

## Association of *MTHFR* C677T Genotype With Ischemic Stroke Is Confined to Cerebral Small Vessel Disease Subtype

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**Background and Purpose**—Elevated plasma homocysteine levels are associated with stroke. However, this might be a reflection of bias or confounding because trials have failed to demonstrate an effect from homocysteine lowering in stroke patients, although a possible benefit has been suggested in lacunar stroke. Genetic studies could potentially overcome these issues because genetic variants are inherited randomly and are fixed at conception. Therefore, we tested the homocysteine levels–associated genetic variant *MTHFR* C677T for association with magnetic resonance imaging–confirmed lacunar stroke and compared this with associations with large artery and cardioembolic stroke subtypes.

**Methods**—We included 1359 magnetic resonance imaging–confirmed lacunar stroke cases, 1824 large artery stroke cases, 1970 cardioembolic stroke cases, and 14448 controls, all of European ancestry. Furthermore, we studied 3670 ischemic stroke patients in whom white matter hyperintensities volume was measured. We tested *MTHFR* C677T for association with stroke subtypes and white matter hyperintensities volume. Because of the established association of homocysteine with hypertension, we additionally stratified for hypertension status.

**Results**—*MTHFR* C677T was associated with lacunar stroke ( $P=0.0003$ ) and white matter hyperintensity volume ( $P=0.04$ ), but not with the other stroke subtypes. Stratifying the lacunar stroke cases for hypertension status confirmed this association in hypertensive individuals ( $P=0.0002$ ), but not in normotensive individuals ( $P=0.30$ ).

**Conclusions**—*MTHFR* C677T was associated with magnetic resonance imaging–confirmed lacunar stroke, but not large artery or cardioembolic stroke. The association may act through increased susceptibility to, or interaction with, high blood pressure. This heterogeneity of association might explain the lack of effect of lowering homocysteine in secondary prevention trials which included all strokes. (*Stroke*. 2016;47:646–651. DOI: 10.1161/STROKEAHA.115.011545.)

**Key Words:** cerebral small vessel disease ■ genetic association ■ homocysteine ■ hypertension  
■ lacunar stroke ■ *MTHFR*

Elevated plasma homocysteine levels (tHcy) have been consistently associated with the risk of ischemic stroke in observational studies.<sup>1</sup> Moreover, experimental studies suggest that increases in total homocysteine aggravates vascular

disease.<sup>2</sup> However, some clinical trials that investigated the benefit of lowering tHcy with B vitamins to reduce the risk of stroke have been negative.<sup>3–6</sup> In contrast, a recent large primary prevention trial in China, the China Stroke Primary

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Prevention Trial (CSPT), which recruited only hypertensive patients, demonstrated a beneficial effect in reducing risk of stroke.<sup>7</sup> Possible reasons for this conflicting evidence include insufficient stroke phenotyping particularly if homocysteine is a predominant risk factor for one type of stroke, dietary folate fortification reducing tHcy in populations in which trials have been performed, interactions between treatment and risk factors, and insufficient treatment duration.

A subtype-specific effect, with elevated homocysteine primarily increasing risk for small vessel disease (SVD) stroke, has been suggested by both epidemiological data and secondary analysis of clinical trials. Case-control studies have suggested that elevated homocysteine is primarily a risk factor for lacunar stroke<sup>8,9</sup> and that there may be heterogeneity even within this subtype, with strongest associations in those SVD cases with multiple lacunar infarcts and confluent leukoaraiosis on magnetic resonance imaging (MRI).<sup>10</sup> Most previous clinical trials that investigated the benefit of lowering homocysteine studied stroke as a combined event lumping together all different etiologies (ie, hemorrhage and ischemic and ischemic stroke subtypes). A secondary analysis in the Vitamins to Prevent Stroke (VITATOPS) trial<sup>3</sup> found a borderline treatment effect in patients with lacunar stroke (hazard ratio 0.80 (95% confidence interval [CI] 0.67–0.96)), whereas an MRI VITATOPS substudy found vitamin-lowering therapy was associated with reduced white matter lesion volume progression in patients with severe white matter lesions.<sup>11</sup> A further possibility is that elevated homocysteine may interact with certain cardiovascular risk factors, and treatment effect may only be detected if these interactions are taken into account; of possible relevance, the positive CSPT trial was only performed in hypertensive individuals.

Another possible explanation for the conflicting epidemiological and clinical trial data is that the association between homocysteine and risk of ischemic stroke is a reflection of reverse causality or residual confounding, that is, elevated tHcy does not play a causal role in stroke pathogenesis but are merely noncausally associated with an increased risk of stroke. Genetic studies have the potential to overcome these issues by using genetic variants associated with elevated tHcy as a proxy for tHcy because the inheritance of genetic variants is random and not influenced by confounding factors. The most often studied genetic variant, showing the strongest association with increased tHcy, is the cytosine (C) to thymine (T) substitution at position 677 of the methylene tetrahydrofolate reductase (*MTHFR*) gene (rs1801133).<sup>12–14</sup> Case-control studies that investigated the association of the *MTHFR* C677T variant with stroke yielded inconsistent results, which is likely because of small sample sizes and the varying stroke phenotypes studied. Meta-analyses have produced conflicting results with an association reported between *MTHFR* and ischemic heart disease and stroke in one study but not with stroke in another.<sup>12,15</sup> Studies using detailed MRI-based stroke phenotyping have suggested the association may be confined to, or strongest in, patients with the lacunar stroke subtype.<sup>10</sup>

Based on the above data, we hypothesised that the *MTHFR* C677T variant may be a specific risk factor for SVD but not for other stroke subtypes. Lacunar infarcts are small and frequently not seen on computed tomography, and therefore, MRI is important for accurate diagnosis.<sup>16</sup> Therefore,

we determined whether the *MTHFR* polymorphism is associated with MRI-confirmed lacunar stroke. We compared these results with similar analyses from patients with cardioembolic and large artery stroke. In addition, we determined whether the same polymorphism was associated with MRI white matter hyperintensities, another marker of SVD. Because of the known association of hypertension with both tHcy and stroke, and in view of the positive results from the recent CSPT study, we also stratified the analyses by hypertension status.

## Methods

### Stroke Populations

We included 1359 MRI-defined lacunar stroke cases from the UK young lacunar stroke DNA study, the Leuven Stroke Study (LSS), and the MRI-confirmed lacunar stroke collaboration (MCLSC), including cohorts from the UK, Germany, Italy, and Australia (Table I in the online-only Data Supplement). Lacunar stroke was defined as a clinical lacunar syndrome<sup>17</sup> with an anatomically corresponding lacunar infarct on MRI (subcortical infarct  $\leq 15$  mm in diameter). All MRI scans were centrally reviewed by one physician (H.S. Markus). Exclusion criteria were stenosis  $>50\%$  in the extra- or intracranial cerebral vessels; cardioembolic source of stroke, defined according to the Trial of Organization 10172 in Acute Stroke Treatment (TOAST) criteria<sup>18</sup> as high or moderate probability; subcortical infarct  $>15$  mm in diameter, as these can be caused by embolic mechanisms (striatocapsular infarcts); any other specific cause of stroke (eg, lupus anticoagulant, cerebral vasculitis, dissection, monogenic forms of stroke, eg, cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy [CADASIL]). Large-artery and cardioembolic stroke cases were obtained from Genetic Risk Factors for Leukoaraiosis Study (GENESIS), LSS, MCLSC, and the Wellcome Trust Case Control Immunochip Consortium (WTCCC2-Immunochip), including cohorts from the UK, Germany, Belgium, Italy, Sweden, Poland, Austria, and Australia. Cases were classified into stroke subtypes according to the pathophysiological TOAST classification,<sup>18</sup> using clinical assessment, as well as brain and vascular imaging where available. Hypertension was defined as prescription of antihypertensives before stroke or systolic blood pressure  $>140$  mmHg or diastolic blood pressure  $>90$  mmHg  $>1$  week post stroke. Fourteen thousand four hundred and forty-eight ancestry-matched controls were obtained from the same geographical location as the cases in each group. A description and characteristics of all cohorts are given in Table I in the online-only Data Supplement.

### White Matter Hyperintensity Volumes Population

The white matter hyperintensity (WMH) volume population ( $n=3670$ ) was derived from the International Stroke Genetics Consortium (ISGC) WMH collaboration. This collaboration measured WMH volumes in patients with ischemic stroke from the UK, Italy, Belgium, Germany, Australia, and USA (Table II in the online-only Data Supplement). Inclusion criteria were  $>18$  years of age, self-reported European ancestry, and a diagnosis of ischemic stroke. Exclusion criteria were any other cause of white matter disease, including CADASIL, vasculitis, and demyelinating and mitochondrial disorders. MRI scans were acquired as part of routine clinical practice for evaluation of ischemic stroke. Fluid-attenuated inversion recovery sequences were primarily used for leukoaraiosis analysis; however, in their absence, T2 sequences were used. All scans were quantitatively graded to obtain a WMH volume, which was normalized for intracranial volume. WMH volume was measured in the hemisphere contralateral to the infarct and doubled to obtain whole brain volumes. Patients with bilateral nonlacunar infarcts were excluded. All neuroimaging analyses have been previously described.<sup>19</sup>

### Genotyping

Direct genotyping of rs1801133 was performed in all cohorts except WTCCC2-Immunochip using commercially available arrays from

Affymetrix or Illumina. In WTCCC2-ImmunoChip, rs1801133 was imputed from the 1000 Genomes integrated variant set (March 2012) using IMPUTE v2.<sup>20</sup> The SNP was imputed with high accuracy (imputation info score = 0.91). The SNP passed genotyping frequency thresholds (>97%) and was in Hardy–Weinberg equilibrium in all groups. To control for population stratification, individuals were removed that did not segregate with Hapmap II European populations based on ancestry informative principal component analysis using EIGENSTRAT software or multidimensional scaling in PLINK software, and ancestry-informative covariates were included in all analyses.<sup>21,22</sup>

## Statistical Analysis

In each cohort, logistic regression was performed to test for association of *MTHFR* C677T with MRI-defined lacunar stroke, cardioembolic stroke, and large artery stroke, assuming an additive model and adjusting for ancestry-informative principal components.

For each cohort, the association between WMH volume and *MTHFR* C677T was determined by performing linear regression of WMH volume on genotype dosages. Results across all cohorts were combined using a fixed-effects inverse variance weighted meta-analysis method. Subsequently, we repeated the analyses for MRI-defined lacunar stroke and WMH volume stratified by hypertension status.

We set our *P* value threshold for the main analyses to *P* < 0.05 and then used a Bonferroni-corrected value (*P* = 0.005) to assess significance in secondary analyses.

## Results

### Stroke Analyses

*MTHFR* C677T was significantly associated with lacunar stroke (odds ratio [OR] 1.20, 95% CI 1.09–1.33; *P* = 0.0003), but not with large-artery stroke (OR 1.01, 95% CI 0.93–1.08; *P* = 0.88) or cardioembolic stroke (OR 1.03, 95% CI 0.96–1.11; *P* = 0.44; Figure 1).

In the lacunar stroke cases, the association was most pronounced in homozygotes (OR 1.48, 95% CI 1.20–1.84 for TT versus CC; *P* = 0.0003 and OR 1.17, 95% CI 1.01–1.36 for CT versus CC; *P* = 0.03).

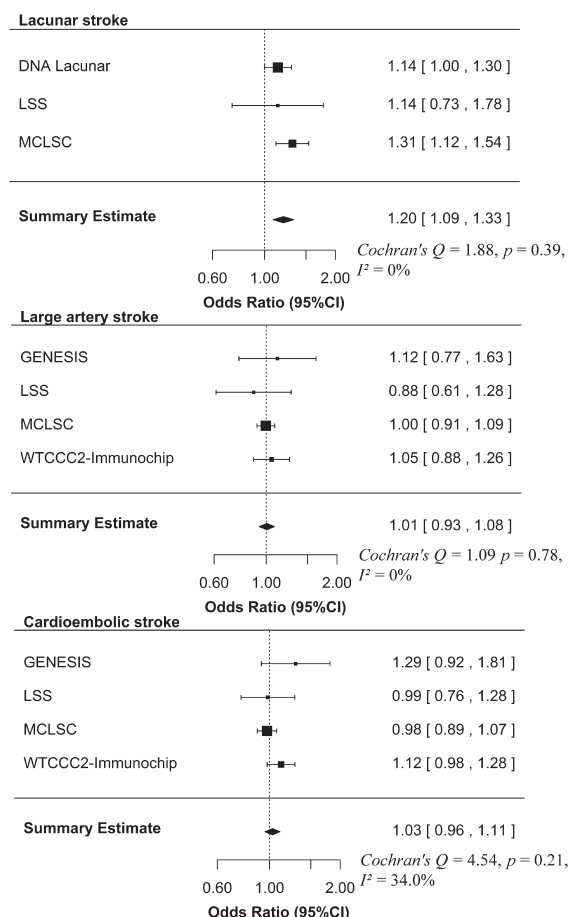
The overall prevalence of hypertension in the lacunar stroke cases was 72.6%. There were no differences in the prevalence of hypertension according to *MTHFR* genotype (72.0% in CC, 73.4% in CT, and 71.2% in TT). Stratifying the lacunar stroke cases for hypertension status demonstrated the association of *MTHFR* C677T with lacunar stroke was present in hypertensives (OR 1.24, 95% CI 1.11–1.38; *P* = 0.0002), but not in normotensives (OR 1.09, 95% CI 0.92–1.29; *P* = 0.30). In hypertensive and normotensive cases separately, the association of *MTHFR* C677T with lacunar stroke was again most pronounced in homozygotes (Table).

### WMH Volumes Analyses

*MTHFR* C677T was significantly associated with WMH volume (OR 1.06, 95% CI 1.01–1.11; *P* = 0.04; Figure 2). In the secondary analyses in which we stratified by hypertension status, there was a borderline association for either hypertensive cases (OR 1.06, 95% CI 1.00–1.13; *P* = 0.05), although this did not pass the Bonferroni-corrected threshold, whereas there was no association in normotensive cases (OR 1.02, 95% CI 0.95–1.11; *P* = 0.57).

## Discussion

In the present study, we showed that the tHcy-associated genetic variant *MTHFR* C677T was associated with lacunar



**Figure 1.** Forest plot for the association of *MTHFR* C677T with stroke subtypes. The size of the box is inversely proportional to the estimate variance of the effect estimator. GENESIS indicates Genetic Risk Factors for Leukoaraiosis Study; LSS, Leuven Stroke Study; MCLSC, magnetic resonance imaging–confirmed lacunar stroke collaboration; and WTCCC2-ImmunoChip, Wellcome Trust Case Control ImmunoChip Consortium.

stroke risk and cerebral SVD, but not for large artery or cardioembolic stroke, and that this association was restricted to patients with hypertension. Thereby, this study supports the hypothesis that homocysteine is a risk factor for specifically SVD and not for the other stroke subtypes.

Previous genetic association studies linking homocysteine to ischemic stroke have produced conflicting results.<sup>15</sup> Such candidate studies may be influenced by publication bias, which is reduced in large multicentre GWAS studies. A recent analysis of 18 SNPs associated with tHcy reported equivocal results in 12389 ischemic stroke cases (METASTROKE).<sup>23</sup> There was no association between any SNPs associated with tHcy and all ischemic stroke or large artery or cardioembolic subtypes, consistent with this study. However, one SNP was associated with lacunar stroke (rs9369898, *MUT*), but no association was found with the *MTHFR* polymorphism.

Our study is the first large-scale study to include MRI-based phenotyping of lacunar stroke. The majority of stroke cases in previous studies have relied on CT brain imaging in combination of a diagnosis of a lacunar stroke syndrome. In ≤50% of cases, a clinical lacunar syndrome is caused by pathologies other than SVD.<sup>24</sup>



**Table. Association Between *MTHFR* C677T Genotypes and MRI-Defined Lacunar Stroke**

<i>MTHFR</i> Genotype	OR	P Value
All lacunar stroke		
CT vs CC	1.17 (95% CI 1.01–1.36)	0.03
TT vs CC	1.48 (95% CI 1.20–1.84)	0.0003
Hypertensive lacunar stroke		
CT vs CC	1.19 (95% CI 1.01–1.40)	0.04
TT vs CC	1.58 (95% CI 1.25–2.00)	0.0001
Normotensive lacunar stroke		
CT vs CC	1.07 (95% CI 0.84–1.37)	0.58
TT vs CC	1.23 (95% CI 0.85–1.78)	0.28

CI indicates confidence interval; MRI, magnetic resonance imaging; and OR, odds ratio.

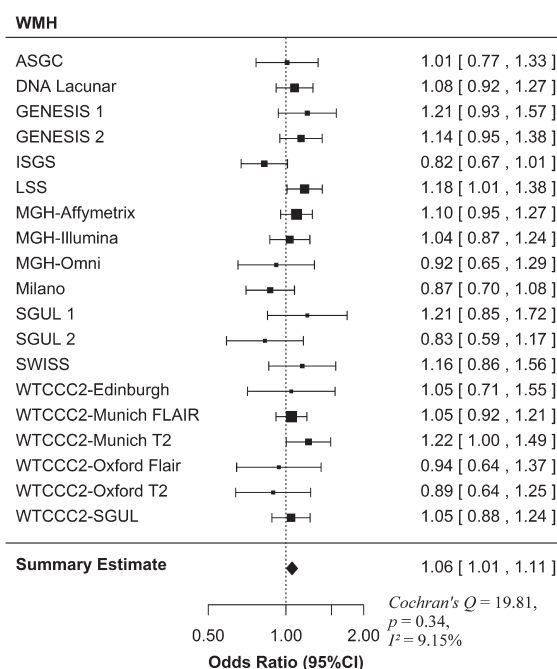
Another possible reason for the conflicting results between different studies is dietary folic acid fortification in certain populations. This was present in some cohorts in METASTROKE and might have attenuated any association with *MTHFR* C677T in METASTROKE.<sup>15</sup> In the present study, folic acid fortification was not used in any of the included stroke cohorts, but was used in some of the included WMH volume cohorts. The association shown between *MTHFR* C677T and WMH volume in the present study might have been attenuated by the inclusion of these folic acid-fortified cohorts.

The SVD-specific effect of *MTHFR* C677T in the present study might reflect the effects of increased tHcy in SVD patients with this genetic variant. Although we did not assess tHcy in the present study and, therefore, cannot draw conclusions on this association in the present study, previous studies support this hypothesis of possible SVD-specific effect of increased tHcy. Secondary analyses of the VITATOPS trial suggested that homocysteine-lowering therapy may be associated with improved outcome in SVD (both lacunar stroke and WMH) but not in other stroke subtypes.<sup>3</sup> In the VITATOPS trial, in which lacunar stroke subtyping was largely based on a clinical stroke syndrome with computed tomography imaging, a borderline significant reduction in recurrent stroke occurred in patients with SVD; based on our results, one could hypothesize that this treatment effect might be stronger in MRI-confirmed lacunar stroke. Consistent with this specific effect in SVD are the results of an MRI sub-study in 359 individuals from VITATOPS; although no association was found in the group as a whole, in a subanalysis of patients with MRI evidence of severe SVD at baseline, B vitamin supplementation was associated with a significant reduction in WMH volume change.<sup>11</sup> Two meta-analyses on previous genetic association studies linking *MTHFR* with WMH on MRI could not confirm an association between *MTHFR* and WMH.<sup>25,26</sup> Individual studies that were included in these meta-analyses had only small number of patients, and it was suggested that much larger studies would be needed to detect an association.<sup>25</sup> In the present study, we included twice as many subjects as the largest study in the previous meta-analyses that assessed WMH on a dichotomous scale and three times as many subjects as the study that assessed WMH volume.

We found that the association of *MTHFR* C677T with lacunar stroke was restricted to hypertensive individuals. One possible explanation for this finding exclusively in hypertensive individuals might be that the association acts through increased susceptibility to, or interaction with, high blood pressure. Interestingly, the recent large primary prevention CSPPT trial showed a benefit of homocysteine-lowering therapy in reducing stroke risk in hypertensive individuals from a population in which folic acid fortification was not occurring.<sup>7</sup>

The major strength of our study is the confirmation of all lacunar strokes by MRI and the relatively large sample size. Furthermore, the approach of using a genetic proxy for tHcy reduces the likelihood of reversed causality and residual confounding compared with previous observational studies. The relationship between tHcy and stroke in observational studies might be confounded by unmeasured or not adequately measured factors (eg, other dietary factors) that are causally associated with stroke. In contrast, genetic studies rely on the fact that genetic variants are fixed from conception and are not influenced by other traits.

Moreover, the stroke cohorts were derived from countries in which folic acid fortification was not implemented at the time of stroke, which maximized the chances of demonstrating an effect for *MTHFR* C677T. We also included an analysis of WMH volume in ischemic stroke patients. Stroke patients represent an enriched population in whom WMH are increased. It has been shown, however, that the genetic factors underlying



**Figure 2.** Forest plot for the association of *MTHFR* C677T with WMH. The size of the box is inversely proportional to the estimate variance of the effect estimator. ASGC indicates Australian Stroke Genetics Collaborative; GENESIS, Genetic Risk Factors for Leukoaraiosis Study; ISGS, Ischemic Stroke Genetics Study; LSS, Leuven Stroke Study; MGH, Massachusetts General Hospital; SGUL, St Georges University of London; SWISS, Sibling with Ischemic Stroke Study; and WTCCC2, Wellcome Trust Case-Control Consortium 2.

WMH in ischemic stroke patients seem to be similar to those in population-based studies of WMH.<sup>19</sup>

A potential limitation of the present study is that we did not have independent replication cohorts available to validate our findings, and therefore, future studies are warranted to confirm these interesting findings. Furthermore, although *MTHFR* C677T is strongly associated with tHcy in other studies,<sup>12,14</sup> we could not also directly assess the association of tHcy with stroke subtype in the present study because tHcy measurements were not available in all of our cohorts.

In summary, we showed that *MTHFR* C677T was associated with lacunar stroke in hypertensive individuals, supporting a possible causal role for homocysteine in the pathogenesis of cerebral SVD. Our results suggest that any future trials investigating the benefit of lowering homocysteine in stroke patients should focus on the SVD subtype and that they should incorporate MRI-based diagnosis.

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### Disclosures

None.

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## Association of *MTHFR* C677T Genotype With Ischemic Stroke Is Confined to Cerebral Small Vessel Disease Subtype

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## SUPPLEMENTAL MATERIAL

### Association of *MTHFR* C677T genotype with ischemic stroke is confined to cerebral small vessel disease subtype

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## Cohorts description

### **UK Young Lacunar Stroke DNA Study (DNA Lacunar)**

DNA Lacunar is a multicentre cohort study, which constitutes a large DNA resource of young patients with well phenotyped lacunar stroke and stroke-free community controls. Between 2005 and 2012, 1030 white patients of European ancestry with lacunar stroke, aged  $\leq 70$  years, were recruited from 72 specialist stroke centres throughout the UK. All patients underwent brain MRI, imaging of the carotid arteries and ECG. Echocardiography was performed when appropriate. All MRI's and clinical histories were reviewed centrally by one experienced stroke physician.

970 Unrelated Caucasian controls, free of clinical cerebrovascular disease, were obtained by random sampling from general practice lists from the same geographical location as the patients. Sampling was stratified for age and sex.

### **MRI-confirmed ischemic stroke collaboration (MCISC)**

#### *Wellcome Trust Case-Control Consortium 2 (WTCCC2)*

The WTCCC2 samples were genotyped as part of the WTCCC 2 ischemic stroke study. Stroke cases were recruited from three centres in the UK (St. George's University London, Oxford and Edinburgh) and one centre in Germany, University and Klinikum Großhadern, Ludwig-Maximilians-University, Munich

*WTCCC2-UK:* The St George's Stroke Study consecutively recruited ischemic stroke patients attending cerebrovascular services in London between 1995 and 2008. All patients had clinically relevant diagnostic workup performed, including brain imaging with computed tomography (CT) and/or magnetic resonance imaging (MRI) as well as ancillary diagnostic investigations including duplex ultrasonography of the carotid and vertebral arteries, echocardiography,

Holter monitoring, magnetic resonance angiography (MRA), CT-angiography (CTA) and blood tests. The Oxford Vascular Study recruited patients with acute ischemic stroke or transient ischemic attack (TIA) with evidence of infarction on brain imaging between 2002 and 2008 as part of a population-based stud. All cases were phenotyped by one experienced stroke neurologist with review of original imaging. The Edinburgh Stroke Study prospectively recruited consecutive stroke inpatients and outpatients between 2002 and 2005. An experienced stroke physician assessed each patient as soon as possible after the stroke, prospectively recording demographic and clinical details, including vascular risk factors and results of brain imaging and other investigations

*WTCCC2-Germany:* The Munich study recruited consecutively between 2002 and 2008, from a single Stroke Unit with a high rate of MR imaging ( $>80\%$ ) (n=1383). All subjects were over 18 years of age, of self-reported European ancestry and with a diagnosis of ischemic stroke classified according to TOAST by an experienced neurologist or stroke physician. All patients had brain imaging as well as ancillary diagnostic investigations where clinically relevant.

Controls for the UK samples were drawn from shared WTCCC controls obtained from the 1958 Birth Cohort. This is a prospectively collected cohort of individuals born in 1958 (<http://www.b58cgene.sgul.ac.uk/>), and ascertained as part of the national child development study (<http://www.cls.ioe.ac.uk/studies.asp?section=000100020003>). Data from this cohort are available as a common control set for a number of genetic and epidemiological studies. For the German samples controls were Caucasians of German origin participating into the population KORAgene study ([www.gsf.de/kora/en/english.html](http://www.gsf.de/kora/en/english.html)). This survey represents a gender- and age stratified random sample of all German residents of the Augsburg area and

consists of individuals 25 to 74 years of age, with about 300 subjects for each 10-year increment. All controls were free of a history of stroke or transient ischemic attack.

#### *Besta Stroke Study (Milano)*

This study includes consecutive Italian patients referred to Besta Institute from 2000 to 2009 with stroke and included in the Besta Cerebrovascular Diseases Registry (CEDIR). Ischemic stroke cases, first ever or recurrent, confirmed on brain imaging, were selected for this study. An experienced stroke neurologist assessed all cases.

#### *Australian Stroke Genetics Collaborative (ASGC)*

Stroke cases comprised European-ancestry patients admitted to four clinical centres across Australia (The Neurosciences Department at Gosford Hospital, Gosford, New South Wales (NSW); the Neurology Department at John Hunter Hospital, Newcastle, NSW; The Queen Elizabeth Hospital, Adelaide; and the Royal Perth Hospital, Perth) between 2003 and 2008. Stroke was defined by WHO criteria as a sudden focal neurologic deficit of vascular origin, lasting more than 24 hours and confirmed by brain imaging. Other investigative tests such as electrocardiogram, carotid Doppler and trans-oesophageal echocardiogram were conducted to define stroke aetiology as clinically appropriate.

#### **Leuven Stroke Study**

Patients with cerebral ischemia, defined as a clinical stroke with imaging confirmation or a TIA with a new ischemic lesion on diffusion weighted MRI, who were admitted to the Stroke Unit of the University Hospitals in Leuven were enrolled. All patients underwent brain imaging and a standardized protocol including carotid ultrasound or CT angiography and cardiac examination (echocardiography and Holter monitoring) in all patients.

Control individuals were selected from the same population and were either spouses of patients with multiple sclerosis, amyotrophic lateral sclerosis or stroke or healthy community dwelling subjects partially from the Leuven University Gerontology Database. Controls either confirmed they never had a stroke or TIA or responded negative to any item of the Verification of Stroke Free Status questionnaire.

#### **GENESIS**

This study recruited patients attending cerebrovascular services at St. George's Hospital, London between 2011-2013. All patients had clinically relevant diagnostic workup performed, including brain imaging with magnetic resonance imaging (MRI) as well as ancillary diagnostic investigations including duplex ultrasonography of the carotid and vertebral arteries, echocardiography, Holter monitoring, magnetic resonance angiography (MRA), CT-angiography (CTA) and blood tests

#### **WTCCC2-ImmunoChip**

##### *Bio-Repository of DNA in Stroke (BRAINS): London*

The Bio-Repository of DNA in Stroke (BRAINS) is an international study recruiting highly phenotyped patients with stroke. For the purposes of the current work all patients were Caucasians. Diagnosis of stroke was confirmed using positive imaging (MRI or CT) and ischemic stroke subtypes were assigned using TOAST criteria, based on clinical, imaging and risk factor data. The cohort has been described in detail elsewhere (Yadav S, Schanz R,

Maheshwari A, et al. Bio-Repository of DNA in stroke (BRAINS): a study protocol. BMC Med Genet 2011; 12: 34).

#### *Glasgow: Scotland*

Patients with ischemic stroke attending the cerebrovascular service of the Western Infirmary, Glasgow, were recruited between 1990 and 2004 as part of an ongoing study of genetic and circulating biomarkers in stroke. All patients underwent brain imaging and extracranial carotid ultrasound in accordance with a standard clinical protocol. The study was approved by the West Ethics Committee.

Controls for the UK samples were drawn from shared WTCCC controls obtained from the 1958 Birth Cohort. This is a prospectively collected cohort of individuals born in 1958 (<http://www.b58cgene.sgul.ac.uk/>), and ascertained as part of the national child development study (<http://www.cls.ioe.ac.uk/studies.asp?section=000100020003>). Data from this cohort are available as a common control set for a number of genetic and epidemiological studies.

#### *Lund Stroke Register, Sweden*

Lund Stroke Register (LSR) since 2001 continuously enrolls patients aged 18 and older with first-ever stroke, living in the primary uptake area of Skane University Hospital, Lund. The study is mainly hospital-based but has a good coverage of the whole geographical population.<sup>28</sup> All included patients are examined with CT/MR or autopsy of the brain. When clinically indicated, the patients are examined with ultrasound imaging of carotid arteries, echocardiography, and angiography. In this study, first-ever ischemic stroke patients from LSR between 2001 and 2004 were included. All patients were assessed by a neurologically trained physician regarding stroke type. Informed consent was obtained from all individuals or when they were not able to respond from their next-of-kin. The study was approved by the Ethics Committee of Lund University. Biobank services were performed at Region Skane Competence Centre (RSKC Malmo), Malmo University Hospital, Malmo, Sweden.

#### *Swedish Control Samples*

Controls for the Lund cases were provided by the Swedish SLE network. Controls were healthy blood donors from the geographical areas of Uppsala, Stockholm and Lund. The studies were approved by the regional ethics boards and all subjects gave their informed consent to participate. Genotyping of the Swedish control samples was performed at the SNP&SEQ technology platform in Uppsala, Sweden ([www.genotyping.se](http://www.genotyping.se)).

#### *Munich: Germany*

Cases were consecutive European Caucasians recruited from a single dedicated Stroke Unit at the Department of Neurology, Klinikum Großhadern, Ludwig-Maximilians-University, Munich. Ischemic stroke subtypes were determined according to TOAST criteria based on relevant clinical and imaging data.

#### *German control samples*

German healthy control individuals were obtained from the PopGen biobank [Krawczak et al., Community Genet 9:55-61, 2006]. Written, informed consent was obtained from all study participants and all protocols were approved by the institutional ethical review committee of the participating centre. The panel is a cross-sectional control cohort from the Kiel area in Northern Germany. More than 300 phenotypes were collected for the cohort and a 3-year follow has recently been completed. All data and biomaterials are accessible via the PopGen biobank. The Genotyping was part of the German National Genome Research Network

(NGFN) GWAS initiative and performed by the Institute of Clinical Molecular Biology (Christian-Albrechts-University of Kiel).

*Poland:Krakow*

Patients were recruited in the stroke unit of the Jagiellonian University in Krakow, Poland (a single-center study). All stroke patients and controls were >18 years of age and were white. All patients had clinically relevant diagnostic workup performed, including brain imaging with computed tomography (CT) (100%) and/or magnetic resonance imaging (MRI) (8%) as well as ancillary diagnostic investigations including duplex ultrasonography of the carotid and vertebral arteries (85.2%), echocardiography (54.8%). MR-angiography, CT-angiography, Holter monitoring, transesophageal echocardiography and blood tests for hypercoagulability were performed where indicated. Patients were classified into etiologic subtypes according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST).

The control group included unrelated subjects taken from the population of southern Poland. Control subjects had no apparent neurological disease based on the findings in a structured questionnaire and a neurological examination. The study was approved by local research ethics committees and informed consent was obtained from all participants.

**White Matter Hypertensities collaboration International Stroke Genetics Consortium**

*St Georges University of London (SGUL)*

This study recruited patients attending cerebrovascular services at St. George's Hospital, London between 2007-2011. All patients had clinically relevant diagnostic workup performed, including brain imaging with magnetic resonance imaging (MRI) as well as ancillary diagnostic investigations including duplex ultrasonography of the carotid and vertebral arteries, echocardiography, Holter monitoring, magnetic resonance angiography (MRA), CT-angiography (CTA) and blood tests

*Massachusetts General Hospital (MGH)*

Cases presenting with ischemic stroke and admitted to the Massachusetts General Hospital (MGH) Stroke Unit through the Emergency Department, or evaluated in the MGH Neurology outpatient clinics, as well as on the inpatient Medical and Vascular Surgical services from January 2003 to July 2008. Ischemic stroke was defined as either (1) a radiographically proven (head CT or MRI) infarct associated with the appropriate clinical stroke syndrome, or (2) a fixed neurological deficit persisting more than 24 hours, consistent with a vascular pattern of involvement and without radiographic evidence of demyelinating or other non-vascular disease. All subjects were evaluated by a neurologist upon presentation and clinical and laboratory data were collected during the admission for qualifying ischemic stroke event. All patients had acute brain imaging as well as ancillary diagnostic investigations: cervical and intracranial vessel imaging using CT or MR angiography (75%), cervical ultrasound (24%), echocardiography (86%), and Holter monitoring (16%).

*Ischemic Stroke Genetics Study (ISGS)*

Ischemic Stroke Genetics Study (ISGS) was a 5-center, prospective, case-control study of first-ever ischemic stroke cases in the United States. All affected individuals had WHO-defined stroke confirmed by a study neurologist to be ischemic on the basis of head CT or brain MRI. Peripheral blood DNA samples were collected between May 2003 and September 2008.



*Sibling with Ischaemic Stroke Study (SWISS)*

This is a prospective, multicentre study of sibling pairs with first-ever or recurrent ischemic stroke. Probands were recruited from 70 clinical centres across the US and Canada. Ischemic stroke affected and unaffected siblings were recruited primarily using proband-initiated contact. All affected individuals had WHO-defined stroke confirmed by a study neurologist to be ischemic on the basis of brain imaging. Peripheral blood DNA samples were collected between October 2000 and December 2009.

## Appendix

### UK Young Lacunar Stroke DNA Study collaborators

*Study managers:* Josie Monaghan; Alan Zanich, Samantha Febrey, Eithne Smith, Jenny Lennon, St George's University of London

*Participating centres (number of enrolled patients per centre; local investigators):*

Aberdeen Royal Infirmary, Aberdeen (12; Mary Macleod). Addenbrooke's Hospital, Cambridge (54; Jean-Claude Baron, Elizabeth Warburton, Diana J Day, Julie White). Airedale General Hospital, Steeton (4; Samantha Mawer). Barnsley Hospital, Barnsley (3; Mohammad Albazzaz, Pravin Torane, Keith Elliott, Kay Hawley). Bart's and the London, London (2; Patrick Gompertz). Basingstoke and North Hampshire Hospital, Basingstoke (13; Elio Giallombardo, Deborah Dellafera). Blackpool Victoria Hospital, Blackpool (11; Mark O'Donnell). Bradford Royal Infirmary, Bradford (1; Chris Patterson). Bristol Royal Infirmary, Bristol (8; Sarah Caine). Charing Cross Hospital, London (12; Pankaj Sharma). Cheltenham General and Gloucester Royal Hospitals, Cheltenham and Gloucester (10; Dipankar Dutta). Chesterfield Royal Hospital, Chesterfield (4; Sunil Punnoose, Mahmud Sajid). Countess of Chester Hospital, Chester (22; Kausik Chatterjee). Derriford Hospital, Plymouth (4; Azlisham Mohd Nor). Dorset County Hospital NHS Foundation Trust, Dorchester (6; Rob Williams). East Kent Hospitals University NHS Foundation Trust, Kent (22; Hardeep Baht, Guna Gunathilagan). Eastbourne District General Hospital, Eastbourne (4; Conrad Athulathmudali). Frenchay Hospital, Bristol (1; Neil Baldwin). Frimley Park Hospital NHS Foundation Trust, Frimley (6; Brian Clarke). Guy's and St Thomas' Hospital, London (14; Tony Rudd). Institute of Neurology, London (25; Martin Brown). James Paget University Hospital, Great Yarmouth (1; Peter Harrison). King's College Hospital, London (16; Lalit Kalra). Leeds Teaching Hospitals NHS Trust, London (125; Ahamad Hassan). Leicester General Hospital and Royal Infirmary, Leicester (9; Tom Robinson, Amit Mistri). Luton and Dunstable NHSFT University Hospital, Luton (16; Lakshmanan Sekaran, Sakthivel Sethuraman, Frances Justin). Maidstone and Tunbridge Wells NHS Trust (3; Peter Maskell). Mayday University Hospital, Croydon (14; Enas Lawrence). Medway Maritime Hospital, Gillingham (5; Sam Sanmuganathan). Milton Keynes Hospital, Milton Keynes (1; Yaw Duodu). Musgrove Park Hospital, Taunton (9; Malik Hussain). Newcastle Hospitals NHS Foundation Trust, Newcastle upon Tyne (12; Gary Ford). Ninewells Hospital, Dundee (5; Ronald MacWalter). North Devon District Hospital, Barnstaple (8; Mervyn Dent). Nottingham University Hospitals, Nottingham (17; Philip Bath, Fiona Hammonds). Perth Royal Infirmary, Perth (2; Stuart Johnston). Peterborough City Hospital, Peterborough (1; Peter Owusu-Agyei). Queen Elizabeth Hospital, Gateshead (5; Tim Cassidy, Maria Bokhari). Radcliffe Infirmary, Oxford (5; Peter Rothwell). Rochdale Infirmary, Rochdale (4; Robert Namushi). Rotherham General Hospital, Rotherham (1; James Okwera). Royal Cornwall Hospitals NHS Trust, Truro (11; Frances Harrington, Gillian Courtauld). Royal Devon and Exeter Hospital, Exeter (22; Martin James). Royal Hallamshire Hospital, Sheffield (1; Graham Venables). Royal Liverpool University Hospital and Broadgreen Hospital, Liverpool (9; Aravind Manoj). Royal Preston Hospital, Preston (18; Shuja Punekar). Royal Surrey County Hospital, Guildford (23; Adrian Blight, Kath Pasco). Royal Sussex County Hospital, Brighton (14; Chakravarthi Rajkumar, Joanna Breeds). Royal United Hospital, Bath (6; Louise Shaw, Barbara Madigan). Salford Royal Hospital, Salford (16; Jane Molloy). Southampton General Hospital, Southampton (1; Giles Durward). Southend Hospital, Westcliff-on-Sea (26; Paul Guyler). Southern General Hospital, Glasgow (34; Keith Muir,

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**Table I** Stroke cohorts

<b>Cohort</b>	<b>N</b>	<b>Mean age (sd)</b>	<b>% Male</b>	<b>% Hypertensive</b>
<i>DNA Lacunar &amp; GENESIS</i>				
Lacunar stroke	998	57.3 (9.6)	704 (71%)	705 (71%)
Large artery stroke	64	70.1 (11.6)	44 (69%)	
Cardioembolic stroke	80	73.5 (14.6)	47 (59%)	
Controls	970	59.7 (4.3)	510 (53%)	NA
<i>MCLSC</i>				
Lacunar stroke	319	68.5 (11.8)	190 (60%)	262 (82%)
Large artery stroke	1338	68.9 (11.9)	904 (68%)	906 (68%)
Cardioembolic stroke	1094	73.7 (13.8)	558 (51%)	761 (70%)
Controls	7623	62.5 (10.2)	4002 (52%)	NA
<i>LSS</i>				
Lacunar stroke	42	65.5 (13.9)	29 (69%)	26 (62%)
Large artery stroke	70		43 (61%)	48 (69%)
Cardioembolic stroke	157		96 (61%)	98 (62%)
Controls	455	55.7 (14.5)	212 (47%)	NA
<i>WTCCC2-ImmunoChip</i>				
Large artery stroke	352	69.1 (12.2)	220 (62%)	234 (67%)
Cardioembolic stroke	639	75.7 (11.8)	339 (53%)	417 (67%)
Controls	5401	NA	2304 (43%)	NA

Abbreviations: DNA Lacunar, UK Young Lacunar Stroke DNA Study; GENESIS, Genetic Risk factors for Leukoaraiosis study; MCLSC, MRI-confirmed lacunar stroke collaboration; LSS, Leuven Stroke Study, WTCCC2-ImmunoChip, Wellcome Trust Case-Control Consortium II ImmunoChip



**Table II** WMH study populations

<b>Centre</b>	<b>Country</b>	<b>N</b>	<b>Mean age (sd)</b>	<b>% Male</b>	<b>% Hypertensive</b>
Milano	Italy	151	57 (14)	60%	57%
WTCCC2-Edinburgh	UK	64	68 (13)	50%	72%
WTCCC2-Munich FLAIR	Germany	447	66 (12)	66%	72%
WTCCC2-Munich T2	Germany	203	67 (12)	55%	67%
WTCCC2-Oxford Flair	UK	65	65 (15)	54%	65%
WTCCC2-Oxford T2	UK	75	67 (13)	59%	68%
WTCCC2-SGUL	UK	323	70 (14)	63%	77%
GENESIS 1	UK	121	67 (14)	67%	62%
GENESIS 2	UK	228	69 (15)	58%	76%
SGUL 1	UK	70	70 (13)	61%	61%
SGUL 2	UK	57	68 (14)	58%	72%
DNA Lacunar	UK	303	57 (9)	72%	68%
LSS	Belgium	361	66 (15)	58%	59%
MGH-Affymetrix	US	476	67 (14)	60%	64%
MGH-Omni	US	84	64 (15)	63%	68%
MGH-Illumina	US	228	66 (15)	64%	61%
ASGC	Australia	96	65 (13)	57%	77%
ISGS	US	207	68 (14)	62%	61%
SWISS	US	111	66 (11)	48%	74%
Overall		3670			

Abbreviations: Milano, Besta Stroke Register; WTCCC2, The Wellcome Trust Case-Control Consortium II; GENESIS, Genetic Risk factors for Leukoaraiosis study; SGUL, St Georges University of London; DNA Lacunar, UK Young Lacunar Stroke DNA Study; LSS, Leuven Stroke Study; MGH, Massachusetts General Hospital; ASGC, Australian Stroke Genetics Collaborative; ISGS, Ischemic Stroke Genetics Study; SWISS, Sibling with Ischaemic Stroke Study