression, and suggests MPO may be a potential therapeutic target for prevention of small vessel-related strokes.

Our findings also lend support to more recent concepts of cerebral small vessel disease pathogenesis stemming from the scrutiny of anatomical descriptions of cerebral microvascular pathology (Wardlaw *et al.*, 2003), as (i) an intrinsic process affecting cerebral small vessels over several years before becoming clinically evident; and (ii) a diffuse cerebrovascular endothelial failure, which precipitates small vessel-related strokes. The process of cerebral arteriolar and capillary endothelial failure result in disruption of normal vessel architecture (lipohyalinosis and fibrinoid

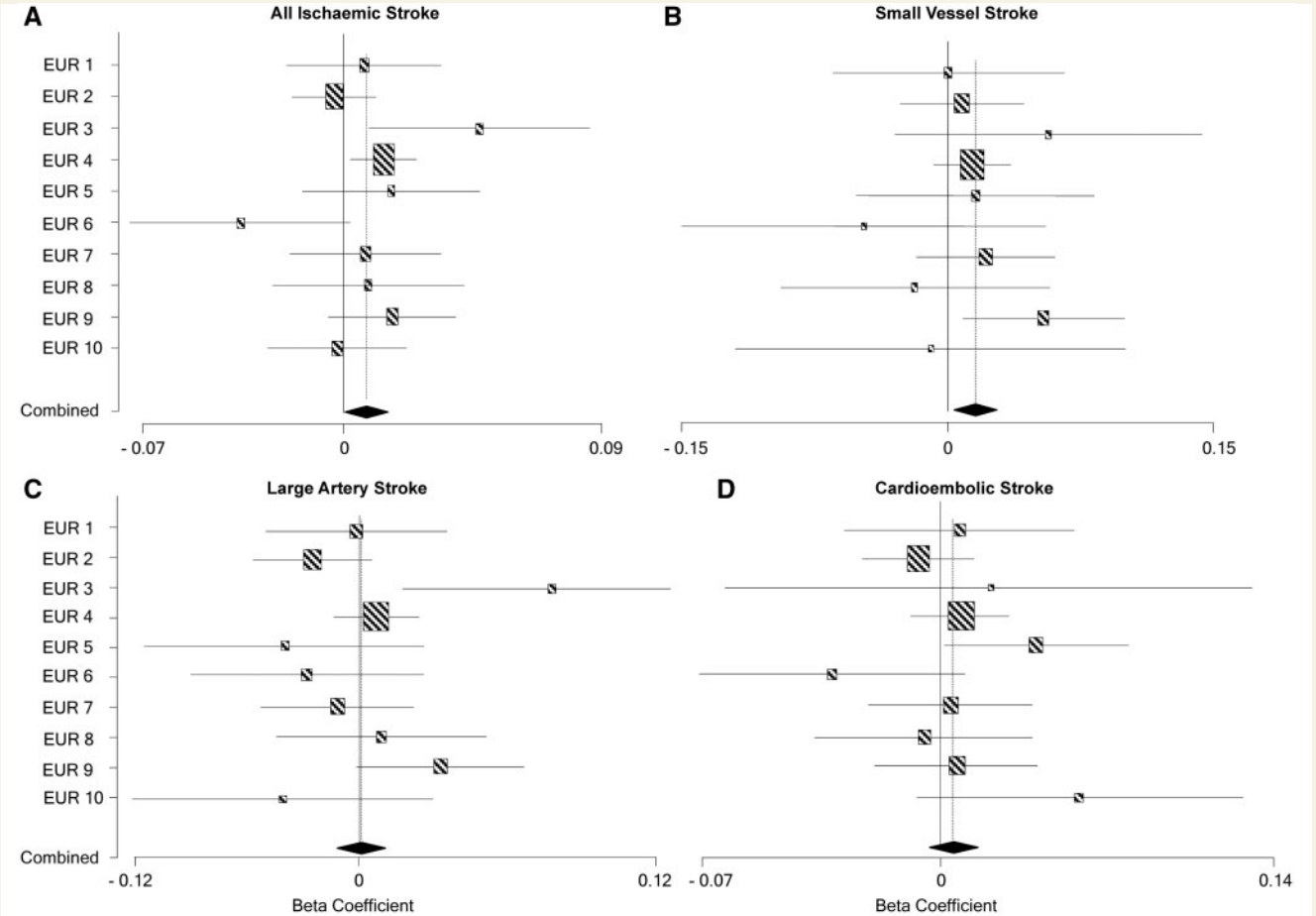

Figure 4 MPO GRS and ischaemic stroke risk by TOAST subtype. (**A**–**D**) MPO genetic risk score meta-analysis results for each additional trait-associated allele by ischaemic stroke subtype in NINDS-SiGN cohorts of European ancestry.

necrosis) (Lammie *et al.*, 1998) producing end results of diffuse, patchy luminal dilatation and narrowing, and stiffened vessel walls with loss of normal autoregulation, frequently observed in cerebral small vessel disease (Stevenson *et al.*, 2010). MPO is an active mediator of endothelial dysfunction (Vita *et al.*, 2004) and may play a crucial role in early, common pathways driving vascular endothelial failure through its pleiotropic role in oxygen radical generation, inflammation, and vasomotor failure (Lau and Baldus, 2006; Rudolph *et al.*, 2012).

MPO acts as the cornerstone of these pathophysiologic mechanisms through a combination of direct oxidative injury to arterial wall, and indirect effects on vascular integrity and function, (i) promoting atherosclerotic plaque formation by driving the core formation of low density lipoprotein (LDL)-rich foamy macrophages; (ii) influencing function, distribution and efflux of serum cholesterol by lipid peroxidation; (iii) destabilizing and rupturing atherosclerotic plaque through matrix metalloproteinase activation; (iv) stimulating local occlusive thrombosis possibly via P-selectin interactions; and (v) impairing vascular reactivity via consumption of endothelium-derived nitric oxide (NO), reducing nitric oxide bioavailability, and impairing vasodilatory and anti-inflammatory properties (Vita *et al.*, 2004; Lau and Baldus, 2006; Nicholls and Hazen, 2009). The effect of elevated circulating MPO in promoting diffuse vascular endothelial and autoregulatory impairment, occurring repeatedly over many years, may result in impaired cerebral function over time due to the native high metabolic demands of the brain (Hawkins and Davis, 2005).

The magnitude of effect sizes for the MPO genetic score in both lacunar stroke and primary intracerebral haemorrhage in our analyses are small per allele. This is not surprising, as each SNP in our genetic score had a mild effect on peripheral MPO levels (estimated 1 pmol/l increase). Given that the average individual in our study had approximately six MPO-increasing alleles, and the circulating MPO levels of an ageing population at risk for coronary artery disease is ~704 pmol/l (IQR 492–1021) by one large community sample (Meuwese *et al.*, 2007), our score can be estimated to explain 1–2% of the variance in serum MPO. However, it is possible that our GRS has a much larger influence on MPO-related biological effects beyond

its action on circulating levels due to pleiotropy, commonly seen in variants associated with traits within the same pathway or disease (Parkes *et al*., 2013).

In addition to the small-observed effect size, our study has additional limitations. Our investigative methodology using genetic score analysis prohibits identification of individual causative variants as it utilizes aggregation of signals to detect associations. We are also unable to confirm causality of elevated serum MPO in small vessel-related strokes via true one-sample Mendelian randomization by integrating biomarker level data in our analysis due to the absence of serum MPO measurements in our study cohorts. However, we undertook our investigative approach precisely to surmount known challenges of serum measurement variability and inaccuracies prevalent in acute biomarker levels. While a two-sample Mendelian randomization approach would theoretically be possible using our available data, the complex inter-relationships between inflammatory pathways and endothelial function make it extremely difficult to eliminate the potential for unmeasured collider relationships. As a result, formal adoption of the MPO GRS as an instrumental variable was not judged to be defensible. Finally, we are also unable to determine the commonality of inflammatory pathways promoting MPO release in small vessel-related stroke risk amongst other racial ancestries as we limited our analysis to populations of European descent from North America and Europe. However, it is likely that there is similar genetic contribution in MPO levels towards stroke risk amongst other races as elevations in serum MPO levels have also been observed to be predictive of increased risk of cardiovascular events in an East Asian population (Liu *et al*., 2012).

This preliminary work demonstrates that genetic variation associated with elevated circulating MPO levels increases risk of small vessel-related strokes represented by primary intracerebral haemorrhage and lacunar stroke. Our findings implicate MPO as an important factor in the development and progression of cerebral small vessel-related strokes, and support the growing body of evidence that inflammation is a key mediator of cerebral arteriolar disease. Furthermore, our results showing restriction of MPO genetic effects to small vessel disease phenotypes despite adequate statistical power in other stroke subtypes suggest a unique inflammatory component in cerebral small vessel pathophysiology. Future studies will be needed to identify additional interactions among components of the MPO-related inflammatory pathway, as well as investigating generalizability of our observations using genetic data from other ancestral populations.

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Conflicts of interest

B.N., Editor-in-Chief, European Stroke Journal; J.R., Spanish Ministry of Health; A.L., Advisory Board: Boeringer Ingelheim, Astra-Zeneca, Bayer. Sponsored lectures: BMS, Pfizer, Boeringer Ingelheim; B.B.W., Associate Editor, Neurology; J.R., Editorial Board, Stroke and Lancet Neurology, NIH; C.D.A., NIH.

Supplementary material

Supplementary material is available at *Brain* online.

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