A Novel MMP12 Locus Is Associated with Large Artery Atherosclerotic Stroke Using a Genome-Wide Age-at-Onset Informed Approach

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters.

Citation

Published Version
doi:10.1371/journal.pgen.1004469

Accessed
February 16, 2015 9:23:25 PM EST

Citable Link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:12785954

Terms of Use
This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA

(Article begins on next page)
A Novel MMP12 Locus Is Associated with Large Artery Atherosclerotic Stroke Using a Genome-Wide Age-at-Onset Informed Approach


1 Stroke and Dementia Research Centre, St George’s University of London, London, United Kingdom, 2Department of Clinical Chemistry, Fimlab Laboratories, Tampere, Finland, 3School of Medicine, University of Tampere, Tampere, Finland, 4Center for Clinical Epidemiology and Biostatistics, School of Medicine and Public Health, University of Newcastle, Callaghan, New South Wales, Australia, 5Hunter Medical Research Institute, New Lambton Heights, New South Wales, Australia, 6Center for Human Genetic Research, Massachusetts General Hospital, Boston, Massachusetts, United States of America, 7Program in Medical and Population Genetics, Broad Institute of Harvard and MIT, Cambridge, Massachusetts, United States of America, 8Laboratory of Neurogenetics, National Institute on Aging, Bethesda, Maryland, United States of America, 9Cardiovascular Health Research Unit, Department of Human Genetics, University of Washington, Seattle, Washington, United States of America, 10Perelman School of Medicine, Division of Translational Medicine and Human Genetics, University of Pennsylvania, Philadelphia, Pennsylvania, United States of America, 11Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland, United States of America, 12Research and Development Program, Veterans Affairs Maryland Health Care System, Baltimore, Maryland, United States of America, 13Department of Neurology and Neurosurgery, Brain Center Rudolf Magnus, University Medical Center Utrecht, Utrecht, The Netherlands, 14Institute for Stroke and Dementia Research, Klinikum der Universität München, Ludwig-Maximilians-Universität, Munich, Germany, 15Division of Clinical Neurosciences and Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, United Kingdom, 16Clinical Neurosciences, University of Cambridge, Cambridge, United Kingdom, 17Department of Surgery, Tampere University Hospital, Tampere, Finland, 18KU Leuven - University of Leuven, Department of Neurosciences, Experimental Neurology - Laboratory of Neurobiology, Leuven, Belgium, 19VIB - Vesalius Research Center, Leuven, Belgium, 20University Hospitals Leuven, Department of Neurology, Leuven, Belgium, 21Department of Clinical Sciences Lund, Neurology, Lund University, Lund, Sweden, 22Department of Neurology and Rehabilitation Medicine, Skåne University Hospital, Lund, Sweden, 23Department of Neurology, Jagiellonian University, Krakow, Poland, 24School of Nursing and Midwifery, University of Newcastle, Callaghan, New South Wales, Australia, 25Centre for Translational Neuroscience and Mental Health, University of Newcastle, Callaghan, New South Wales, Australia, 26Institute of Cardiovascular and Medical Sciences, University of Glasgow, Glasgow, United Kingdom, 27Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands, 28Imperial College Cerebrovascular Research Unit (ICCRU), Imperial College London, London, United Kingdom, 29Department of Cerebrovascular Disease, Fondazione Istituto di Ricovero e Cura a CarattereScientifico (IRCCS) Istituto Neurologico Carlo Besta, Milan, Italy, 30Stroke Prevention Research Unit, Nuffield Department of Clinical Neuroscience, University of Oxford, Oxford, United Kingdom, 31Department of Medical Genetics, University Medical Centre, Utrecht, The Netherlands, 32Division of Genetics, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, United States of America, 33Department of Biostatistics and Epidemiology, University of Pennsylvania, Philadelphia, Pennsylvania, United States of America, 34Center for Non-Communicable Diseases, Karachi, Pakistan, 35Department of Neurology, Mayo Clinic, Jacksonville, Florida, United States of America, 36Munich Cluster for Systems Neurology (SyNergy), Ludwig-Maximilians-Universität, Munich, Germany, 37Department of Medical & Molecular Genetics, King’s College London, London, United Kingdom, 38Social, Genetic and Developmental Psychiatry Centre, Institute of Psychiatry, King’s College London, London, United Kingdom

Abstract

Genome-wide association studies (GWAS) have begun to identify the common genetic component to ischaemic stroke (IS). However, IS has considerable phenotypic heterogeneity. Where clinical covariates explain a large fraction of disease risk, covariate informed designs can increase power to detect associations. As prevalence rates in IS are markedly affected by age, and younger onset cases may have higher genetic predisposition, we investigated whether an age-at-onset informed approach could detect novel associations with IS and its subtypes; cardioembolic (CE), large artery atherosclerosis (LAA) and small vessel disease (SVD) in 6,778 cases of European ancestry and 12,095 ancestry-matched controls. Regression analysis to identify SNP associations was performed on posterior liabilities after conditioning on age-at-onset and affection status. We sought further evidence of an association with LAA in 1,881 cases and 50,817 controls, and examined mRNA expression levels of the nearby genes in atherosclerotic carotid artery plaques. Secondly, we performed permutation analyses to evaluate the extent to which age-at-onset informed analysis improves significance for novel loci. We identified a novel association with an MMP12 locus in LAA (rs660599; p = 2.5 × 10⁻⁷), with independent replication in a second population (p = 0.0048, OR(95% CI) = 1.18(1.05–1.32); meta-analysis p = 2.6 × 10⁻⁹). The nearby gene, MMP12, was significantly overexpressed in carotid plaques compared to atherosclerosis-free control arteries (p = 1.2 × 10⁻⁷; fold change = 335.6). Permutation analyses demonstrated improved significance for associations when accounting for age-at-onset in all four stroke phenotypes (p<0.001). Our results show that a covariate-informed design, by adjusting for age-at-onset of stroke, can detect variants not identified by conventional GWAS.
Introduction

Genome-wide association studies (GWAS) in ischaemic stroke have begun to identify the common genetic variants that confer risk of the disease. However, there is considerable heterogeneity present in stroke phenotypes: GWAS analyses have primarily looked at three main subtypes: cardioembolic (CE), large artery atherosclerosis (LAA) and small vessel disease stroke (SVD). Within these subtype analyses, numbers of cases are smaller, but the expectation is that the effects of SNPs identified within the subtypes will be considerably larger. Indeed, all validated GWAS SNPs for ischaemic stroke to date have been stroke subtype-specific [1,2,3,4,5], indicating the importance of subtyping of cases.

Clinical risk factors are important in stroke; as many as 77% of first-ever stroke patients are hypertensive [6], and other factors such as diabetes mellitus and elevated serum cholesterol confer a considerable proportion of disease risk [7]. These risk factors increase in prevalence in older age groups, suggesting older stroke patients may have a reduced stroke-specific genetic contribution.


Editor: Timothy M. Frayling, University of Exeter Medical School, United Kingdom

Received December 21, 2013; Accepted May 14, 2014; Published July 31, 2014

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

Funding: The principal funding for this study was provided by the Wellcome Trust, as part of the Wellcome Trust Case Control Consortium 2 project (085475/B/08/Z and 085475/Z/08/Z and WT084724/MA). HMS is supported by an NIH Senior Investigator award and the NIH Biomedical Research Centre at Cambridge. We acknowledge support from the National Institutes of Health Research Biomedical Research Centre at Guy’s and St Thomas’ NHS Foundation Trust in partnership with King’s College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health. The PROMISE Study (The Netherlands) was made possible, in part, by a Complementation Grant to PIWdB from the Biobanking and Biomolecular Resources Research Infrastructure in the Netherlands (BBMRI-NL). SA is supported in part by a grant from the Netherlands Heart Foundation (grant no. 2005B031) and a grant from the Dutch Brain Foundation (project 2008(1.10). The Australian Stroke Genetics Collaboration (ASGC) Australian population control data was derived from the Hunter Community Study. We also thank the University of Newcastle for funding and the men and women of the Hunter region who participated in this study. This research was funded by grants from the Australian National and Medical Health Research Council (NHMRC) Project Grant ID: 560257, the Australian National Heart Foundation (NHF Project Grant ID: G 045 1623), the University of Newcastle, the Gladys M Brawn Festival committee and the Vincent Fairfax Family Foundation in Australia. EGH is supported by the Australian NHMRC Fellowhip scheme. The Genetics of Early Onset Stroke (GEOS) Study, Baltimore, USA was supported by the National Institutes of Health Genes, Environment and Health Initiative (GEI) Grant U01 HG004436, as part of the GENEA consortium under GEI, with additional support provided by the Mid-Atlantic Nutrition and Obesity Research Center (P30 DK072488); and the Office of Research and Development, Medical Research Service, and the Baltimore Geriatrics Research, Education, and Clinical Center of the Department of Veterans Affairs. Genotyping services were provided by the Johns Hopkins University Center for Inherited Disease Research (CIDR), which is fully funded through a federal contract from the National Institutes of Health to the Johns Hopkins University (contract number HHSN268200782096C). Assistance with data cleaning was provided by the GENEVA Coordinating Center (U01 HG004446; PI Bruce Weaver). Study recruitment and assembly of datasets were supported by a Cooperative Agreement with the Division of Adult and Community Health, Centers for Disease Control and by grants from the National Institute of Neurological Disorders and Stroke (NINDS) and the NIH Office of Research on Women’s Health (R01 NS45012, U01 NS069208-01). The Heart and Vascular Health Study (HVH) research reported in this article was funded by NHLBI grants R01 HL085251 and R01 HL073410. The Ischemic Stroke Genetics Study (IGSS)/Siblings With Ischemic Stroke Study (SWISS) study was supported in part by the Intramural Research Program of the National Institute on Aging, NIH project Z01 AG000954-06, IGSS/0055 used samples and clinical data from the NIH-NINDS Human Genetics Resource Center and Cell Line Repository (http://ccr.coordin.org/ninds), human subjects protocol numbers 2003-081 and 2004-147. IGSS/ SWISS used stroke-free participants from the Baltimore Longitudinal Study of Aging (BSLA) as controls. The inclusion of BLSA samples was supported in part by the Intramural Research Program of the National Institute on Aging, NIH project Z01 AG-00015-50, human subjects protocol number 2003-078. The IGSS study was funded by NIH-NINDS Grant R01 NS-42733 (JFM, P.I.). The SWISS study was funded by NIH-NINDS Grant R01 NS-39987 (JFM, P.I.). This study utilized the high-performance computational capabilities of the Biowulf Linux cluster at the NIH (http://biowulf.nih.gov). The MGH Genes Affecting Stroke Risk and Outcome Study (MGH-GASROS) GASROS was supported by The National Institute of Neurological Disorders and Stroke (U01 NS069208), the American Heart Association/Bugher Foundation Centers for Stroke Prevention Research 0775010N, the National Institutes of Health and National Heart, Lung, and Blood Institute’s STAMPED genomics program (R01 HL087676) and a grant from the Dutch Heart Institute for Research. The Broad Center for Genome Research is supported by grant US4 RR020278 from the National Center for Research resources. Milano - Basta Stroke Register Collection and genotyping of the Milan cases within CEDIR were supported by Annual Research Funding of the Italian Ministry of Health (Grant Numbers: RC 2007/LR6, RC 2008/LR6; RC 2009/LR8; RC 2010/ LR8; PF6 LSHM-CT-2007-032773 for the PROCARDIS control samples. The Wellcome Trust Case-Control Consortium 2 (WTCCC2) The principal funding for the WTCCC2 study was provided by the Wellcome Trust Case Control Consortium, as part of the Wellcome Trust Case Control Consortium (W084726/08/Z and WT084724/MA). The Stroke Association provided additional support for collection of some of the St George’s, London cases. The Oxford cases were collected as part of the Oxford Medical Research Council, Stroke Association, Dunhill Medical Trust, National Institute of Health Research (NIHR) and the NIHR Biomedical Research Centre, Oxford. PMR has a Wellcome Trust Senior Investigator Award and an NIHR Senior Investigator Award. The Edinburgh Stroke Study was supported by the Wellcome Trust (clinician scientist award to CS), and the Binks Trust. Sample processing occurred in the Genetics Core Laboratory of the Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh. This work was supported by grants received from the German Federal Ministry of Education and Research (BMBF) in the context of the eMed program (eAtheroSysMed) and the FP7 European Union project CVgenes@Target (261123) to Martin Dichgans. Much of the neuroimaging occurred in the Scottish Funding Council Brain Imaging Research Centre (www.sbcrc.ed.ac.uk). Assistance with data cleaning was provided by the Vascular Dementia Research Foundation. This work made use of data and samples generated by the 1958 Birth Cohort (NCDS). Access to these resources was enabled via the 58READIE Project funded by Wellcome Trust and Medical Research Council of the Munich cases and data analysis was supported by the Vascular Dementia Research Foundation. This work made use of data and samples generated by the 1958 Birth Cohort Biomedical Resource is available at http://www2.le.ac.uk/ projects/birthcohort. Tampere Vascular Study (TVS) was supported by the European Union 7th Framework Programme funding for the AtheroRemo project (201668), the Finnish Foundation of Cardiovascular Research (TL), the Finnish Cultural Foundation, the Tampere University Hospital Medical Fund (grant 406768) and the Foundation of Clinical Chemistry. Immunochip case data for UK, Polish, Belgium, German and Swedish cohorts was generated by the Sanger Centre, Cambridge UK as part of the Wellcome Trust Case Control Consortium 2 project (085475/B/08/Z and 085475/Z/08/Z and WT084724/MA), as were the UK and Polish control cohorts. German Control Genotypes was provided through the POPGEN Consortium. Swedish Immunochip Control Samples were provided by the Swedish SLE network and the Uppsala Bioreource. Belgian Immunochip Control Sample Genotypes were provided through the efforts of the International Multiple Sclerosis Genetics Consortium (ISGC). The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.
* Email: mtraylor@sgul.ac.uk
* These authors contributed equally to this work
† Members of Wellcome Trust Case Control Consortium 2 are listed in the Text S1.

PLOS Genetics | www.plosgenetics.org 2 July 2014 | Volume 10 | Issue 7 | e1004469
Author Summary

Ischaemic stroke places an enormous burden on global healthcare. However, the disease processes that lead to stroke are not fully understood. Genome-wide association studies have recently established that common genetic variants can increase risk of ischaemic stroke and its subtypes. In this study, we aimed to identify novel genetic associations with ischaemic stroke and its subtypes by addressing the fact that younger onset cases may have a stronger genetic component, and using this information in our analyses. We identify a novel genetic variant on chromosome 11 (rs660599), which is associated with increased risk of large artery stroke. We also show that mRNA expression of the nearest gene (MMP12) is higher in arteries with the disease process underlying large artery stroke (atherosclerosis). Finally, we evaluate our novel analysis approach, and show that our method is likely to identify further associations with ischaemic stroke.

Indeed, IS is uncommon in individuals below middle age, but increases greatly in prevalence beyond the age of 65 [8], with a lifetime risk of 1 in 5 for women and 1 in 6 for men [9].

Under the assumptions of the liability threshold model, the low prevalence of IS in younger age ranges suggests that individuals who do suffer strokes in this age group are likely to have an increased genetic predisposition. This is supported by family history data; with stronger family history seen in younger onset cases [10,11,12], and twin studies [13], which suggest that early onset cases may have higher heritability. We recently showed stronger effects for all stroke-associated SNPs in younger age groups, found evidence genome-wide that a significant number of SNPs show stronger association p-values when the oldest cases are removed, and showed increased pseudoheritability estimates for younger onset cases in certain stroke subtypes, thereby supporting this hypothesis [14]. However, the question of how best to integrate this information into GWAS analyses of ischaemic stroke remains unanswered. Previous GWAS have analysed younger subsets of ischaemic stroke cases [1,15], but this approach may not be optimal for existing GWAS datasets if the increase in odds ratios for SNPs in younger cases are not sufficient to justify discarding a large proportion of the ascertained cases. All previous young onset analyses have been restricted to all ischaemic stroke cases versus controls; this may be particularly relevant given that all known loci for ischaemic stroke to date are for stroke subtypes [16].

A recent publication [17], outlined a novel method of informing genetic association analyses on important clinical covariates. Using the liability threshold model in conjunction with estimates of disease prevalence for individuals with specific clinical covariates, the method estimates posterior disease liabilities for each individual in a GWAS, and uses these liabilities in regression analyses to test for association with genome-wide SNPs. This approach avoids issues due to multiple testing across age-at-onset thresholds, and provides a simple solution that is rooted is previous epidemiological research. In the present study, we extend the clinical covariate informed analysis approach to imputed genotypes, informing our analyses on the age-at-onset to identify novel variants associated IS. We perform a genome-wide analysis with four stroke phenotypes (IS, CE, LAA, SVD), and then determine the utility of the approach in ischaemic stroke GWAS, testing whether SNPs increase in significance.

Results

Association analysis

We performed age-at-onset informed association analysis for a total of 6,778 ischaemic stroke cases and 12,095 controls across four ischaemic stroke phenotypes; all IS and the three major subtypes: CE, LAA, and SVD (Table 1); with 1,637, 1,316, and 1,108 cases in the CE, LAA and SVD analyses respectively. With the exception of the young Milanese cohort, the age-at-onset distributions were similar in all cohorts (Table S3).

We identified a group of twenty SNPs proximal to MMP3 and MMP12 on chromosome 11 in the LAA subtype that met our criteria for replication. The strongest associated of these was rs662558 (p = 1.4×10^{-7}), a SNP that is in 1000 Genomes, but not HapMap II. Therefore, to enable replication in existing METASTROKE datasets, which were imputed to HapMap II, we selected the most strongly associated SNP from the HapMap II panel, which was in perfect LD with the lead SNP in our discovery meta-analysis (rs660599; uninform, p = 1.6×10^{-6}; informed, p = 2.5×10^{-7}; Figure 1) [16]. We found no evidence of between-study heterogeneity at either SNP (Cochran’s Q p = 0.22 and p = 0.19 for rs662558 and rs660599, respectively). The evidence of an age-at-onset effect at rs660599 was p = 0.011 (from permutations). We calculated age-at-onset quartiles for all large artery stroke cases from the discovery cohorts, and used these to evaluate this region at different age-at-onset thresholds. The median age-at-onset was 71 years, and the interquartile range was between 61 and 78 years. Post-hoc analyses of rs660599 in the discovery cohorts using logistic regression (full details in Text S2) showed considerably stronger associations in younger age-at-onset quartiles (Q1; OR(95% CI) = 1.83 (1.46–2.30), Q1–Q2; 1.56 (1.33–1.83), Q1–Q3; 1.30 (1.14–1.49), Q1–Q4; 1.30 (1.15–1.46)). No other regions met our criteria for replication.

Replication analysis

The associated locus was evaluated in a further 1,881 large artery stroke cases and ancestry matched controls in 9 cohorts from METASTROKE (Table 2). We found evidence for replication of the SNP (rs660599) in all large artery stroke cases of European Ancestry (p = 0.0048, OR(95% CI) = 1.18 (1.05–1.32)). Combining this result with the discovery p-value gave a genome-wide significant p-value of 2.6×10^{-8} (Table 3). Secondly, we used the Han and Eskin random effects meta-analysis approach to evaluate the association [18] after including a further 355 cases and 1,390 controls of Pakistani ancestry. The evidence for replication in this sample was p = 0.0063, giving an overall p-value of 3.4×10^{-8}. Age-at-onset information was available across all age-at-onset quantiles for a subset of the replication studies (1,240 cases, 9,238 controls; ASGC, HVH, ISGS/SWISS, MGH-GASROS, Utrecht). We evaluated the SNP (rs660599) in these studies at different age-at-onset quantiles using logistic regression, meta-analysing as previously. We again found the strongest effects in the youngest age quantile, consistent with a stronger effect in younger onset cases (Q1; OR(95% CI) = 1.27 (1.02–1.57), Q1–Q2; 1.18 (1.00–1.39), Q1–Q3; 1.2 (1.05–1.40), Q1–Q4; 1.22 (1.07–1.41)).

mRNA expression in carotid plaques

mRNA expression of the two proximal genes, MMP3 and MMP12 was analysed from 29 carotid, 15 abdominal aorta, 24 femoral plaques, and 28 atherosclerosis free left internal thoracic artery controls. MMP12 expression was upregulated in carotid plaques compared with left internal thoracic artery controls (P = 1.2×10^{-15}, fold change [FC] = 335.6). It was also upregulated in femoral plaques (P = 3.2×10^{-13}, FC = 306.0) and abdominal
plaques ($P = 5.0 \times 10^{-11}$; FC = 399.3) compared with controls. Conversely, $MMP3$ was not significantly overexpressed in carotid, femoral or abdominal plaques versus controls ($p > 0.05$).

**Regulatory information from ENCODE**

Eight SNPs were identified that were perfect proxies ($r^2 = 1$) with the associated SNP (rs660599) in the region. Seven of the SNPs were in an intergenic region between $MMP3$ and $MMP12$, while one fell within an intron of $MMP12$. We investigated the evidence that any of these SNPs are functional variants using RegulomeDB [19]. Of the eight SNPs, we found strong evidence that one of these SNPs (rs586701) affects binding. The SNP overlaps both CHIP-seq and DNA-seq peaks from ENCODE analyses, indicating that there is open chromatin in the region, and therefore that the SNP is likely to be functional. There is also evidence from a separate CHIP-seq analysis that the SNP affects protein binding [20], and evidence from multiple sources that the SNP overlaps a predicted motif [21,22,23]. Histone modifications were observed in CHIP-seq experiments from ENCODE in a number of cell types, including Human umbilical vein endothelial (Huvec) cells. Two other SNPs (rs17368582, rs2276109) in moderate LD with the associated SNP ($r^2 = 0.64$) have been previously shown to directly influence $MMP12$ expression by affecting the affinity of an AP-1 binding site in the $MMP12$ promoter region [24,25]. Using RegulomeDB, we found further evidence from ENCODE that one of these SNPs (rs2276109) is indeed functional, giving evidence that the associated locus in this analysis is likely to affect $MMP12$ expression through altered

![Figure 1. LocusZoom plot of $MMP12$ association using age-at-onset informed approach.](image)

SNPs are colored based on their correlation ($r^2$) with the labeled top SNP, which has the smallest $P$ value in the region. The fine-scale recombination rates estimated from 1000 Genomes (EUR) data are marked in light blue, with genes marked below by horizontal blue lines. Arrows on the horizontal blue lines show the direction of transcription, and rectangles are exons. SNP $p$-values are from the discovery meta-analysis only with the exception of rs660599, for which the given $p$-value indicates the overall evidence for association from the discovery and replication cohorts.

doi:10.1371/journal.pgen.1004469.g001

<table>
<thead>
<tr>
<th>Study Population</th>
<th>IS</th>
<th>CE</th>
<th>LAA</th>
<th>SVD</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium – Immunochip</td>
<td>396</td>
<td>147</td>
<td>57</td>
<td>49</td>
<td>319</td>
</tr>
<tr>
<td>Germany-Immunochip</td>
<td>421</td>
<td>127</td>
<td>101</td>
<td>-</td>
<td>2,355</td>
</tr>
<tr>
<td>Krakow – Immunochip</td>
<td>384</td>
<td>119</td>
<td>33</td>
<td>28</td>
<td>255</td>
</tr>
<tr>
<td>Sweden – Immunochip</td>
<td>796</td>
<td>246</td>
<td>56</td>
<td>183</td>
<td>997</td>
</tr>
<tr>
<td>UK – Immunochip</td>
<td>867</td>
<td>130</td>
<td>152</td>
<td>257</td>
<td>1,790</td>
</tr>
<tr>
<td>Germany – WTCCC2</td>
<td>1,174</td>
<td>330</td>
<td>346</td>
<td>106</td>
<td>797</td>
</tr>
<tr>
<td>UK – WTCCC2</td>
<td>2,374</td>
<td>474</td>
<td>498</td>
<td>460</td>
<td>5,175</td>
</tr>
<tr>
<td>Milano</td>
<td>366</td>
<td>64</td>
<td>73</td>
<td>25</td>
<td>407</td>
</tr>
<tr>
<td>Total (Discovery)</td>
<td>6,778</td>
<td>1,637</td>
<td>1,316</td>
<td>1,108</td>
<td>12,095</td>
</tr>
</tbody>
</table>

IS, all ischaemic stroke; CE, cardioembolic stroke; LAA, large artery stroke; SVD, small vessel disease.
doi:10.1371/journal.pgen.1004469.t001
transcription. Detailed results for all analysed SNPs are given in Table S1. Additionally, we investigated if these SNPs (rs17368582, rs2276109, rs586701) were associated with IS, and performed age-at-onset informed analysis and subsequent generated 1000 permutations of age-at-onset within each centre, to establish stringent p-value thresholds in the case control discovery data set. We compared the age-at-onset informed approach in permutation analyses for SNPs that met p-value thresholds in the observed age-at-onset informed meta-analysis compared to the permutations. At this p-value selection threshold decreased, the summed Z score statistic became less significant in each stroke type, possibly reflecting lower overall power when fewer SNPs are included, even as these SNPs may have larger average effects. Further details are seen from the median proportion of SNPs more significant in the age-at-onset informed analysis than in the permutations (Figure 2, blue points, left hand axis). For CE and LAA stroke, the proportions increased with more stringent p-value thresholds (from 52.1% to 56.3% for LAA) with more stringent p-value thresholds (from 52.1% to 56.3% for p<0.05 and p<0.00005 thresholds in CE, and from 51.4% to 56.0% for p<0.05 and p<0.00005 thresholds in LAA). Interestingly, in the all ischaemic stroke analysis the median proportion of SNPs more significant in the observed results than permutations dropped from 55.1% for SNPs with p<0.05 to 49.2% for only SNPs with p<0.00005. This result may indicate a reduced proportion of true associations at stricter p-value thresholds for all ischaemic stroke compared to the subtypes, which is consistent with the observation that all common variants associated with stroke are for stroke subtypes, rather than for the phenotype of all ischaemic stroke [16].

The previously reported GWAS associations from a recent ischaemic stroke meta-analysis [9,21, HDAC9, PITX2, ZFHX3] were all found to be more significant using the age-at-onset informed approach than the uninformed analysis (Figure 3). The increase in significance ranged from over half an order of magnitude.

Table 3. Evidence for association of A allele of rs660599 (chromosome 11; Base position 102,234,967) with large artery atherosclerotic stroke and all ischaemic stroke.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>SNP</th>
<th>RAF</th>
<th>p-value (discovery)</th>
<th>OR (95% CI) (EUR replication)</th>
<th>p-value (EUR replication, overall)</th>
<th>p-value (ALL replication, overall)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAA</td>
<td>rs660599</td>
<td>0.19</td>
<td>2.5 × 10^{-7}</td>
<td>1.18 (1.05–1.32)</td>
<td>0.0048, 2.6 × 10^{-8}</td>
<td>0.0063, 3.4 × 10^{-8}</td>
</tr>
<tr>
<td>IS</td>
<td></td>
<td></td>
<td>3.2 × 10^{-4}</td>
<td>1.05 (1.00–1.11)</td>
<td>0.050, 1.9 × 10^{-4}</td>
<td>0.098, 3.6 × 10^{-4}</td>
</tr>
<tr>
<td>CE</td>
<td></td>
<td></td>
<td>0.13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SVD</td>
<td></td>
<td></td>
<td>0.30</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

LAA, large artery stroke; IS, all ischaemic stroke; SNP, single nucleotide polymorphism; RAF, risk allele frequency; OR, odds ratio; 95% CI, 95% confidence interval; EUR, meta-analysis in individuals of European ancestry alone; ALL, trans-ethnic meta-analysis of all individuals. Forest plots of effect sizes and standard errors for each replication centre are given in Figures S3, S4.

doi:10.1371/journal.pgen.1004469.0003
magnitude ($7.9 \times 10^{-9}$ to $1.5 \times 10^{-9}$ for rs879324 in ZFHX3, CE), to under half an order of magnitude ($5.7 \times 10^{-9}$ to $2.5 \times 10^{-9}$ for rs2107595 in HDAC9, LVD). To ensure these analysis methods were comparable, we calculated genomic inflation factors and plotted QQ-plots. These were similar in the standard and the age-at-onset informed approach (Table S4, Figure S1, S2). For these four associated SNPs, we further used the permuted data sets to assess the observation of increased significance in the age-at-onset informed analysis. We compared the observed meta-analysis p-value to those from the permutations, generating an empirical p-value by dividing the number of permutations more significant than the observed results by the number of permutations. In LAA stroke, we observed a significant age-at-onset effect ($p = 0.018$, 0.011 and 0.002 for the HDAC9, MMP12 and 9p21-associated SNPs in Figure 3, respectively). Similarly, for CE, we observed a significant age-at-onset effect for rs879324 ($ZFHX3$, $p = 0.026$), and a near-significant effect in rs6843082 ($PITX2$, $p = 0.081$). This result provides further evidence that risk variants associated with ischaemic stroke subtypes have a stronger role in younger onset cases, and suggests that the age-at-onset informed approach will produce improved significance when the magnitude of genetic effects are stronger in younger onset cases.

**Discussion**

We used a large GWAS dataset to evaluate the utility of an age-at-onset informed analysis approach to ischaemic stroke, and to identify novel variants associated with ischaemic stroke phenotypes. We identified a novel MMP12 locus that is associated with large artery atherosclerotic stroke, and verified that the age-at-onset informed approach produces improved significance for loci associated with each of the stroke phenotypes studied, as well as demonstrating that it increased the significance of four previous GWAS associations with ischemic stroke, all without systematic inflation of the test statistic. Importantly, the novel associated SNP would not have been identified using a standard logistic regression framework.

We identified a group of SNPs proximal to Matrix Metalloproteinase 12 (MMP12) that showed increased significance when using the age-at-onset informed approach. The increase in significance from the equivalent uninformed analysis was of almost an order of magnitude (from $p = 1.6 \times 10^{-6}$ to $p = 2.3 \times 10^{-7}$ for rs660599). We took a single SNP from this region forward for replication in an independent dataset, finding further evidence that the region is associated with large artery stroke. Two SNPs (rs17368382, rs2276109) in this LD-block have previously been shown to directly influence MMP12 expression by...
show increased activity in atheromatous plaques [31]. Macrophage invasion [27,28,29], promote angiogenesis [30], and key role in atherosclerosis. They are thought to promote the Matrix Metalloproteinase (MMP) family of proteases, which affect the affinity of an AP-1 binding site in the MMP12 promoter region [24,25], and another variant in this block (rs17361668) is associated with increased fibrinogen levels, leading to an increased risk of developing advanced carotid atherosclerotic lesions, and an increased risk of myocardial infarction. We identified a second functional candidate (rs586701), which falls within both CHIP-seq and DNA-seq peaks from ENCODE, and is in complete LD with the associated SNP in our analysis.

We investigated mRNA expression of MMP12 and MMP3 in carotid atherosclerotic plaques in individuals from the Tampere Vascular Study. MMP12 was overexpressed in diseased tissue compared to healthy controls, while no significant difference was found for the nearby gene, MMP3. MMP12 is a member of the Matrix Metallloproteinase (MMP) family of proteases, which are capable of degrading extracellular matrix proteins, and have a prominent role in atherosclerosis. They are thought to promote macrophage invasion [27,28,29], promote angiogenesis [30], and show increased activity in atheromatous plaques [31]. MMP12 deletions are associated with smaller, more stable lesions in the brachiocephalic artery of rabbits [32], and reduced elastin degradation in the aortic arch [33], indicating that MMP12 may have a role in destabilising plaques. Studies in humans have found MMP12 is localized to the core of advanced plaques, in macrophages with decreased arginase-I expression [34], that MMP12 localizes selectively to macrophages at the borders of the lipid core [35], and that MMP12 is significantly overexpressed in ruptured plaques when compared with thick or thin cap plaques, or with plaques with pathological intimal thickening [36]. This indicates that MMP12 is likely be involved in late-stage plaque instability; our study suggests that genetic variation impacts on this process.

Secondly, we performed extensive permutation analyses to assess the utility of the age-at-onset informed approach genome-wide. In each phenotype studied we found evidence that SNPs were more strongly associated using the approach than would be expected by chance, indicating that multiple risk variants are likely to be more common in younger onset cases. The significance was strongest when more SNPs were included in the analysis, which likely reflects the cumulative impact of age-at-onset effects on many SNPs. An alternative explanation might be that the increased significance for lower p-value thresholds is the result of the cumulative effects of subtle confounding. However, this is unlikely because any subtle biases will also be present in the permutations, and should therefore not affect the significance of the results. This result supports observations from family history and prospective cohort studies, which have observed stronger effects in younger onset cases [6,11]. Furthermore, all known associations with stroke were more significant using the age-at-onset informed approach. The increase in significance was around half an order of magnitude (e.g from p = 7.9 × 10−9 to 1.5 × 10−9 for ZFHX3, Figure 2), and was significant in all but one locus, as assessed by permutation. Taken together, these results indicate that age-at-onset is an important measure to stratify stroke cases, and show that, as expected by theory [17], integrating this information into association studies is likely to increase power to identify novel loci when the relative contribution of genetic is dependent on age-at-onset.

Our study has limitations. We used imputed data from the Immunochip platform, meaning we only had access to ~40% of the genome across all centres. Secondly, cases were drawn from a number of international centres, meaning that despite efforts to standardize phenotyping, we cannot rule out differences in screening and clinical ascertainment.

Of complex diseases, IS has a particularly large degree of heterogeneity, exemplified by the fact that all validated associations identified to date have been within subtypes defined by clinical and radiological information. Further heterogeneity by risk factor and clinical covariate profiles is likely to exist, but the optimal method of incorporating this information into analyses remains an unanswered question. Our results indicate that a
covariate-informed design, conditioning on age-at-onset of stroke, can unearth further associated variants. We provide evidence for this by identifying an association with a novel \textit{MMP12} locus in large artery stroke, supported by increased mRNA expression of the implicated gene in carotid plaques. GWAS in ischaemic stroke have begun to identify the genetic component of the disease, but these results are not yet clinically useful. Our study suggests that a more refined approach to analysis of genetic data, incorporating covariate information, is an important step in this process, and will help to ensure success in future GWAS.

\section*{Materials and Methods}

\subsection*{Ethics statement}
All studies were approved by their local ethics committees; all patients gave informed consent.

\subsection*{Description of datasets}
The initial dataset consisted of 6,778 ischaemic stroke cases of European ancestry and 12,095 ancestry-matched controls from the Wellcome Trust Case-Control Consortium II project in ischaemic stroke [1], as well as a cohort from Milan, Italy [16]. These included 2,858 cases and 5,716 matched controls genotyped using the Immunochip platform; and 3,940 cases genotyped using either the Illumina 610 k or 660 k platforms matched with 6,379 controls genotyped on the Illumina Human 1.2M Duo (UK), Illumina Human 550 k (German) and Illumina 610 k platforms (Italian) (Table 1). The Immunochip cases were described in the previous WTCCC2 ischaemic study, where they formed the replication effort [1], as well as in a recent paper [37]. Genotyping of the five Immunochip case cohorts on the commercially available Immunochip array (Illumina, San Diego, CA, USA) was performed at the Sanger Centre, Hinxton, Cambridge UK. Swedish controls were provided and genotyped by the Swedish SLE network, Uppsala, Sweden. Belgian control samples were provided through the efforts of the International Multiple Sclerosis Genetics Consortium (IMSGC). German controls were derived from the PopGen biobank, [38]. UK controls were derived from the 1958 Birth cohort. Any of the 1958 Birth controls overlapping with those from the WTCCC2 datasets, as assessed by IBD estimates, were removed prior to analysis. Standard quality control procedures were undertaken on all centres, before centre-wise imputation to the 1000 Genomes phase 1 integrated variant set (March 2012), using IMPUTE v2.2.0 [39,40]. SNPs with poor imputation quality (\textit{info}<0.3) or low minor allele frequency (\textit{MAF}<0.01) were discarded.

Ischemic stroke was defined as a typical clinical syndrome with radiological confirmation; ascertainment cases were classified into individual stroke subtypes using the Trial of Org 10172 in acute stroke (TOAST) criteria in all centres [41]. Age-at-onset was defined as age at first hospital admission for stroke; where this information was unavailable, age at blood draw was used (7.3\% of cases). The age-at-onset and gender distributions of the populations are given in Table S3. Age-at-onset quantiles were calculated from all the cases from the discovery datasets in the four stroke phenotypes (all IS and the three stroke subtypes: CE, LAA, SVD) and these were used to evaluate associated loci at different age-at-onset thresholds.

\subsection*{Association analysis}
The prevalence of ischaemic stroke by age was obtained from a recent publication [9]; gender-specific estimates were averaged, and prevalences within each of the stroke subtypes were assumed to be approximately 20\% of the overall total, similar to proportions seen in population-based studies [42]. We modeled phenotype data using a continuous unobserved quantitative trait called the disease liability, which we used to approximate the effect of age-at-onset on the liability scale, based on estimates of ischaemic stroke prevalence by age from epidemiological data (full details in Text S2). We developed two models for our analysis; one based on the prevalence rates for all ischaemic stroke cases, and secondly for the three stroke subtypes. We used these models to calculate posterior mean liabilities after conditioning on age-at-onset for the four stroke phenotypes separately. Controls were modeled in the same way, but were assumed to take the posterior mean from the lower (unaffected) portion of the distribution in the liability threshold model. Where age data was missing, individuals were assigned the median age value. Full descriptions of the models used and the formulae used to calculate posterior mean liabilities are given in Text S2. Regression was then performed on posterior liabilities by multiplying the number of samples by the squared correlation between the expected genotype dosage and posterior mean liabilities for each of the discovery cohorts in the four ischaemic stroke phenotypes (CE, LAA, SVD, IS), following a previous approach [17]. Ancestry-informative principal components were included where appropriate (6 of 8 centres), using the EIGENSTRAT procedure [43]. All analysis was performed using the R statistical software.

The results from each centre were meta-analysed for each of the four phenotypes using Stouffer’s Z-score weighted approach, as implemented in METAL [44]. Genomic control was used to correct for any residual inflation due to population stratification [45]. Between-study heterogeneity was assessed using Cochran’s Q statistic. We considered only SNPs present in at least 75\% of the cases, and with no evidence of heterogeneity (Cochran’s Q p-value>0.001). All SNPs analysed were either genotyped or imputed in both the Immunochip and the genome-wide datasets. After meta-analysis, the resulting p-values were compared with the equivalent values from an unconditioned analysis. For SNPs more significant in the age-at-onset informed analysis and with p<5×10^{-6}, we determined the evidence of a true age-at-onset effect by generating 1000 permutations of age-at-onset and rerunning the age-at-onset informed analysis, meta-analysing as previously. We calculated an empirical p-value by dividing the number of permuted observations showing greater significance in the meta-analysis than the observed results by the number of permutations. Any novel SNP with a meta-analysis p<5×10^{-6} and evidence of an age-at-onset effect at p<0.05 were taken forward for replication. We set the experiment-wide significance threshold at p<5×10^{-6}.

\subsection*{Replication analysis}
Replication of an associated variant was performed in a further 10 cohorts from METASTROKE. Nine of the centres used a cross-sectional design, while one was a large prospective, population based cohort (ARIC). Nine of the centres were of European ancestry, while one consisted of individuals of Pakistani ancestry (RACE) (Table 2). All centres used a case-control methodology; centres with a cross sectional design used logistic regression to model the association of genotype dosages from imputation with the dichotomous outcome of ischaemic stroke and prospective cohorts used Cox proportional-hazards models to evaluate time to first stroke, fitting an additive model relating genotype dose to the stroke outcome. European ancestry replication centres were meta-analysed using a fixed effects inverse-variance weighted method. To assess the evidence for association of the SNP for replication samples of all ancestries, we
performed a trans-ethnic meta-analysis using a random-effects model to control for any resulting heterogeneity [18]. To evaluate the overall evidence for association, the results of the discovery and replication analyses were combined using Fisher’s Method.

mRNA expression in carotid atherosclerotic plaques

Expression of the two genes proximal to the associated variant was tested in atherosclerotic plaques from the Tampere Vascular study [27,46,47,48,49]. Carotid, femoral, and aortic atherosclerotic plaques constituting the intima and inner media were prospectively obtained between 2003 and 2009 from patients fulfilling the following inclusion criteria: (1) carotid endarterectomy attributable to asymptomatic or symptomatic >70% carotid stenosis, or (2) femoral or (3) aortic endarterectomy with aortoiliac or aortobifemoral bypass attributable to symptomatic peripheral arterial disease. Whole thickness left internal thoracic artery samples obtained during coronary artery bypass surgery and identified as being microscopically atherosclerosis free were used as controls. The patients were consecutively recruited and stratified according to indication for surgery. All open vascular surgical procedures were performed at the Division of Vascular Surgery and Heart Center, Tampere University Hospital.

Fresh tissue samples were immediately soaked in RNALater solution (Ambion Inc) and homogenized using an Ultra-Turrax T80 homogenizer (IKA). RNA was extracted with the Trizol reagent (Invitrogen) and miRNEasy Mini-Kit (Qiagen) with the RNAse-Free DNase Set (Qiagen) according to manufacturer instructions. The RNA isolation protocol was validated by analyzing the integrity of the RNA with the RNA 6000 Nano Chip Kit (Agilent). The expression levels were analyzed with an Illumina HumanHT-12 v3 Expression BeadChip (Illumina). In brief, 300–500 ng of RNA was reverse transcribed in cRNA and biotin-UTP labeled using the IlluminaTotalPrep RNA Amplification Kit (Ambion), and 1500 ng of cRNA was then hybridized to the Illumina HumanHT-12 v3 Expression BeadChip.

The BeadChips were scanned with the Illumina iScan system. After background subtraction, raw intensity data were exported using the Illumina Genome Studio software. Further data processing was conducted by means of R language and appropriate Bioconductor modules. Data were log2-transformed, and robust multichip average and robust spline normalization (rma_rsn) were used. Accuracy of the expression array was validated with qRT-PCR [50]. mRNA Expression levels in the tissues were determined; a fold change statistic was estimated between the two tissues, and significance was calculated using a t test.

Regulatory information using RegulomeDB

Recent evidence indicates that a significant proportion of GWAS SNPs fall within regions that are likely to affect binding of nearby proteins, such as transcription factor binding sites [31,32]. We used the RegulomeDB database to access regulatory information from ENCODE and other existing publications [19], investigating the evidence that the SNPs in the associated locus have a regulatory function. First, the linkage-disequilibrium (LD) patterns amongst the most strongly associated SNPs were determined. We then used PLINK to determine the LD structure of the associated region, using LD-patterns from the 85 Utah residents from the 1000 Genomes project [33,34]. All SNPs with $r^2$ > 0.6 were identified within a 2,000 kb window from the index SNP. All of the SNPs identified were then investigated using RegulomeDB to determine the evidence that any of the SNPs have a regulatory function.

Evaluation of age-at-onset informed approach

Permutation analysis was performed to evaluate the age-at-onset informed approach, to show that including age at onset information directly led to the increased significance, due solely to inclusion of age-at-onset information at tested SNPs. First, we identified a set of SNPs enriched for true association in the case control analysis of ischaemic stroke and subtypes. An expanded set of discovery and METASTROKE studies were analysed using standard case control methods and subsequent meta-analysis (see Table S2). SNPs with $p < 0.05$ and no evidence of heterogeneity ($p > 0.0001$) were extracted and pruned for LD ($300$ kb window, $r^2 < 0.25$), leaving a set of almost independent SNPs for further analysis. Each retained SNP represented the most significant association in each LD block, as determined by the “clump” procedure in PLINK, based on LD patterns from the CEU individuals from 1000 Genomes. The number of SNPs used in each analysis is given in Table S5. These SNP subsets were derived for ischaemic stroke, and for each stroke subset and then used in the age-at-onset informed analysis. Analysis was performed as previously for each stroke subtype using the age-at-onset informed method within studies and meta-analysis across studies (giving observed results, as obtained above). We then performed a permutation study to obtain the expected distribution of $p$-values at these SNPs. Age at onset for cases was permuted within stroke subtypes within each study, and then the data were re-analysed, for 1000 permutations. Two summary statistics were constructed: (1) within permutations, we compared $p$-values from analysis of permuted age at onset with $p$-values from the observed data, and tabulated the proportion of SNPs with increased significance in the observed data set than in the permuted data set; across permutations, we calculated the median proportion of SNPs with increased significance in the observed data; (2) Within permutations, we converted each SNP $p$-value to a Z score and summed the absolute value of the Z score across SNPs (sumZ). An empirical $p$-value for the age-informed analysis was calculated from the proportion of simulated data sets where sumZ exceeded the value in the observed analysis. This analysis was performed at SNP subsets defined from four SNP $p$-value thresholds in the discovery and METASTROKE studies: $p < 0.05$, $p < 0.005$, $p < 0.0005$, and $p < 0.00005$.

Finally, we assessed the evidence of an age-at-onset effect at the four stroke loci identified in the METASTROKE ischaemic stroke collaboration [9p21, HDAC9, PITX2, ZFHX3] [16]. For each SNP, we generated an empirical $p$-value from the proportion of permutations showing stronger association than in the observed age-at-onset informed analysis.

Supporting Information

Figure S1 QQ-plots for cardioembolic stroke and all ischaemic stroke analyses. QQ-plots of expected $p$-values (x-axis) against observed $p$-values (y-axis) for analyses of (clockwise from top left) cardioembolic stroke (age-at-onset informed), cardioembolic stroke (uninformed), all ischaemic stroke (uninformed), all ischaemic stroke (age-at-onset informed). Lambda values for each plot are given in Table S4. (DOCX)

Figure S2 QQ-plots for large artery atherosclerotic stroke and small vessel disease stroke analyses. QQ-plots of expected $p$-values (x-axis) against observed $p$-values (y-axis) for analyses of (clockwise from top left) large artery stroke (age-at-onset informed), large artery stroke (uninformed), small vessel stroke (uninformed), small
vessel stroke (age-at-onset informed). Lambda values for each plot are given in Table S4.

(DOCX)

**Figure S3** Forest plot of SNP effects for rs660599 in the large artery atherosclerotic stroke replication populations. ASGC, the Australian Stroke Genetics collaboration; deCODE, deCODE genetics; GEOS, the Genetics of early onset stroke study; HVH, the heart and vascular health study; ISGS/SWISS, the Ischaemic stroke genetics study/Siblings with Ischaemic stroke study; MGH-GASROS, Massachusetts General Hospital – Genetics affecting stroke risk and outcome. PROMiSe, Prognostic modeling in ischaemic stroke study; RACE, Risk Assessment of Cerebrovascular Events study.

(DOCX)

**Figure S4** Forest plot of SNP effects for rs660599 in the large artery atherosclerotic stroke replication populations for cases with age <61 years. ASGC, the Australian Stroke Genetics collaboration; HVH, the heart and vascular health study; ISGS/SWISS, the Ischaemic stroke genetics study/Siblings with Ischaemic stroke study; MGH-GASROS, Massachusetts General Hospital – Genetics affecting stroke risk and outcome. PROMiSe, Prognostic modeling in ischaemic stroke study.

(DOCX)

**Table S1** Results from RegulomeDB, showing the evidence that SNPs in the associated MMP12 region have a regulatory function. Scores indicate the following degrees of evidence: Score 2, TF binding; Score 3, DNase peak; Score 4, TF binding + DNase peak; Score 5, TF binding + DNase peak + DNase footprint; Score 6, TF binding + DNase peak + DNase footprint. “No data” indicates that RegulomeDB holds no information about the given SNP, meaning there currently exists no evidence to suggest that the SNP has a regulatory function. In some cases this may indicate that the SNP falls within a protein-coding region. SNP, single nucleotide polymorphism.

(DOCX)

**Table S2** Expanded set of populations used to generate SNPs with p<0.05 to evaluate the age-at-onset informed approach. ARIC, The Atherosclerosis Risk in Communities study; ASGC, Australian Stroke Genetics Collaborative; CHS, Cardiovascular Health Study; FHS, Framingham Heart Study; HPS, Heart Protection Study; HVH, The Heart and Vascular Health Study; ISGS/SWISS, The Ischemic Stroke Genetics Study/Sibbling with Ischaemic Stroke Study; MGH-GASROS, The MGH Genes Affecting Stroke Risk and Outcome Study; WTCCC2, The Wellcome Trust Case-Control Consortium II Munich; WTCCC2-UK, The Wellcome Trust Case-Control Consortium II UK; RACE, Risk Assessment of Cerebrovascular Events Study, Pakistan.

(DOCX)

**Table S3** Age and gender distributions of populations. ARIC, The Atherosclerosis Risk in Communities study; ASGC, Australian Stroke Genetics Collaborative; CHS, Cardiovascular Health Study; FHS, Framingham Heart Study; HPS, Heart Protection Study; HVH, The Heart and Vascular Health Study; ISGS/SWISS, The Ischemic Stroke Genetics Study/Sibbling with Ischaemic Stroke Study; MGH-GASROS, The MGH Genes Affecting Stroke Risk and Outcome Study; WTCCC2, The Wellcome Trust Case-Control Consortium II Munich; WTCCC2-UK, The Wellcome Trust Case-Control Consortium II UK; RACE, Risk Assessment of Cerebrovascular Events Study, Pakistan. IS, all ischaemic stroke; CE, cardioembolic stroke; LAA, large artery stroke; SVD, small vessel disease.

(DOCX)

**Table S4** Genomic inflation (λ) rates for discovery populations for age-at-onset informed and uninformed approaches. IS, all ischaemic stroke; CE, cardioembolic stroke; LAA, large artery stroke; SVD, small vessel disease.

(DOCX)

**Table S5** Number of SNPs used in evaluation of age-at-onset informed approach. IS, all ischaemic stroke; CE, cardioembolic stroke; LAA, large artery stroke; SVD, small vessel disease.

(DOCX)

**Text S1** Membership of Wellcome Trust Case Control Consortium 2 (WTCCC2).

(DOCX)

**Text S2** Liability threshold models.

(DOCX)

**Acknowledgments**

The authors thank all study staff and participants for their important contributions, and METASTROKE for granting access to study data.

**Author Contributions**

Conceived and designed the experiments: MT CML HSM. Performed the experiments: MT KMM. Contributed reagents/materials/analysis tools: LLK RM CS SB VT RL AL AS JM MM MY AA FS JRA GBB PMR PIW dB JCB DS SJK BDM JR JFM CL MD TL. Wrote the paper: MT CML HSM.

**References**


MMP12 and Large Artery Atherosclerotic Stroke


