Common NOTCH3 Variants and Cerebral Small-Vessel Disease

Loes C.A. Rutten-Jacobs, PhD*; Matthew Traylor, PhD*; Poneh Adib-Samii, MBBS; Vincent Thijs, MD; Cathie Sudlow, MD; Peter M. Rothwell, MD; Giorgio Boncoraglio, MD; Martin Dichgans, MD; Steve Bevan, PhD; James Meschia, MD; Christopher Levi, MD; Natalia S. Rost, MD; Jonathan Rosand, MD; Ahamad Hassan, MRCP; Hugh S. Markus, MD

Background and Purpose—The most common monogenic cause of cerebral small-vessel disease is cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, caused by NOTCH3 gene mutations. It has been hypothesized that more common variants in NOTCH3 may also contribute to the risk of sporadic small-vessel disease. Previously, 4 common variants (rs10404382, rs1043994, rs10423702, and rs1043997) were found to be associated with the presence of white matter hyperintensity in hypertensive community-dwelling elderly.

Methods—We investigated the association of common single nucleotide polymorphisms (SNPs) in NOTCH3 in 1350 patients with MRI-confirmed lacunar stroke and 7397 controls, by meta-analysis of genome-wide association study data sets. In addition, we investigated the association of common SNPs in NOTCH3 with MRI white matter hyperintensity volumes in 3670 white patients with ischemic stroke. In each analysis, we considered all SNPs within the NOTCH3 gene, and within 50-kb upstream and downstream of the coding region. A total of 381 SNPs from the 1000 genome population with a mean allele frequency >0.01 were included in the analysis. A significance level of P<0.0015 was used, adjusted for the effective number of independent SNPs in the region using the Galway method.

Results—We found no association of any common variants in NOTCH3 (including rs10404382, rs1043994, rs10423702, and rs1043997) with lacunar stroke or white matter hyperintensity volume. We repeated our analysis stratified for hypertension but again found no association.

Conclusions—Our study does not support a role for common NOTCH3 variation in the risk of sporadic small-vessel disease. *(Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.114.008540.)*

Key Words: CADASIL ■ cerebral small vessel diseases ■ genetic association studies ■ stroke, lacunar

Cerebral small-vessel disease (SVD) accounts for nearly one quarter of all ischemic strokes and is an important cause of dementia. Lacunar infarction and white matter hyperintensities (WMH) on MRI are lesions commonly seen in SVD. Genetic factors have been suggested to play an important role in SVD.1-3 Several monogenic causes of SVD have been described, the most common of which is cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). CADASIL is caused by mutations in the NOTCH3 gene, and among the main features are recurrent ischemic strokes and white matter lesions on MRI.4 Besides CADASIL, causing mutations, it has been suggested that more common variants in NOTCH3 may also contribute to the risk of sporadic SVD.5 This study in a community-dwelling elderly cohort, the Austrian Stroke Prevention Study, found 4 common single
nucleotide polymorphism (SNP) polymorphisms at the NOTCH3 gene (rs10404382, rs1043994, rs10423702, and rs1043997) to be associated with the presence of WMH. However, these associations seemed to be restricted to hypertensive subjects. In contrast, another study in 120 patients with lacunar stroke found no association between 2 common NOTCH3 SNPs (rs3815188 and rs1043994) and the presence of WMH.8 One other study investigated the association between common NOTCH3 variation and ischemic stroke in white patients.7 This study identified the SNP rs78501403 to be associated with ischemic stroke, but power was lacking to investigate this association in the SVD subtype. Lacunar infarcts are small and frequently not seen on computed tomography; therefore, MRI is important for accurate diagnosis.

To test the hypothesis that common NOTCH3 variation is associated with SVD, we investigated the association of common variants in NOTCH3 with both clinical and MRI-confirmed lacunar stroke and with WMH lesion volume quantified on MRI.

**Methods**

**Lacunar Stroke Population**

Lacunar stroke cases were obtained from cohorts from the United Kingdom, Germany, and Belgium (n=1350; aged, 60 years [SD, 11]; 68% men; Table I in the online-only Data Supplement). Lacunar stroke was defined as a clinical lacunar syndrome9 with a compatible lesion on MRI (subcortical infarct ≤15 mm in diameter). Exclusion criteria were as follows: stenosis >50% in the extra- or intracranial cerebral vessels; cardioembolic source of stroke, defined according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria10 as high or moderate probability; subcortical infarct >15 mm in diameter, as these can be caused by embolic mechanisms (stria-atrocapsular infarcts); any other specific cause of stroke (eg, lupus anticoagulant, cerebral vasculitis, and dissection). A description of all cohorts is given in the online-only Data Supplement. Controls (n=7397) for the United Kingdom and German analyses were derived from population cohorts and were therefore not confirmed to be stroke free. Belgian controls were ascertained from the local population.

SVD stroke subtype, classified using the TOAST criteria,10 and leukoaraiosis grading using the semiquantitative Fazekas scale was performed with central review of all MRI scans by 1 physician (H.S.M.). The Fazekas scale has been shown to reflect pathological severity of SVD in a postmortem validation study.11 In addition, lacunar infarcts were determined as high signal lesions <1.5 cm diameter on acute diffusion-weighted imaging sequences or fluid attenuated inversion recovery or low signal lesions on T1 sequences.

A preplanned secondary analysis was performed in those SVD cases with confluent leukoaraiosis (Fazekas grade >2) or multiple lacunar infarcts (n=717; 53%), as cases of CADASIL present with this phenotype, and compared with the controls (n=7397).

**WMH Volumes Population**

The WMH volumes population (n=3670) was derived from ischemic stroke cohorts from United Kingdom, Italy, Belgium, Germany, Australia, and United States (Table II in the online-only Data Supplement). Inclusion criteria were as follows: aged >18 years, self-reported European ancestry, and a diagnosis of ischemic stroke. Exclusion criteria were CADASIL, vasculitis, and demyelinating and mitochondrial disorders. For the present study, we included all available patients with ischemic stroke from each cohort who met the inclusion and exclusion criteria and had MRI and genome-wide association study data available. MRI scans were acquired as a 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 infarcts and doubled to obtain whole brain volumes. All neuroimaging analyses have been previously described.12

**Genotyping**

Genotyping of all cohorts was performed on commercially available arrays from Affymetrix or Illumina. All cohorts performed extensive quality control steps before imputation, removing SNPs showing significant departure from Hardy-Weinberg equilibrium, high levels of missingness or low minor allele frequency. Individuals were removed that did not segregate with Hapmap II European populations based on ancestry informative principal component (PC) analysis using EIGENSTRAT software package or multidimensional scaling in PLINK software package.13 In addition, individuals with high levels of missingness or heterozygosity were excluded. All data sets were imputed to 1000 genomes integrated variant set (March 2012) using IMPUTE version 2.14

**Lacunar Stroke Analyses**

We analyzed binary case/control status for each lacunar stroke population using a score test, as implemented in SNPTTEST version 2.5. Imputed genotype probabilities were taken into account using a missing data likelihood score test or an expectation-maximization method for SNPs with low mean allele frequency or high uncertainty. The first 2 ancestry informative PCs, age and sex were included as covariates in the model where possible (sex and PC1, PC2 only in the UK-Wellcome Trust Case-Control Consortium-2 and Germany-Wellcome Trust Case-Control Consortium-2 studies; Table I in the online-only Data Supplement). We meta-analyzed the 4 cohorts using a fixed-effects inverse variance-weighted method as implemented in METAL.15 We first performed analyses using an additive model and then under dominant and recessive models.

**WMH Volumes Analysis**

The association between WMH volume and each autosomal SNP was determined by performing linear regression of WMH volume on genotype dosages using PLINK version 1.07.15 SNPs with PLINK INFO (information content metric) score >0.7 or mean allele frequency <0.01 were removed from further analyses. We used genomic inflation to evaluate inflation of test statistics in each center.16 Results across all centers were combined using a fixed-effects inverse variance-weighted method using METAL.15 Heterogeneity was assessed using Cochran $q$ statistic. After the meta-analysis, we considered only SNPs present in >12 centers, and with heterogeneity $P>0.001$, for analysis.

**NOTCH3 SNPs Analyzed and Assessment of Statistical Significance**

In each analysis, we considered all SNPs within the NOTCH3 gene, and within 50-kb upstream and downstream of the coding region. A total of 381 SNPs from the 1000 genomes population with mean allele frequency >0.01 were included in the analysis. We used the Galway method to estimate the effective number of independent SNPs in the region,17 based on the linkage disequilibrium patterns from European individuals in the 1000 genomes population.18 This method has been shown to give the best agreement with random permutations. Using the method, we estimated there to be 34 effective independent SNPs in the region. Therefore, we set our $P$ value threshold for each analysis to $P<0.0015$. Power calculations were conducted using the Genetic Power Calculator for a case–control study of discrete traits under an additive disease risk model and a disease prevalence of 0.2% for lacunar stroke.18
Results

Lacunar Stroke Analyses

We first tested for an association of any NOTCH3 SNP with lacunar stroke under an additive model. No SNP met our criteria for statistical significance for association with lacunar stroke. Results for all SNPs in the region by chromosomal position are given in Figure 1A.

We then performed secondary analyses under recessive and dominant models. Again, none of these SNPs met our criteria for statistical significance (Figure 1A and 1B in the online-only Data Supplement). All SNPs had \( P > 0.005 \) in all analyses.

We next tested for association of any NOTCH3 SNP in those patients with lacunar stroke who also had confluent leukoaraiosis or multiple lacunar infarcts (n=717) under an additive model.

No SNP met our criteria for statistical significance for association with lacunar stroke with confluent leukoaraiosis or multiple lacunar infarcts (Figure 1B). The associations for the SNPs that were identified in previous studies are given in the Table. Power calculations showed that we had >95% power to replicate these findings (Table III in the online-only Data Supplement).

Secondary analyses under recessive and dominant models also revealed no significant associations (Figure IIA and IIB in the online-only Data Supplement). All SNPs had \( P > 0.005 \) in all analyses.

WMH Volumes Analysis

Similarly, no SNP met our criteria for statistical significance for association with WMH volumes. Results for all SNPs in
the region by genomic position are given in Figure 2. Forest plots showing the associations of rs10404382, rs1043994, rs10423702, and rs1043997 with WMH per cohort are shown in the Figure IIIA–IIID in the online-only Data Supplement.

We repeated the latter analysis in only hypertensive subjects (n=2466), but again none of the SNPs met our criteria for statistical significance (Figure IV in the online-only Data Supplement).

**Discussion**

Mutations in the *NOTCH3* gene cause CADASIL, a hereditary form of SVD. Common variants in the *NOTCH3* gene have been suggested to also confer risk of sporadic SVD. To test this hypothesis, we analyzed *NOTCH3* in an imputed genome-wide association study data set of 1350 cases and 7397 controls. We found no evidence that common variants in *NOTCH3* associated with risk of lacunar stroke or WMH.

Our observation is in contrast to a recent study in a community-dwelling elderly cohort, the Austrian Stroke Prevention study, which found 4 common variants at the *NOTCH3* gene to be associated with the presence of WMH although only in hypertensive subjects. The SNP that showed the strongest association, rs10404382, was replicated within the Cohorts for Heart and Ageing Research in Genomic Epidemiology (CHARGE) Consortium. The associations found in the Austrian Stroke Prevention study were all confined to hypertensive subjects. In the present study, we failed to replicate any of these findings in the present study, even when only hypertensive patients were studied. There might be several

**Table. Association of Single Nucleotide Polymorphisms Reported in Previous Publications With WMH and Lacunar Stroke**

|                | WMH                        | Lacunar Stroke | Lacunar Stroke With Leukoaraiosis or Multiple Lacunar Infarcts |
|----------------|----------------------------|----------------|---------------------------------------------------------------|
|                | MAF | OR (95% CI)* | P Value | MAF | OR (95% CI)* | P Value | MAF | OR (95% CI)* | P Value |
| Schmidt et al5 |     |              |         |     |              |         |     |              |         |
| rs10404382     | 0.12| 1.04 (0.97–0.11) | 0.33 | 1.08 (0.92–1.26) | 0.36 | 1.05 (0.87–1.27) | 0.61 |
| rs1043994      | 0.12| 1.03 (0.96–1.11) | 0.34 | 1.08 (0.92–1.26) | 0.33 | 1.06 (0.88–1.29) | 0.54 |
| rs10423702     | 0.12| 1.04 (0.97–1.11) | 0.31 | 1.07 (0.92–1.25) | 0.39 | 1.05 (0.87–1.27) | 0.63 |
| rs1043997      | 0.13| 1.04 (0.97–1.11) | 0.23 | 1.10 (0.95–1.28) | 0.20 | 1.10 (0.91–1.32) | 0.31 |
| Ross et al7    |     |              |         |     |              |         |     |              |         |
| rs78501403     | <0.01|† | ... | † | ... | † | ... | † |
| rs61749020     | 0.02| 1.32 (0.83–2.10) | 0.25 | 1.31 (0.75–2.29) | 0.33 |
| rs3815188      | 0.22| 1.11 (0.68–1.81) | 0.66 | 1.31 (0.73–2.35) | 0.37 |

CI indicates confidence interval; MAF, mean allele frequency; OR, odds ratio; and WMH, white matter hyperintensity.

*The reported associations are adjusted for age, sex, and the first 2 ancestry informative principal components.
†These variants were not genotyped in the specific populations because of a too low MAF.

**Figure 2.** Association of common *NOTCH3* variants with white matter hyperintensity volumes. SNP indicates single nucleotide polymorphism.
explanations for the discrepancy between the results of the Austrian Stroke Prevention study and the present study. First, our negative finding might be because of a type II error. The 4 SNPs associated with the presence of WMH in hypertensive patients had an odds ratio between 2.1 and 3.2. We performed power calculations, and we had an estimated 100% power to detect these associations in our study (Table III in the online-only Data Supplement) This makes type II error unlikely.

Second, there are differences in the populations studied. WMH lesion volume in our study was measured in clinical ischemic stroke populations, whereas the populations in the Austrian Stroke Prevention study and the CHARGE consortium were community-dwelling elderly free from stroke. WMH are more frequent in patients with a history of stroke than in healthy age-matched individuals.19 It might be that the underlying pathology of WMH differs between patients with a history of stroke and community-dwelling elderly free from stroke. However, it is likely that, at least to some extent, there is an overlap in pathology because the WMH-associated locus 17q25, which was previously identified in the CHARGE consortium, was successfully replicated in the WMH populations used in the present study.11

Third, methods to assess WMH volume differed between studies. Grading of WMH was done in a similar manner in the present study and Austrian Stroke Prevention study because both used the Fazekas scale and the same cutoff for the presence of WMH.

WMH volume measurements in the present study were done using a similar semiautomatic method in all cohorts, with a good agreement between the 2 main reading centers (intraclass correlation coefficient, 0.95; 95% confidence interval, 0.91–0.97; n=50). Also in the Austrian Stroke Prevention study, a semiautomatic method was used to measure the WMH volume. In the CHARGE consortium, WMH volume measurements were done using 2 different approaches; in most cohorts, either an automatic or a semiautomatic method is used, but in some cohorts, a semiquantitative rating scale was used.

Fourth, statistical analysis differed between the studies. In the Austrian Stroke Prevention, analyses were adjusted for several potential confounding risk factors: age, sex, diabetes mellitus, and cardiac disease. We repeated the analysis for association with the 4 SNPs detected in the previous study, adjusted for age, sex, hypertension, and diabetes mellitus in a subset of the patients with lacunar stroke and available data on hypertension and diabetes mellitus status (Table IV in the online-only Data Supplement). The additional adjustment for hypertension and diabetes mellitus did not significantly change the estimates. In addition, population structure is an important source of confounding to account for in genetic association studies.12 In contrast to the analysis reported to be done in Austrian Stroke Prevention study and its replication in CHARGE, we accounted for population structure in our analysis by including 2 ancestry informative PCs in every analysis.

Furthermore, we applied a correction for multiple testing in the analysis of the present study, based on the effective number of independent SNPs in the studied region (Galwey method).17 In the Austrian Stroke Prevention study, no correction for multiple testing was used in their analyses, which enhances the possibility of false-positive findings.

One other study investigated the association between common NOTCH3 variants and ischemic stroke and revealed an association for 1 SNP, rs78501403.2 Unfortunately, we could not investigate this SNP in our study because the minor allele frequency of this SNP is generally <0.01% in white populations and therefore the SNP was not present in the 1000 genomes population to which we imputed our data to.14 Surprisingly, this SNP had a minor allele frequency of 3.3% in the white population in this previous study. Consistent with our finding, 1 previous study in lacunar stroke found no association between 2 common NOTCH3 SNPs and the presence of WMH.6

There are several limitations in this study. We used the approach of genotyping and then imputing all cohorts to the 1000 genomes population. Although this method provides good performance for identifying common variants, the quality of imputation can drop at mean allele frequency <5%, meaning we cannot rule out associations at these frequencies. Similarly, the size of some of the WMH cohorts was small, meaning low-frequency variants could not be assessed in this analysis.

In summary, our results do not support a role for common NOTCH3 variation in the risk of sporadic SVD.

Acknowledgments
We thank all study staff and participants for their important contributions. Study-specific acknowledgments are reported in the online-only Data Supplement.

Sources of Funding
Collection of the UK Young Lacunar Stroke DNA Study (DNA lacunar) was primarily supported by the Wellcome Trust (WT072952) with additional support from the Stroke Association (TSA 2010/01). Genotyping of the DNA lacunar samples, and Dr Traylor, was supported by a Stroke Association Grant (TSA 2013/01). Funding for the genotyping at Massachusetts General Hospital was provided by the Massachusetts General Hospital-Deane Institute for the Integrative Study of Atrial Fibrillation and Stroke and the National Institute of Neurological Disorders and Stroke (U01 NS069208). Dr Rutten-Jacobs was supported by a project grant from the Stroke Association/Heather Heart Foundation grant (TSA BHF 2010/01). Dr Adib-Samii was supported by a Medical Research Council (United Kingdom) training fellowship. Drs Markus and Bevan were supported by the National Institute for Health Research Cambridge University Hospitals Comprehensive Biomedical Research Centre. Dr Markus was supported by a National Institute for Health Research Senior Investigator award. Dr Thijs was supported by a Clinical Investigator Grant from the scientific research fund, Fonds Wetenschappelijk Onderzoek Flanders. Dr Rost was supported by a National Institute of Neurological Disorders and Stroke grant (R01 NS082285-01). The sponsors of the study had no role in the study design, data collection, data analysis, interpretation, writing of the article, or the decision to submit the article for publication.

Disclosures
None.

References
1. Atwood LD, Wolf PA, Heard-Costa NL, Massaro JM, Beiser A, D’Agostino RB, et al. Genetic variation in white matter hyperintensity...
volume in the Framingham Study. Stroke. 2004;35:1609–1613. doi: 10.1161/01.STR.0000129643.77045.10.

2. Carmelli D, DeCarli C, Swan GE, Jack LM, Reed T, Wolf PA, et al. Evidence for genetic variance in white matter hyperintensity volume in normal elderly male twins. Stroke. 1998;29:1177–1181.

3. Turner ST, Jack CR, Fornage M, Mosley TH, Boerwinkle E, de Andrade M. Heritability of leukoaraiosis in hypertensive sibships. Hypertension. 2004;43:483–487. doi: 10.1161/01.HYP.0000123032.26158.92.

4. Hervé D, Chabriat H. CADASIL. J Geriatr Psychiatry Neurol. 2010;23:269–276. doi: 10.1177/0891988710383570.

5. Schmidt H, ZEGINIG M, Wiltgen M, Freudenberger P, Petrovic K, Cavalieri M, et al; CHARGE consortium Neurology working group. Genetic variants of the NOTCH3 gene in the elderly and magnetic resonance imaging correlates of age-related cerebral small vessel disease. Brain. 2011;134(Pt 11):3384–3397. doi: 10.1093/brain/awr252.

6. Dong Y, Hassan A, Zhang Z, Huber D, Dalageorgou C, Markus HS. Yield of screening for CADASIL mutations in lacunar stroke and leukoaraiosis. Stroke. 2003;34:203–205.

7. Ross OA, Soto-Ortolaza AI, Heckman MG, Verbeeck C, Serie DJ, Rayaprolu S, et al. NOTCH3 variants and risk of ischemic stroke. PLoS One. 2013;8:e75035. doi: 10.1371/journal.pone.0075035.

8. Bamford J, Sandercock P, Dennis M, Burn J, Warlow C. Classification and natural history of clinically identifiable subtypes of cerebral infarction. Lancet. 1991;337:1521–1526.

9. Adams HP Jr, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke, 1993;24:35–41.

10. Fazekas F, Kleistn R, Offenbacher H, Schmidt R, Kleinert G, Payer F, et al. Pathologic correlates of incidental MRI white matter signal hyperintensities. Neurology. 1993;43:1683–1689.
Common NOTCH3 Variants and Cerebral Small-Vessel Disease
Loes C.A. Rutten-Jacobs, Matthew Traylor, Poneh Adib-Samii, Vincent Thijs, Cathie Sudlow, Peter M. Rothwell, Giorgio Boncoraglio, Martin Dichgans, Steve Bevan, James Meschia, Christopher Levi, Nataliå S. Rost, Jonathan Rosand, Ahamad Hassan and Hugh S. Markus

Stroke. published online May 7, 2015;

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2015/05/07/STROKEAHA.114.008540
Free via Open Access

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2015/05/08/STROKEAHA.114.008540.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Common NOTCH3 variants and cerebral small vessel disease

Loes CA Rutten-Jacobs, PhD1*, Matthew Traylor, PhD1*, Poneh Adib-Samii, MBBS2, Vincent Thijs, MD3, Cathie Sudlow, FRCP4, Peter M Rothwell, FMedSci5, Giorgio Boncoraglio, MD6, Martin Dichgans, MD7, Steve Bevan, PhD1, James Meschia, MD8, Christopher Levi, MD9, Natalia S Rost, MD10, Jonathan Rosand, MD10, Ahamad Hassan, MRCP11, Hugh S Markus, FRCP1

Affiliations
1 University of Cambridge, Department of Clinical Neurosciences, Cambridge, UK
2 Stroke and Dementia Research Centre, St George’s University of London, London, UK
3 KU Leuven Department of Experimental Neurology and Leuven Research Institute for Neuroscience and Disease, University of Leuven, and Laboratory of Neurobiology, Vesalius Research Center, VIB, Leuven, Belgium
4 Division of Clinical Neurosciences, Neuroimaging Sciences and Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK
5 Stroke Prevention Research Unit, Nuffield Department of Neuroscience, University of Oxford, UK
6 Department of Cerebrovascular Diseases, Fondazione IRCCS Istituto Neurologico "Carlo Besta", Milano, Italy
7 Institute for Stroke and Dementia Research, Klinikum der Universität München, Ludwig-Maximilians-University Munich, Germany
8 Department of Neurology, Mayo Clinic, Jacksonville, USA
9 Centre for Clinical Epidemiology and Biostatistics, Hunter Medical Research Institute and School of Medicine and Public Health, University of Newcastle, NSW, Australia
10 Center for Human Genetic Research and Department of Neurology, Massachusetts General Hospital, Boston, USA
11 Department of Neurology, Leeds General Infirmary, Leeds Teaching Hospitals NHS Trust, Leeds, UK

Corresponding Author:
Loes Rutten-Jacobs
Department of Clinical Neurosciences
University of Cambridge
Cambridge
CB2 0QQ
LR406@medschl.cam.ac.uk
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 ≤ 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.

**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 study. 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.b58gene.sgu.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 KORAgem study (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.

**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.

**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.

**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

**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

**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%).

**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.

**Ischemic Stroke Genetics Study (ISGS)**

Ischemic Stroke Genetics Study (ISGS) was a 5-center, prospective, case-control study of first-ever ischemic stroke cases. 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.
Specific acknowledgements for UK Young Lacunar Stroke DNA Study (DNA Lacunar)

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 Sannuganathan). 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 Guylar). Southern General Hospital, Glasgow (34; Keith Muir, Wilma Smith). St George’s Hospital, London (108; Hugh Markus). St Helier Hospital, Carshalton (10; Val Jones). Stepping Hill Hospital, Stockport (4; Shivakumar Krishnamoorthy). Sunderland Royal Hospital, Sunderland (1; Nikhil Majumdar). The Royal
Bournemouth Hospital, Bournemouth (15; Damian Jenkinson). The Walton Centre, Liverpool (15; Richard White). Torbay Hospital, Torquay (19; Debs Kelly). University Hospital Aintree, Liverpool (19; Ramesh Durairaj). University Hospital of North Staffordshire, Stoke-on-trent (16; David Wilcock). Wansbeck General Hospital and North Tyneside Hospital, Ashington and North Shields (6; Christopher Price). West Cumberland Hospital, Whitehaven (6; Olu Orugun, Rachel Glover). West Hertfordshire Hospital, Watford (20; David Collas). Western General Hospital, Edinburgh (12; Cathie Sudlow). Western Infirmary, Glasgow (33; Kennedy R. Lees, Jesse Dawson). Wycombe Hospital and Stoke Mandeville, High Wycombe (20; Dennis Briley and Matthew Burn). Yeovil District Hospital, Yeovil (46; Khalid Rashed). York Teaching Hospital, York (1; John Coyle).
### Table I Lacunar stroke study population

| Centre                        | Country    | N    | Mean age (sd) | % Male   |
|-------------------------------|------------|------|---------------|----------|
| DNA Lacunar patients          | UK         | 1013 | 57.2 (9.5)    | 720 (71.1) |
| DNA Lacunar controls          |            | 970  | 59.7 (4.3)    | 510 (52.6%) |
| Germany WTCCC2 patients       | Germany    | 37   | 65.2 (9.6)    | 28 (75.7) |
| Germany WTCCC2 controls       |            | 797  | -             | 409 (51.3) |
| UK WTCCC2 patients            | UK         | 258  | 69.1 (11.7)   | 109 (42.2) |
| UK WTCCC2 controls            |            | 5175 | -             | 2564 (49.5) |
| Leuven patients               | Belgium    | 42   | 65.5 (13.9)   | 29 (69%)  |
| Leuven controls               |            | 455  | 55.7 (14.5)   | 212 (46.6%) |
| Overall patients              |            | 1350 |               |           |
| Overall controls              |            | 7397 |               |           |

Abbreviations: DNA Lacunar, UK Young Lacunar Stroke DNA Study; Germany WTCCC2, The Wellcome Trust Case-Control Consortium II Munich; UK WTCCC2, The Wellcome Trust Case-Control Consortium II UK; Leuven, Leuven Stroke Study
Table II WMH study populations

| Centre                        | Country      | N   | Mean age (sd) | % Male | % Hypertensive |
|-------------------------------|--------------|-----|---------------|--------|---------------|
| 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%           |
| Leuven                        | 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; Leuven, Leuven Stroke Study; MGH, Massachusetts General Hospital; ASGC, Australian Stroke Genetics Collaborative; ISGS, Ischemic Stroke Genetics Study; SWISS, Sibling with Ischaemic Stroke Study
Table III  Estimated power in the present study to detect an association of the common SNPs in *NOTCH3* with lacunar stroke

|               | MAF | OR* | Estimated power | n cases needed for 80% power |
|---------------|-----|-----|-----------------|-----------------------------|
| rs10404382    | 0.12| 1.75| >99%            | 236                         |
| rs1043994     | 0.12| 1.68| >99%            | 282                         |
| rs10423702    | 0.12| 1.70| >99%            | 267                         |
| rs1043997     | 0.13| 1.48| >99%            | 531                         |

Abbreviations: MAF, minor allele frequency; OR, odds ratio

*Odds ratio’s for the presence of white matter hyperintensities, reported in the study by Schmidt et al.1

Power calculations were conducted using the Genetic Power Calculator2 for a case-control study of discrete traits under an additive disease risk model and a disease prevalence of 0.2% for lacunar stroke.
Table IV Association of four common SNPs in NOTCH3 in DNA lacunar, adjusted for principal components 1 and 2 and age and sex (model 1) and adjusted for the factors in model 1 plus hypertension and diabetes (model 2).

|          | Model 1 |          | Model 2 |          |
|----------|---------|----------|---------|----------|
|          | MAF     | OR (95% CI) | p   | OR (95% CI) | p   |
| rs10404382 | 0.12    | 1.03 (0.84-1.27) | 0.78 | 1.03 (0.83-1.27) | 0.80 |
| rs1043994 | 0.12    | 1.01 (0.83-1.25) | 0.90 | 1.01 (0.82-1.25) | 0.91 |
| rs10423702 | 0.12    | 1.03 (0.84-1.27) | 0.78 | 1.03 (0.83-1.27) | 0.80 |
| rs1043997 | 0.13    | 0.99 (0.81-1.21) | 0.93 | 0.99 (0.81-1.21) | 0.93 |
Figure I Association of common NOTCH3 variants with lacunar stroke under dominant (A) and recessive (B) models by genomic position
Figure II  Association of common *NOTCH3* variants with lacunar stroke with leukoaraiosis under dominant (A) and recessive (B) models by genomic position
Figure III-A Forest plot for the association of the single nucleotide polymorphism rs10404382 with WMH

The size of the box is inversely proportional to the estimate variance of the effect estimator.
**Figure III-B** Forest plot for the association of the single nucleotide polymorphism rs1043994 with WMH

The size of the box is inversely proportional to the estimate variance of the effect estimator.
**Figure III-C** Forest plot for the association of the single nucleotide polymorphism rs10423702 with WMH

| Study             | Odds Ratio (95% CI)          |
|-------------------|----------------------------|
| ASGC              | 1.09 [0.67, 1.76]           |
| DNA Lacunar       | 1.25 [1.00, 1.57]           |
| GENESIS 1         | 0.74 [0.46, 1.17]           |
| GENESIS 2         | 1.02 [0.77, 1.35]           |
| ISSS              | 1.03 [0.76, 1.39]           |
| Leuven            | 0.97 [0.77, 1.22]           |
| MGH-Affymetrix    | 1.01 [0.63, 1.22]           |
| MGH-Illumina      | 0.91 [0.69, 1.20]           |
| MGH-Omni          | 0.72 [0.44, 1.20]           |
| Milano            | 1.25 [0.92, 1.69]           |
| SGUL 1            | 1.22 [0.69, 2.18]           |
| SGUL 2            | 0.88 [0.51, 1.51]           |
| SWISS             | 0.90 [0.60, 1.36]           |
| WTCCC2-Edinburgh  | 1.42 [0.85, 2.38]           |
| WTCCC2-Munich FLAIR | 1.08 [0.89, 1.31]         |
| WTCCC2-Munich T2  | 0.68 [0.47, 1.01]           |
| WTCCC2-Oxford Flair | 0.46 [0.25, 0.86]         |
| WTCCC2-Oxford T2  | 1.00 [0.58, 1.72]           |
| WTCCC2-SGUL       | 1.25 [0.80, 1.96]           |
| **Summary Estimate** | 1.04 [0.97, 1.11]       |

The size of the box is inversely proportional to the estimate variance of the effect estimator.
Figure III-D Forest plot for the association of the single nucleotide polymorphism rs1043997 with WMH

The size of the box is inversely proportional to the estimate variance of the effect estimator.
**Figure IV** Forest plot for the association of the single nucleotide polymorphism rs10404382 with WMH in only hypertensive patients.

The size of the box is inversely proportional to the estimate variance of the effect estimator.
References

1. Schmidt H, Zeginigg M, Wiltgen M, Freudenberger P, Petrovic K, Cavalieri M, et al. Genetic variants of the NOTCH3 gene in the elderly and magnetic resonance imaging correlates of age-related cerebral small vessel disease. *Brain*. 2011;134:3384-3397

2. Purcell S, Cherny SS, Sham PC. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics*. 2003;19:149-150