SUPPLEMENTAL MATERIAL

Common NOTCH3 variants and cerebral small vessel disease

Loes CA Rutten-Jacobs, PhD[1*], Matthew Traylor, PhD[1*], Poneh Adib-Samii, MBBS[2], Vincent Thijs, MD[3], Cathie Sudlow, FRCP[4], Peter M Rothwell, FMedSci[5], Giorgio Boncoraglio, MD[6], Martin Dichgans, MD[7], Steve Bevan, PhD[1], James Meschia, MD[8], Christopher Levi, MD[9], Natalia S Rost, MD[10], Jonathan Rosand, MD[10], Ahamad Hassan, MRCP[11], Hugh S Markus, FRCP[1]

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] KULeuven 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 $\leq$ 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.

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 KORAgen 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 andTunbridge 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, 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]

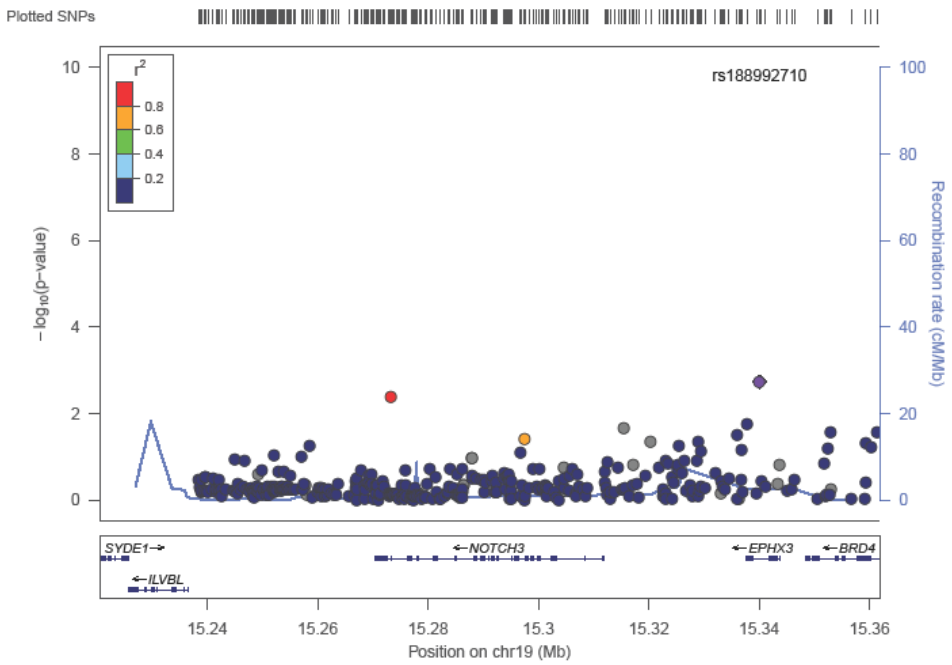
Power calculations were conducted using the Genetic Power Calculator[2] for a case-control study of discrete traits under an additive disease risk model and a disease prevalence of 0.2% for lacunar stroke.

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

A



B

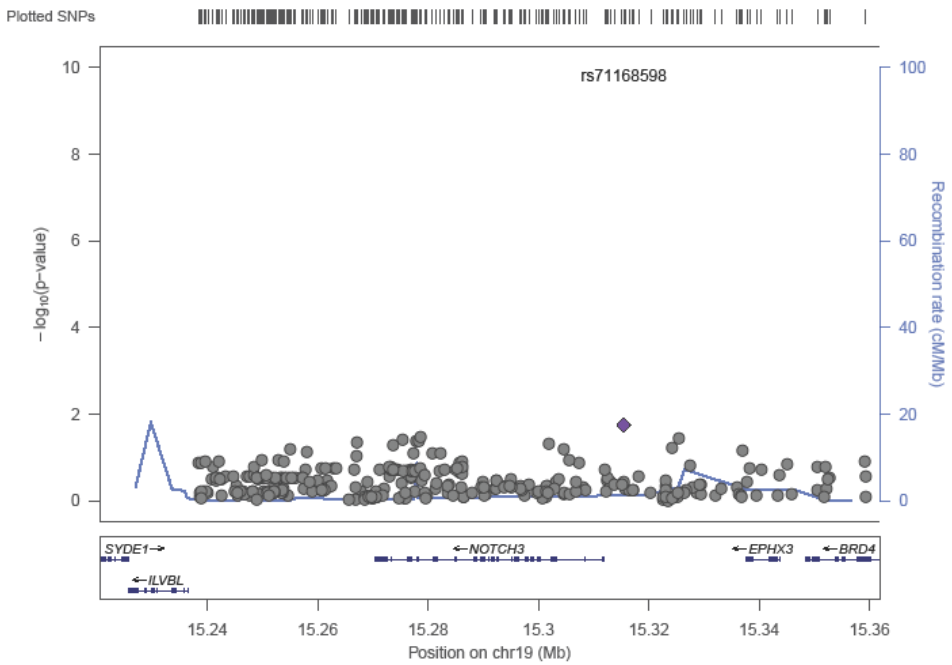
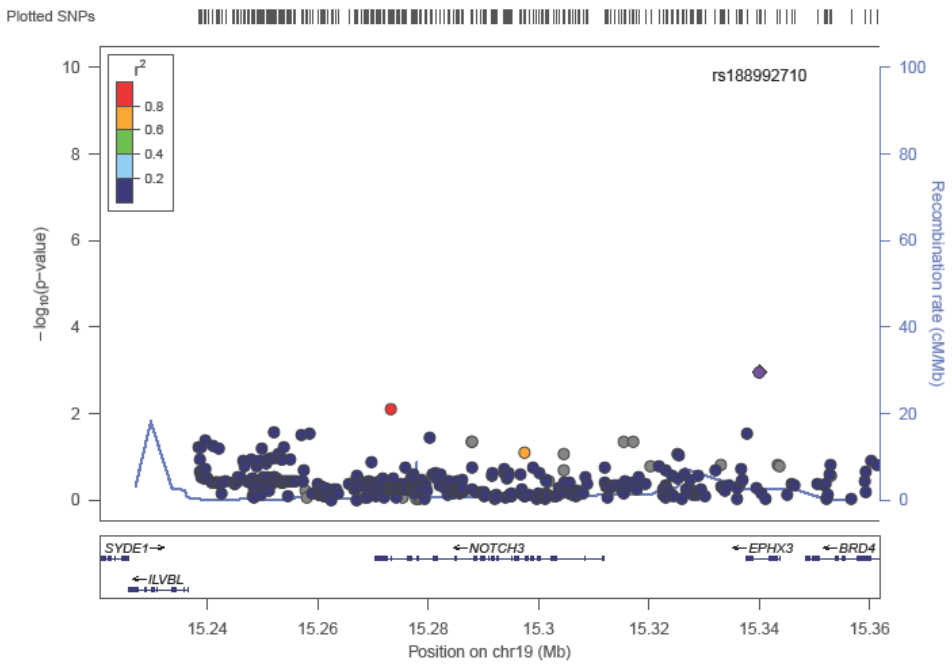


Figure II Association of common *NOTCH3* variants with lacunar stroke with leukoaraiosis under dominant (A) and recessive (B) models by genomic position

A



B

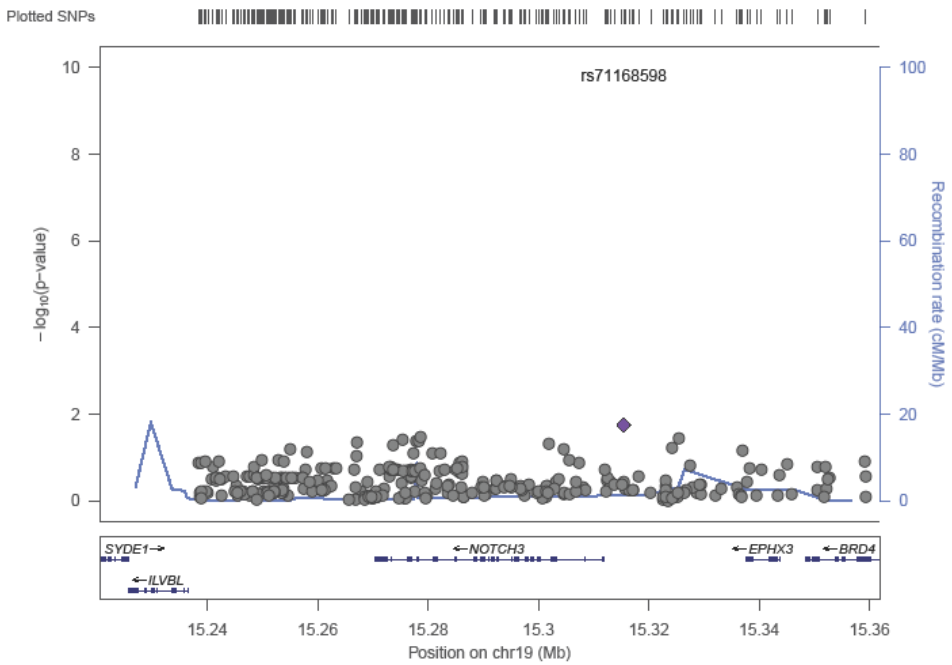
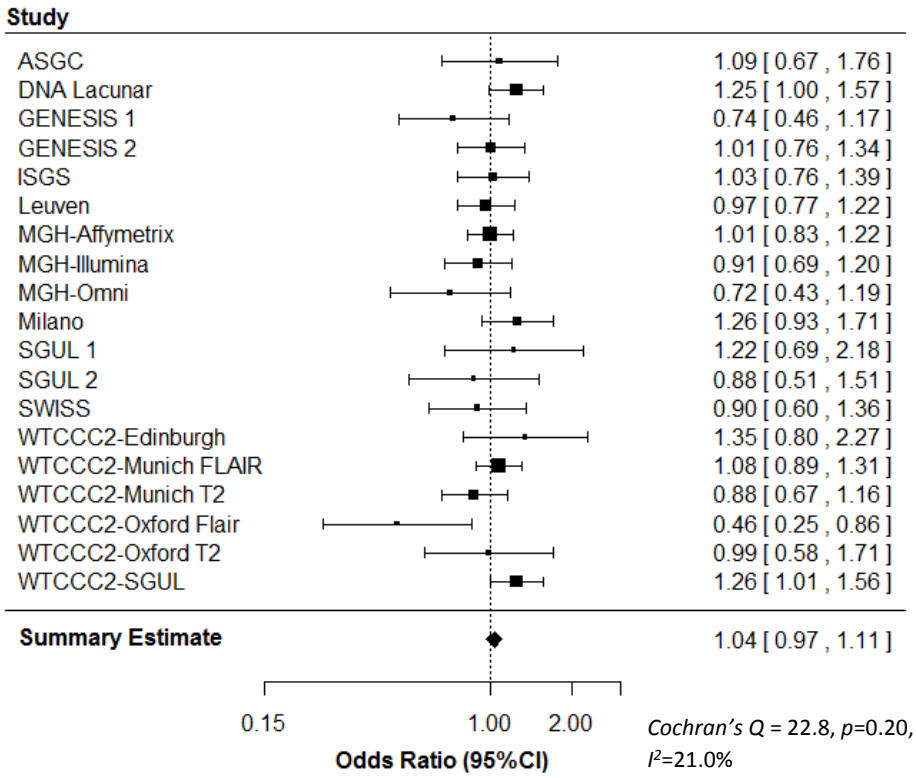
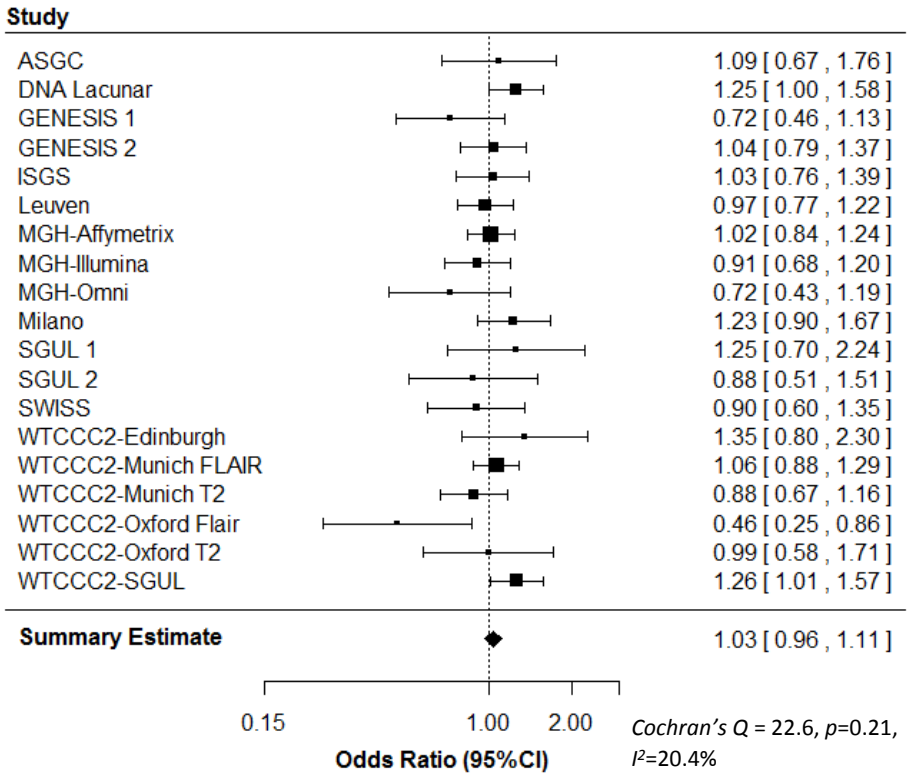


Figure III-A Forest plot for the association of the single nucleotide polymorphism rs10404382 with WMH



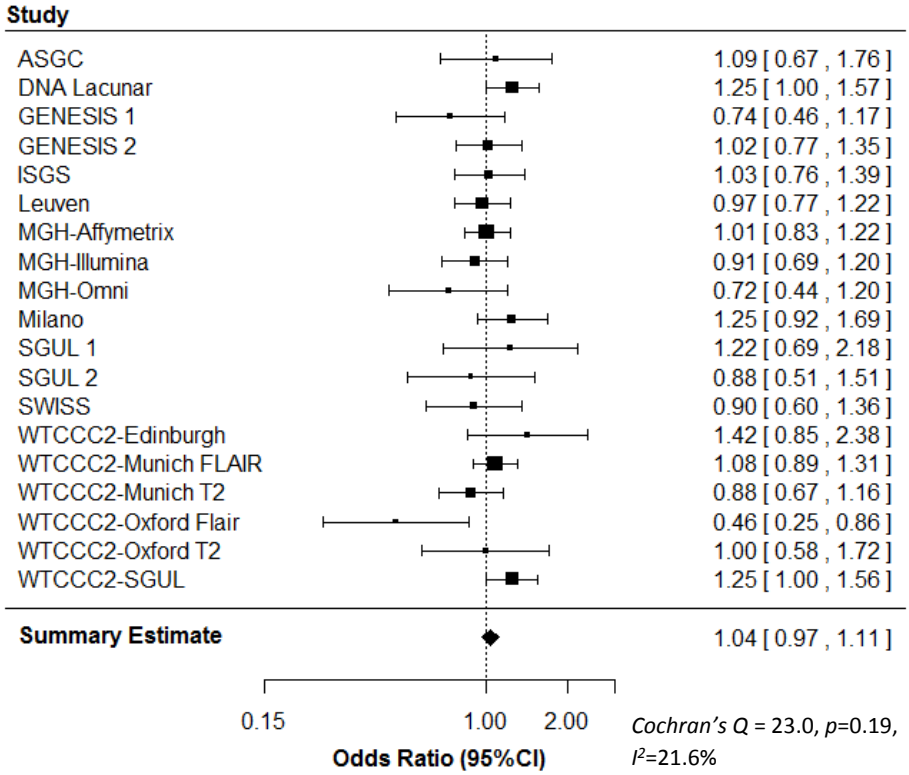
The size of the box is inversely proportional to the estimate variance of the effect estimator.

Figure III-B Forest plot for the association of the single nucleotide polymorphism rs1043994 with WMH



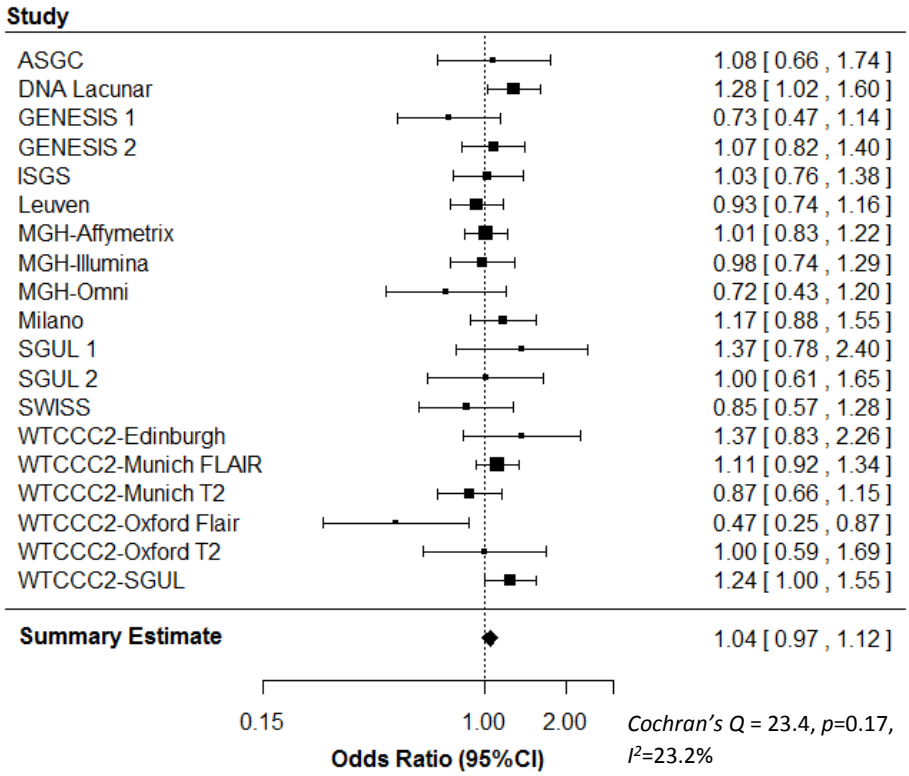
The size of the box is inversely proportional to the estimate variance of the effect estimator.

Figure III-C Forest plot for the association of the single nucleotide polymorphism rs10423702 with WMH



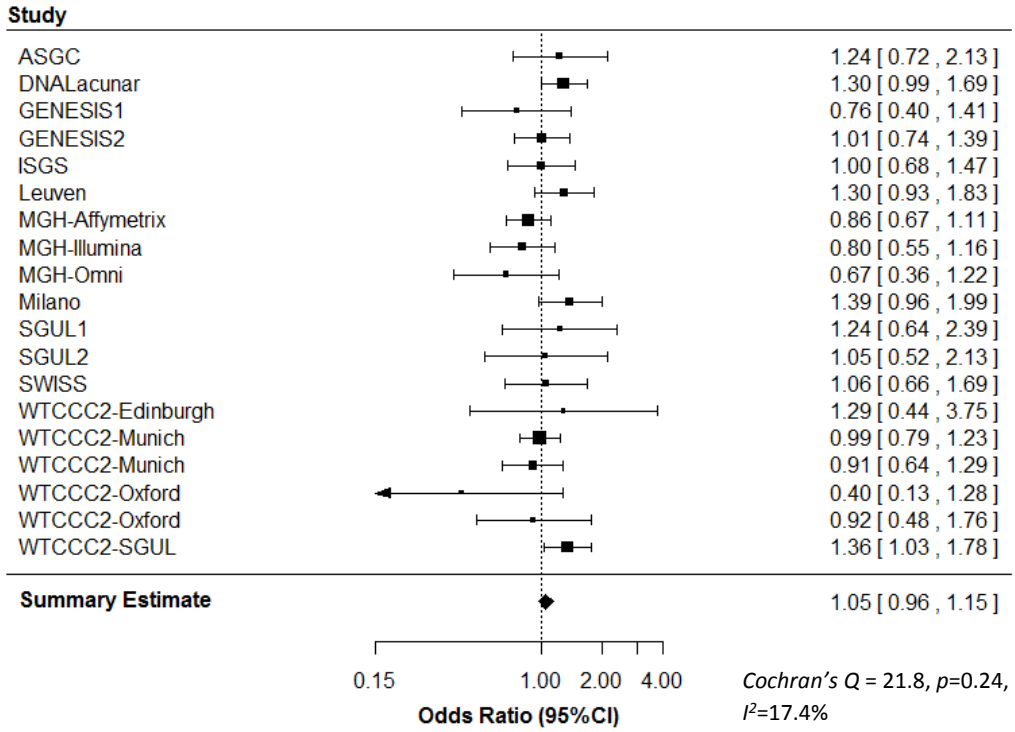
The size of the box is inversely proportional to the estimate variance of the effect estimator.

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