Sequence Variants on Chromosome 9p21.3 Confer Risk for Atherosclerotic Stroke

Andreas Gschwendtner, MD,[1] Steve Bevan, PhD,[2] John W. Cole, MD, MS,[3] Anna Plourde, BA,[4] Mar Matarin, PhD,[5] Helen Ross-Adams, PhD,[6] Thomas Meitinger, MD,[7] Erich Wichmann, MD, PhD,[8,9] Braxton D. Mitchell, PhD,[3] Karen Furie, MD, MPH,[4] Agnieszka Slowik, MD, PhD,[10] Stephen S. Rich, PhD,[11] Paul D. Syme, MD,[12] Mary J. MacLeod, PhD,[6] James F. Meschia, MD,[13] Jonathan Rosand, MD, MSc,[4] Steve J. Kittner, MD, MPH,[3] Hugh S. Markus, FRCP,[2] Bertram Müller-Myhsok, MD,[14] Martin Dichgans, MD,[1] on behalf of the International Stroke Genetics Consortium

Objective: Recent studies have identified a major locus for risk for coronary artery disease and myocardial infarction on chromosome 9p21.3. Stroke, in particular, ischemic stroke caused by atherosclerotic disease, shares common mechanisms with myocardial infarction. We investigated whether the 9p21 region contributes to ischemic stroke risk.

Methods: In an initial screen, 15 single nucleotide polymorphisms (SNPs) covering the critical genetic interval on 9p21 were genotyped in samples from Southern Germany (1,090 cases, 1,244 control subjects) and the United Kingdom (758 cases, 872 control subjects, 3 SNPs). SNPs significantly associated with ischemic stroke or individual stroke subtypes in either of the screening samples were subsequently genotyped in 2,528 additional cases and 2,189 additional control subjects from Europe and North America.

Results: Genotyping of the screening samples demonstrated associations between seven SNPs and atherosclerotic stroke (all $p < 0.05$). Analysis of the full sample confirmed associations between six SNPs and atherosclerotic stroke in multivariate analyses controlling for demographic variables, coronary artery disease, myocardial infarction, and vascular risk factors (all $p < 0.05$). The odds ratios for the lead SNP (rs1537378-C) were similar in the various subsamples with a pooled odds ratio of 1.21 (95% confidence interval, 1.07–1.37) under both fixed- and random-effects models ($p = 0.002$). The point estimate for the population attributable risk is 20.1% for atherosclerotic stroke.

Interpretation: The chromosome 9p21.3 region represents a major risk locus for atherosclerotic stroke. The effect of this locus on stroke appears to be independent of its relation to coronary artery disease and other stroke risk factors. Our findings support a broad role of the 9p21 region in arterial disease.

Ann Neurol 2009;65:531–539

Stroke is the second most frequent cause of death and a major cause of disability worldwide.[1,2] Stroke is causatively heterogeneous. The majority of patients have ischemic stroke (IS), which can be further subdivided according to stroke mechanisms. Major categories include atherosclerotic (ie, large artery) stroke, cardioembolic stroke, and small vessel stroke. There is substantial evidence for a genetic contribution to IS risk.[3] However, the responsible genetic variants are still largely unknown.[4,5]

Recently, genomewide association studies have identified a major locus for risk for coronary artery disease (CAD) and myocardial infarction (MI) on chromosome 9p21.3.[6–9] This locus was uniformly identified as the strongest genetic signal for CAD in four independent screens and was subsequently confirmed in addi-

From the [1]Department of Neurology, Klinikum Großhadern, Ludwig-Maximilians-Universität, Munich, Germany; [2]Centre for Clinical Neuroscience, St. George's, University of London, London, United Kingdom; [3]Department of Neurology, University of Maryland, School of Medicine, and Veterans Affairs Medical Center, Baltimore, MD; [4]Stroke Service and Center for Human Genetics Research, Massachusetts General Hospital, Boston, MA; [5]Molecular Genetics Section, Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, MD; [6]Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen, United Kingdom; [7]Institute of Human Genetics and [8]Institute of Epidemiology, Helmholtz Zentrum München; [9]Institute of Medical Informatics, Biometry and Epidemiology, Ludwig-Maximilians-Universität, Munich, Germany; [10]Department of Neurology, Jagiellonian University, Medical College, Kraków, Poland; [11]Center for Public Health Genomics, University of Virginia, Charlottesville, VA; [12]Department of Clinical and Surgical Sciences, University of Edinburgh, Edinburgh, United Kingdom; [13]Department of Neurology, Mayo Clinic, Jacksonville, FL; and [14]Max-Planck-Institute of Psychiatry, Munich, Germany.

Address correspondence to Dr Dichgans, Marchioninistrasse 15, 81377 München, Germany. E-mail: martin.dichgans@med.uni-muenchen.de

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Received Aug 31, 2008, and in revised form Oct 19. Accepted for publication Oct 31, 2008.

Published in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.21590

tional cohorts.[10–12] The consistency of this finding coupled with a high frequency of the risk allele has attracted great attention all the more because the risk contributed by 9p21 was found to be independent of conventional vascular risk factors.

A sequence variant (rs10757278-G) in the same chromosomal region was subsequently shown to be associated with both abdominal aortic aneurysms (AAAs) and intracranial aneurysms (IAs), suggesting an even broader role of the 9p21 region in arterial disease.[13]

Stroke, in particular, atherosclerotic stroke, shares common risk factors and pathophysiological mechanism with CAD and MI,[14,15] thus rendering the 9p21 region a strong candidate for stroke risk. Only recently, several small studies have looked for an association between sequence variants on 9p21 and IS risk.[13,16,17] These studies have been inconclusive partly because of limited sample size and because stroke subtypes such as atherosclerotic stroke were not considered separately.

Drawing on the resources of the International Stroke Genetics Consortium, we assembled one of the largest collections of IS patients to date to investigate whether genetic variation at 9p21.3 is associated with risk for IS and, in particular, the subtype of atherosclerotic stroke. Furthermore, because CAD and MI are themselves risk factors for stroke,[18,19] we sought to confirm whether any relation between the locus and risk for stroke was independent of CAD.

Subjects and Methods

Study Population

We studied subjects collected by six different centers across Europe and North America (Department of Neurology, Klinikum Großhadern, Munich, Germany; Clinical Neurosciences, St. Georges, London, United Kingdom; Department of Neurology, University of Maryland at Baltimore, Baltimore, MD; Department of Neurology, Mayo Clinic, Jacksonville, FL; Massachusetts General Hospital, Boston, MA; and University of Aberdeen, Aberdeen, United Kingdom) (Table 1 and Supplementary Material). Events with deficits lasting less than 24 hours with corresponding evidence of an acute ischemic infarct on neuroimaging were included. Strokes occurring as an immediate consequence of trauma or associated with subarachnoid hemorrhage and strokes caused by cerebral venous thrombosis were excluded. Stroke subtypes were classified at each participating center using the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification system.[20] The ethics committees of each study site approved the study protocol, and all participants gave written informed consent.

Selection of Single Nucleotide Polymorphisms

For the screening phase, we selected 10 single nucleotide polymorphisms (SNPs) showing the strongest association with CAD or MI in earlier genomewide association studies[6,7,11,13,21] (Fig 1), as well as 5 additional SNPs covering the critical genetic interval on 9p21.[11,22]

Genotyping

Genotyping was performed on four different platforms. After completing local quality checks, all data were transferred to the central site (Munich) for central quality control and data analysis. Genotyping in the Munich and Boston samples was done in Munich using the iPlex Gold chemistry on a MassARRAY platform (Sequenom, San, Diego, CA). The SNP assays were designed with the Sequenom Assay Design 3.1 software. In the Baltimore-Washington Young Stroke Study (BWYSS) and Jacksonville samples, SNP genotyping was performed on-site with TaqMan technology (Applied Biosystems, Foster City, CA). The reaction protocol was specified according to manufacturer's instructions included with each individual primer set. The London and Aberdeen samples were genotyped by KBiosciences (http://www.kbioscience-.co.uk; Essex, United Kingdom), using a combination of their patented competitive allele specific PCR (KASPar) assays designed with proprietary Primer Picker software and TaqMan technology. Genotyping was successful in all samples and for all SNPs except for rs496892, which failed in the London sample for technical reasons. The genotyping call rates ranged from 97.8 to 99.2%. All SNPs are named according to the forward/T strand orientation as in dbSNP.

Statistical Analysis

Testing for a deviation from Hardy–Weinberg equilibrium was done using the exact test for Hardy–Weinberg equilibrium from the R package "genetics," while correcting for multiple comparisons. Analysis for an association of the SNPs studied with the phenotypes of interest (IS as a whole, stroke subtypes, CAD, and MI) was done using logistic regression implemented in the general linear model procedure in R 2.7.1. (http://cran.r-project.org). Genotypes were coded as numbers of the minor alleles, thus implementing Armitage's test for trend. Odds ratios (ORs) and confidence intervals were also obtained from logistic regression while including the following possible confounders in the model: age (age at onset for cases and age at recruitment for control subjects), sex, ethnicity, center, CAD and MI, and vascular risk factors. Meta-analysis, including testing for heterogeneity, was done using the R package rmeta (http://cran.r-project.org/web/packages/rmeta/). To determine the most likely causative SNPs, we performed logistic regression conditioning on the most significant SNP. Testing for genetic models was done using a likelihood ratio testing procedure based on likelihoods obtained from logistic regression.[23] Haplotype analysis was performed using Haploview Version 4.1 in linkage disequilibrium blocks delineated using the four-gamete rule. The linkage disequilibrium structure in the German control population is given in Figure 1 and Supplementary Figure 1. Multiple testing corrections were performed using Sidak's method based on a total of 15 SNPs, thus not limiting our analyses to the 7 SNPs entering stage 2 and also allowing joint analysis of the total sample.[24] Post hoc power analysis was done using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/). For an allele frequency of the risk allele of 0.64 and an allelic OR of 1.2, the power of this study to find a significant effect at a type I error level of 0.00333 (=0.05/15) was 70%.

Table 1. **Baseline Characteristics of the Study Population Stratified by Affection Status**

Subject Characteristics	Munich Sample		London Sample		Baltimore-Washington Young Stroke Study		Ischemic Stroke Genetics Study		Boston Sample		Aberdeen Sample	
	Cases	Control Subjects	Cases	Control Subjects	Cases	Control Subjects	Cases	Control Subjects	Cases	Control Subjects	Cases	Control Subjects
N	1,090	1,244	758	872	652	718	603	435	608	519	607	517
Male subjects, n (%)	672 (61.7)	773 (62.1)	444 (58.6)	498 (57.1)	351 (53.8)	346 (48.2)	256 (42.5)	163 (37.5)	335 (55.0)	249 (48.0)	334 (55.0)	265
Female subjects, n (%)	418 (38.3)	471 (37.9)	314 (41.4)	374 (42.9)	301 (46.2)	373 (51.9)	347 (57.5)	272 (62.5)	274 (45.0)	270 (52.0)	273 (45.0)	252
Ethnicity												
White, n (%)	1,090 (100)	1,244 (100)	758 (100)	872 (100)	327 (50.2)	384 (53.5)	445 (73.8)	314 (72.2)	549 (90.3)	498 (96.0)	607 (100)	517 (100)
Black, n (%)	—	—	—	—	275 (42.2)	271 (37.7)	139 (23.0)	106 (24.4)	22 (3.6)	8 (1.5)	—	—
Other, n (%)	—	—	—	—	50 (7.6)	63 (8.8)	19 (3.2)	15 (3.4)	37 (6.1)	13 (2.5)	—	—
Mean (SD) age, yr	65.4 (13.5)	62.4 (10.9)	66.0 (13.2)	65.3 (8.8)	41.1 (7.3)	39 (7.1)	64.6 (13.8)	60.7 (14.9)	65.2 (15.7)	66.8 (9.3)	69.6 (12.2)	67.1 (9.0)
Ancestry	Southern Germany		United Kingdom		Baltimore-Washington Area, USA		USA and Canada		Boston, USA		Aberdeen and Borders, Scotland, UK	
Stroke subtype												
Atherosclerotic stroke, n (%)	318 (29.2)	—	237 (31.3)	—	64 (9.8)	—	120 (19.9)	—	111 (18.3)	—	170 (28.0)	—
Cardioembolic stroke, n (%)	283 (26.0)	—	126 (16.6)	—	125 (19.2)	—	135 (22.4)	—	23 (38.7)	—	113 (18.6)	—
Small vessel stroke, n (%)	107 (9.8)	—	136 (17.9)	—	97 (14.9)	—	124 (20.6)	—	63 (10.4)	—	226 (37.2)	—
Other determined cause, n (%)	48 (4.4)	—	5 (0.7)	—	80 (12.3)	—	24 (4.0)	—	55 (9.0)	—	—	—
Undetermined cause, n (%)	334 (30.6)	—	254 (33.5)	—	286 (43.9)	—	200 (33.2)	—	144 (23.7)	—	98 (16.1)	—
Coronary artery disease, n (%)	156 (14.3)	—	93 (12.3)	48 (5.5)	64 (9.8)	30 (4.2)	132 (21.9)	38 (8.7)	119 (19.6)	35 (6.7)	237 (39.0)	102 (19.7)
Myocardial infarction, n (%)	119 (10.9)	—	80 (10.6)	48 (5.5)	33 (5.0)	4 (0.6)	107/602 (17.8)	21/434 (4.8)	39 (6.4)	15 (2.9)	29 (4.8)	11/317 (3.5)
Cardiovascular risk factor												
Hypertension, n (%)	673 (61.7)	758 (60.9)	567 (74.8)	510/869 (58.7)	274/648 (42.3)	128/716 (17.9)	434 (72)	170/433 (39.3)	363 (59.7)	243 (46.8)	351 (57.8)	229 (44.3)
Former or current smoker, n (%)	301 (27.6)	671 (53.9)	552/757 (38.4)	505/870 (58.0)	290/651 (44.5)	191/708 (27.0)	399 (66.2)	218 (50.1)	373/593 (62.9)	253/517 (48.9)	259 (42.7)	299 (57.8)
Diabetes mellitus, n (%)	248 (22.8)	79 (6.4)	119/754 (15.8)	38/867 (4.4)	109/650 (16.8)	35/716 (5.0)	157 (26)	59 (13.6)	116 (19.1)	34 (6.6)	89 (14.7)	22 (4.3)
Hypercholesterolemia, n (%)	415 (38.1)	712 (57.2)	534/756 (70.6)	339/519 (65.3)	171/644 (26.6)	169/712 (23.7)	297/600 (49.5)	138/430 (32.1)	222 (36.5)	225 (43.4)	350/603 (58)	312 (60.3)

In general, conventional risk factors are defined as follows: Diabetes mellitus was defined as glucose level > 130mg/dl, known diabetes, or receiving treatment. Hypertension was defined as systolic blood pressure > 140mmHg, diastolic blood pressure > 90mmHg, or receiving treatment for these conditions. Hyperlipidemia was defined as total cholesterol level > 200mg/dl, low-density lipoprotein cholesterol level > 130mg/dl, or receiving lipid-lowering treatment.
SD = standard deviation.

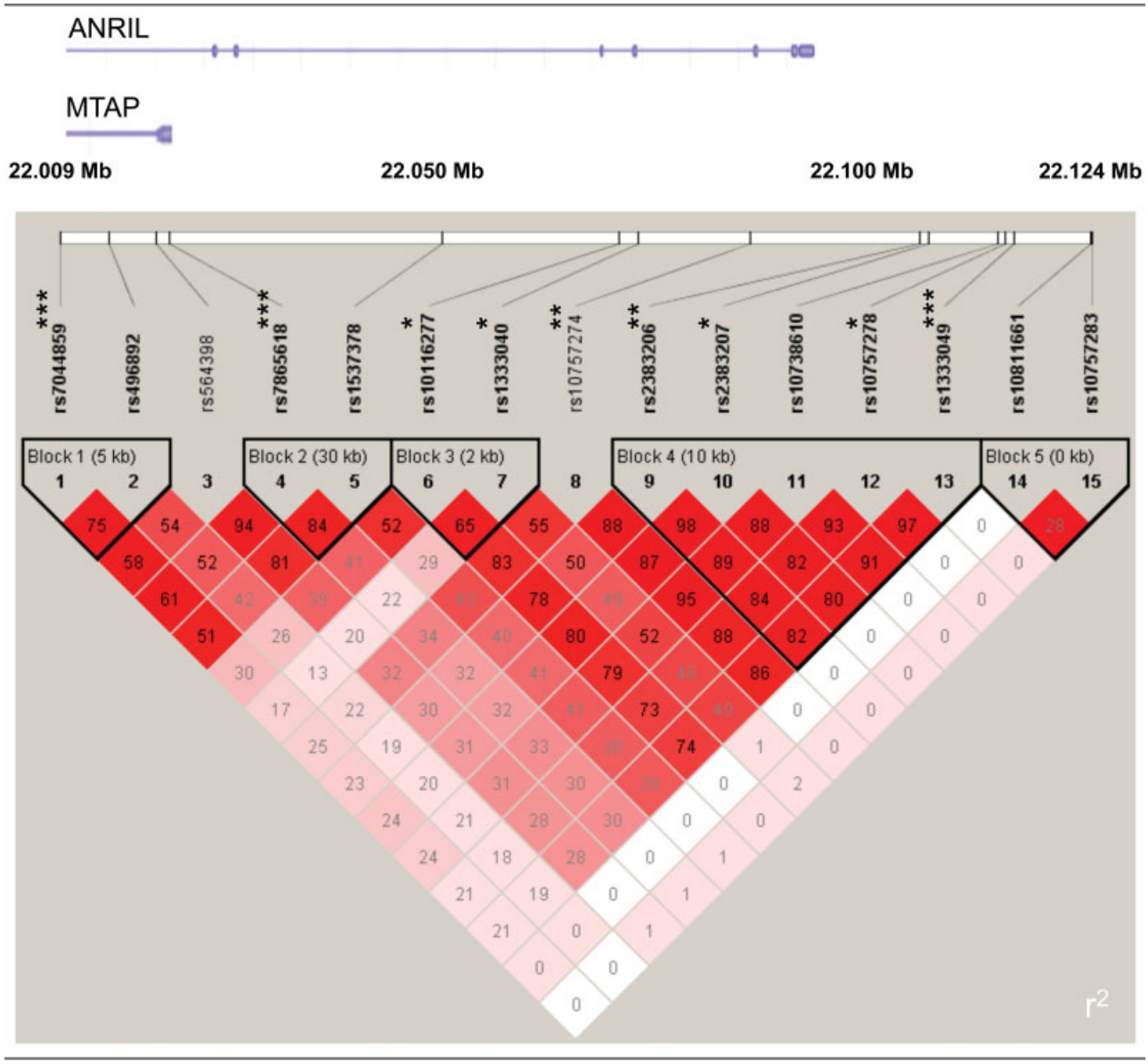

Fig 1. Linkage disequilibrium (LD) structure and genomic locations of known genes in the chromosome 9p21.3 region: Genomic locations are given according to the Reference Sequence collection of the National Center for Biotechnology information (NCBI). Data are derived from the Munich control sample (n = 1,244). Blocks were delimited using the four-gamete rule. LD is shown through values of r^2. *Figure created with Haploview software. The lead single nucleotide polymorphisms (SNPs) of former studies on CAD/MI are marked as follows: *Helgadottir and colleagues, 2007[6]; **McPherson and colleagues, 2007[7]; ***Samani and colleagues, 2007.[9]*

Role of the Funding Sources

The funding sources had no influence on the design and conduct of the study, writing of the manuscript, and decision to submit.

Results

The majority of our cases and control subjects were of European origin. As expected, the relative distribution of atherosclerotic stroke differed across sites, ranging in frequency from 9.8 to 31.3% (see Table 1). In addition, the frequency with which stroke cases had a history of MI or CAD ranged from 4.8 to 9.8% and 17.8 to 39%, respectively.

As an initial step, we genotyped 15 SNPs covering the critical genetic interval on 9p21.3 (see Fig 1) in 1,090 cases and 1,244 control subjects from Southern Germany. Of the 15 SNPs, 6 (rs7044859, rs496892, rs564398, rs7865618, rs1537378, rs2383207) were associated with atherosclerotic stroke (all $p < 0.05$) (see Supplementary Table 1), whereas no associations were found with IS in general or other stroke subtypes (data not shown). Genotyping of rs1333040, rs2383207,

and rs10757278 done in parallel in 758 IS cases and 872 control subjects from the United Kingdom demonstrated associations between two SNPs (rs2383207 and rs10757278) and risk for atherosclerotic stroke (all $p < 0.05$) (see Supplementary Table 1), whereas again no associations were found with other stroke subtypes (data not shown).

The seven SNPs (rs7044859, rs496892, rs564398, rs7865618, rs1537378, rs2383207, and rs10757278) associated with risk for atherosclerotic stroke in either of the two screening samples were subsequently genotyped in four additional samples from North America and Scotland adding 2,528 cases and 2,189 control subjects (see Table 1). Genotyping in the UK sample was completed for the remaining SNPs. There was no significant deviation from Hardy–Weinberg equilibrium in any of the study groups.

Because of the observed association with atherosclerotic stroke in the screening samples, we focused on this stroke subtype in subsequent analyses. Joint analysis of the full sample comprising 4,376 cases and 4,305 control subjects demonstrated significant associations between 6 of the 7 SNPs and atherosclerotic stroke in multivariate analyses including age, sex, ethnicity, and center as covariates (Table 2). Importantly, these associations remained significant when including CAD and MI as covariates. In addition, these associations remained significant when adding vascular risk factors as covariates (rs10757278 was no longer significant, whereas rs7044859 became significant) (see Table 2). None of the interaction terms between SNPs and CAD, MI, or vascular risk factors approached significance.

Further analyses identified rs1537378 as the lead SNP among the polymorphisms tested because none of the SNPs in Table 2 displayed effects independent of rs1537378. Importantly, ORs were similar in all subsamples defined by ethnicity with an overall estimate of 1.19 (95% confidence interval [CI], 1.06–1.33; $p = 0.003$). A meta-analysis restricted to samples with at least 50 ethnically homogeneous individuals showed similar results (Fig 2; see Supplementary Table 2) with a pooled OR of 1.21 (95% CI, 1.07–1.37) under both fixed- and random-effects models ($p = 0.002$). Interestingly, the results did not differ significantly between individuals of European and African American descent, suggesting that the effect of the SNP on risk for atherosclerotic stroke was similar across ethnicities. We also examined whether effects may differ across centers. There was no evidence for heterogeneity, when assessed within the meta-analysis ($p = 0.987$). Furthermore, ORs were similar across subgroups divided by sex, age, and vascular risk factors (see Supplementary Fig 2). We further examined whether there may be interactions between the culprit SNPs and CAD/MI or vascular risk factors. None of the interaction terms between SNPs and CAD, MI, or vascular risk factors approached significance. Testing for different modes of inheritance in the joint data set demonstrated that the best fitting model was a recessive model for the C-allele of rs1537378, although the recessive model was not significantly better than the allele dosage model.

The effect of rs1537378 appeared to be restricted entirely to the subset of atherosclerotic stroke. There were no significant associations between any of the seven SNPs and the risk for other stroke subtypes, with the exception of a nominally significant association between rs1537378 and stroke of undetermined cause, which encompasses multiple competing causative factors including atherosclerosis ($p = 0.039$) (see Supplementary Table 3). In addition, there were associations between two SNPs and risk for overall IS (rs564398, $p = 0.0328$; rs1537378, $p = 0.0114$), although ORs were lower than for atherosclerotic stroke. When atherosclerotic strokes were excluded from the analysis, these associations disappeared, consistent with the hypothesis that the risk for stroke contributed by this locus is restricted to the subtype of atherosclerotic stroke. The point estimate for the population attributable risk was 20.1% for atherosclerotic stroke and 4% for overall IS.

A total of 741 (16.3%) subjects with IS and 243 (5.6%) control subjects had a history of MI or CAD. To confirm the results of prior studies, we repeated our analysis after reclassifying the overall group of cases and control subjects according to MI and CAD status. As expected, several SNPs were significantly associated with risk for MI or CAD in the overall sample (see Supplementary Table 4). The ORs were similar in magnitude and identical in direction to those previously reported.[11] The strongest signal was obtained with variant rs10757278-G. This SNP is highly correlated with rs1333049 ($r^2 > 0.95$) and rs2383207 ($r^2 \geq 0.8$), the lead SNPs in prior studies of MI/CAD[6,9] (see Fig 1).

Haplotype analysis confirmed the single locus results without adding further information, because of high r^2 values between the associated SNPs (see Fig 1). The linkage disequilibrium structure was found to differ markedly between different ethnicities (see Supplementary Fig 3).

Discussion

Cardiovascular disease including stroke is the most frequent cause of death and a major cause of disability worldwide.[1] This study demonstrates that the recently identified locus for CAD on chromosome 9p21.3 is also implicated in risk for atherosclerotic stroke. This relation between the locus and risk for stroke appears to be mediated through mechanisms that are not dependent on the presence of MI, CAD, or other vascular risk factors. The observed ORs are substantial, and the population attributable risk is high, suggesting that

Table 2. **Association to Atherosclerotic Stroke on 9p21 in the Overall Sample**

SNP	Alleles	Risk Allele	Cases (N)	Control Subjects (N)	Frequency of Risk Allele		OR (95% CI)	*p*
					Cases	Control Subjects		
Atherosclerotic Stroke, Corrected for Age, Sex, Ethnicity, and Center								
rs7044859	A/T	A	970	4,256	0.4985	0.4899	1.10 (0.99–1.22)	0.0666
rs496892	A/G	G	731	3,360	0.5739	0.5531	1.15 (1.02–1.29)	0.0214
rs564398	A/G	A	964	4,190	0.6494	0.6227	1.19 (1.07–1.33)	0.0009
rs7865618	A/G	A	962	4,272	0.6398	0.6181	1.19 (1.07–1.32)	0.0011
rs1537378	C/T	C	961	4,202	0.6675	0.6423	1.21 (1.09–1.35)	0.0005
rs2383207	A/G	G	962	4,260	0.5889	0.5700	1.17 (1.06–1.30)	0.0025
rs10757278	A/G	G	952	4,262	0.5047	0.4693	1.12 (1.02–1.24)	0.0239
Atherosclerotic Stroke, Corrected for Age, Sex, Ethnicity, Center, and CAD and MI								
rs7044859	A/T	A	970	4,256	0.4985	0.4899	1.10 (0.99–1.21)	0.0774
rs496892	A/G	G	731	3,360	0.5739	0.5531	1.15 (1.02–1.29)	0.0218
rs564398	A/G	A	964	4,190	0.6494	0.6227	1.19 (1.07–1.32)	0.0014
rs7865618	A/G	A	962	4,272	0.6398	0.6181	1.18 (1.06–1.32)	0.0019
rs1537378	C/T	C	961	4,202	0.6675	0.6423	1.20 (1.07–1.34)	0.0011
rs2383207	A/G	G	962	4,260	0.5889	0.5700	1.17 (1.05–1.30)	0.0034
rs10757278	A/G	G	952	4,262	0.5047	0.4693	1.11 (1.01–1.23)	0.0396
Atherosclerotic Stroke, corrected for Age, Sex, Ethnicity, Center, CAD and MI, and vascular risk factors								
rs7044859	A/T	A	969	4,240	0.4979	0.4896	1.12 (1.00–1.24)	0.0409
rs496892	A/G	G	730	3,346	0.5733	0.5528	1.16 (1.02–1.30)	0.0181
rs564398	A/G	A	963	4,175	0.6490	0.6225	1.18 (1.06–1.32)	0.0034
rs7865618	A/G	A	961	4,256	0.6394	0.6181	1.18 (1.06–1.32)	0.0033
rs1537378	C/T	C	960	4,187	0.6672	0.6424	1.19 (1.06–1.33)	0.0031
rs2383207	A/G	G	961	4,244	0.5890	0.5700	1.16 (1.04–1.29)	0.0083
rs10757278	A/G	G	951	4,246	0.5047	0.4694	1.11 (0.99–1.23)	0.0618

Shown are all single nucleotide polymorphisms (SNPs) in the region on 9p21 that showed nominally significant association to atherosclerotic stroke in one of the two screening samples (see Supplementary Table 1).
OR = odds ratio; CI = confidence interval; CAD = coronary artery disease; MI = myocardial infarction.

the 9p21.3 region is a major locus for atherosclerotic stroke. Our findings add to recent observations in patients with AAAs and IAs.[13] Together, these findings show a key role of the chromosome 9p21.3 region in arterial disease.

The validity of our findings is supported by several

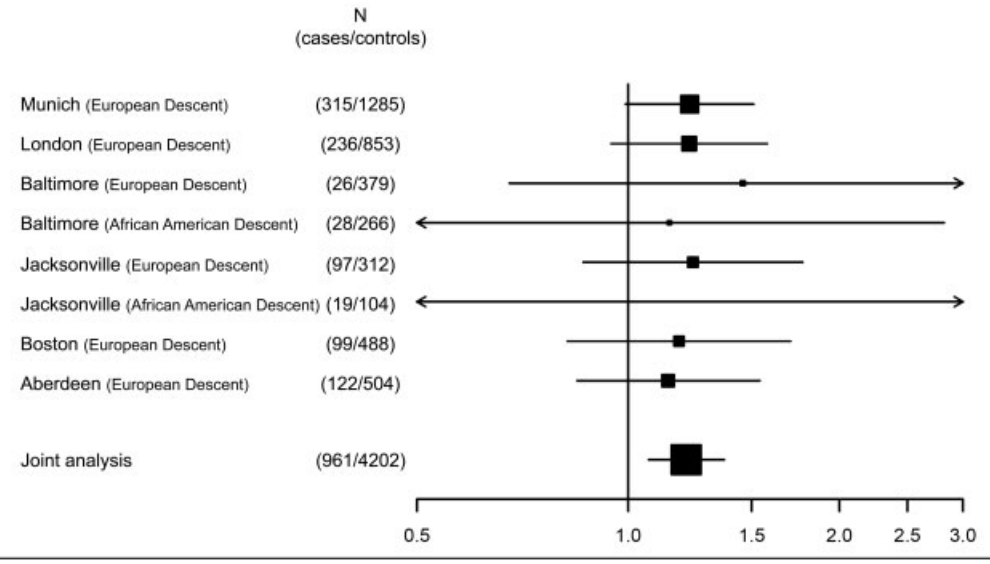

Fig 2. Forest plot of the allele dosage risk for the lead single nucleotide polymorphism (SNP; rs1537378-C) in subgroups defined by center and ethnicity: Odds ratios and 95% confidence intervals are shown on a natural log scale. Solid squares *are scaled in proportion to sample sizes. Samples totalling less than 50 ethnically homogeneous individuals are not included.*

observations. First, associations between SNPs on 9p21 and atherosclerotic stroke were detected in both screening samples and were subsequently confirmed in the meta-analysis of the overall sample totalling 4,376 IS cases and 4,305 control subjects. Second, the ORs for the lead SNP (rs1537378) were remarkably consistent across subgroups including various populations from different geographical regions and ethnic backgrounds (see Fig 2 and Supplementary Fig 2). Third, associations in the overall sample were robust when we controlled for potential confounders, as well as intermediate variables. Fourth, the signal was strictly confined to a single stroke subtype both in the screening samples and in the overall population, suggesting that the signal is clearly delimited and not related to stroke in general.

Significant associations were found with several SNPs covering a genomic interval of more than 100kb. It is evident from the distribution of associated SNPs in this region that the causally responsible variant remains to be identified. The genetic interval overlaps with exons 18 to 24 of *ANRIL,* a newly annotated gene encoding a large antisense noncoding RNA[25] (see Fig 1). *ANRIL* has recently been shown to be expressed in human atheromatous vessels including both AAA and carotid endarterectomy samples.[12] It was further found to be expressed in isolated vascular endothelial cells, monocyte-derived macrophages, and coronary smooth muscle cells, all of which have a role in atherosclerosis. As for most noncoding RNA, the cellular function of *ANRIL* is still unknown. However, it appears from the earlier findings that *ANRIL* represents a good candidate for atherosclerosis risk. The genetic interval on 9p21 further overlaps with exon 5 of a splicing variant of the methylthioadenosine phosphorylase *(MTAP)* gene and is relatively close to the coding sequences of genes for two cyclin-dependent kinase inhibitors, *CDKN2a* (encoding p16INK4a) and *CDKN2B* (encoding p15INK4b). These genes play a key role in regulating cell proliferation, cell senescence, and apoptosis,[26] and may be implicated in atherosclerosis through their role in transforming growth factor-β–induced growth inhibition.[27,28] Of note, there is evidence for a coordinated transcriptional regulation of *ANRIL, p16/CDKN2A,* and *p15/CDKN2B,* as well as other genes in the 9p21.3 region.[25] Further work is needed to determine whether the association between atherosclerotic stroke and the 9p21 region is mediated through these genes or other pathways possibly through long-range regulatory effects.

Apart from demonstrating an association with atherosclerotic stroke, we also replicated the reported association between 9p21.3 and both MI and CAD. The associated alleles and the mode of inheritance (recessive model, data not shown) match well with those previously reported for this phenotype, and the ORs are close to those found in recent studies.[6,13]

Stroke, most notably atherosclerotic stroke, shares common risk factors and mechanisms with MI and CAD.[15] Furthermore, there is substantial comorbidity between stroke, CAD, and MI. Between 20 and 40% of stroke patients have an abnormal cardiac stress response with the rate being greatest in patients with atherosclerotic stroke.[15,29] Carotid intima-media thickness, a quantitative marker, and intermediate phenotype for early atherosclerosis strongly correlates with both CAD and risk for MI.[30,31] Thus, one might speculate that the 9p21.3 region contributes to vascular disease by promoting atherosclerosis.

However, several observations suggest the mechanisms are more complex. First, in this study, associations with atherosclerotic stroke and CAD/MI were found to be independent from each other (see Table 2 and Supplementary Table 4), suggesting that the underlying processes do not run strictly in parallel. Second, the two lead SNPs for CAD (rs10757278-G) and atherosclerotic stroke (rs1537378) identified here are more than 70kb apart. Accumulating data suggest that these two lead SNPs are likely to represent the same signal, each serving as a marker for the same causal variant.[11] Thus, the difference in associated SNPs may just be coincidental. Alternatively, however, this observation might reflect differences in the genetic architecture of atherosclerotic stroke and CAD/MI with regard to the 9p21.3 region as previously documented for CAD and type 2 diabetes.[13] Third, it was recently shown that the 9p21.3 region is also implicated in risk for AAA and IA, which are pathologically distinct from atherosclerosis.[13] In fact, atherosclerosis is not considered a risk factor for IA.[32,33] Together, these findings raise the possibility that the chromosome 9p21 region has a broader role in large-artery disease possibly by impacting on vascular remodelling or repair rather than atherosclerosis per se.[34–36] This concept would agree with results from a recent study that found no association of the rs1333049 genotype with carotid intima-media thickness in 3,572 population-based subjects from Finland.[37] Further studies are needed to determine the mechanisms by which the chromosome 9p21.3 region affects vascular risk. Importantly, the observed effects of 9p21 on atherosclerotic stroke were found to be independent from established risk factors for cardiovascular disease. This finding agrees with similar data for CAD and MI showing that the effects of 9p21 on MI and CAD are not mediated by known vascular risk factors.[7,9]

The strongest associations were seen with rs1537378. This SNP was also significant in the overall group of IS patients and in patients with stroke of "undetermined" cause. Most likely, these associations likewise reflect an association with atherosclerotic stroke because both categories include patients with atherosclerotic stroke, and there was no significant signal for any of the other stroke subtypes including cardioembolic stroke and small vessel stroke. In accord with this, ORs were in the same direction but lower than for atherosclerotic stroke, which would be expected if the results were driven by atherosclerotic stroke.

Our findings may resolve some of the inconsistencies among recent studies that have looked for an association between sequence variants on 9p21 and IS risk.[13,16,17] These studies provided no[13,16] or marginally[17] significant evidence for an independent association between SNPs on 9p21.3 and IS as a whole. However, sample sizes were limited (between 249 and 705 IS cases), and stroke subtypes such as atherosclerotic stroke were not considered separately. This study documents the need for large sample sizes particularly when the ORs are moderate and associations are limited to stroke subtypes.

This study also has potential limitations. First, genotyping involved different methods and platforms that, in theory, might have impacted on our findings. However, we consider this possibility unlikely for the following reasons: (1) cases and control subjects were numerically well balanced on all three platforms; (2) ORs were similar across all sites and platforms (see Fig 2); and (3) ORs were similar for six neighboring SNPs, which can thus be considered technical replicates. Second, samples were ascertained through different protocols without central phenotyping. Conceivably, this might have affected the results. Yet again, we found no evidence for a center effect, suggesting that differences in case ascertainment and phenotyping are less relevant. Finally, we cannot exclude that CAD and MI remained underdiagnosed in our stroke patients, which, in turn, could have contributed to the association with stroke. However, a major bias appears unlikely. For one, this study replicated the known association with CAD and MI, which suggests that the level of phenotyping was good. Second, there was no evidence for an interaction between any of the SNPs and CAD or MI in multivariate analyses when considering stroke as the dependent variable. Regardless of this relation, the association between SNPs on 9p21 region and atherosclerotic stroke remains considerable and important.

The consistency of associations across a broad range of subjects and vascular conditions in conjunction with the substantial increase in IS risk, particularly in homozygous individuals, suggests that genotyping of this locus may have considerable clinical utility in risk prediction provided that these findings are supported in further large population-based studies. Future studies will determine whether genotyping of the 9p21 region adds to already established scores for cardiovascular risk such as the Framingham risk score.[38] Identifying individuals at risk for cardiovascular events may impact on preventive strategies. In this regard, these findings may have therapeutic implications.

In conclusion, this study demonstrates an unexpectedly broad role of the chromosome 9p21 region in arterial disease. Identification of the molecular pathways and biological mechanisms may offer new perspectives for therapeutic interventions.

This study was supported by the German Research Foundation (Di722/3-1 and KFGK1027 and KFGK1028 to MD); German Ministry of Education and Research (PGE-S04T13 and PGE-S04T10 to EW); National Institute of Neurologic Disorders and Stroke (K23NS042720 to KF; P50NS051343 to KF and JR; R01NS42733 to JFM); Deane Institute for Integrative Research in Atrial Fibrillation and Stroke (KF and JR); The Stroke Association (TSA2007/04 to MJM, HRA and PDS), Scottish Chief Scientist's Office (CZG/4/1/26 to MJM and PDS).

Note: the Grant from the Deane institute has no specific grant number.

We thank all the participants for agreeing to donate DNA for the study.

References

1. Murray CJ, Lopez AD. Mortality by cause for eight regions of the world: Global Burden of Disease Study. Lancet 1997;349: 1269–1276.
2. Donnan GA, Fisher M, Macleod M, et al. Stroke. Lancet 2008; 371:1612–1623.
3. Touze E, Rothwell PM. Heritability of ischaemic stroke in women compared with men: a genetic epidemiological study. Lancet Neurol 2007;6:125–133.
4. Dichgans M. Genetics of ischaemic stroke. Lancet Neurol 2007;6:149–161.
5. Matarin M, Brown WM, Scholz S, et al. A genome-wide genotyping study in patients with ischaemic stroke: initial analysis and data release. Lancet Neurol 2007;6:414–420.
6. Helgadottir A, Thorleifsson G, Manolescu A, et al. A common variant on chromosome 9p21 affects the risk of myocardial infarction. Science 2007;316:1491–1493.
7. McPherson R, Pertsemlidis A, Kavaslar N, et al. A common allele on chromosome 9 associated with coronary heart disease. Science 2007;316:1488–1491.
8. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 2007;447: 661–678.
9. Samani NJ, Erdmann J, Hall AS, et al. Genomewide association analysis of coronary artery disease. N Engl J Med 2007; 357:443–453.
10. Larson MG, Atwood LD, Benjamin EJ, et al. Framingham Heart Study 100K project: genome-wide associations for cardiovascular disease outcomes. BMC Med Genet 2007;8(suppl 1):S5.
11. Schunkert H, Gotz A, Braund P, et al. Repeated replication and a prospective meta-analysis of the association between chromosome 9p21.3 and coronary artery disease. Circulation 2008; 117:1675–1684.
12. Broadbent HM, Peden JF, Lorkowski S, et al. Susceptibility to coronary artery disease and diabetes is encoded by distinct, tightly linked SNPs in the ANRIL locus on chromosome 9p. Hum Mol Genet 2008;17:806–814.
13. Helgadottir A, Thorleifsson G, Magnusson KP, et al. The same sequence variant on 9p21 associates with myocardial infarction, abdominal aortic aneurysm and intracranial aneurysm. Nat Genet 2008;40:217–224.
14. Pasternak RC, Criqui MH, Benjamin EJ, et al. Atherosclerotic Vascular Disease Conference: Writing Group I: epidemiology. Circulation 2004;109:2605–2612.
15. Adams RJ, Chimowitz MI, Alpert JS, et al. Coronary risk evaluation in patients with transient ischemic attack and ischemic stroke: a scientific statement for healthcare professionals from the Stroke Council and the Council on Clinical Cardiology of the American Heart Association/American Stroke Association. Circulation 2003;108:1278–1290.
16. Zee RY, Ridker PM. Two common gene variants on chromosome 9 and risk of atherothrombosis. Stroke 2007;38:e111.
17. Matarin M, Brown M, Singleton A, et al. Whole genome analysis suggests ischemic stroke and heart disease share an association with polymorphisms on chromosome 9p21. Stroke 2008; 39:1586–1589.
18. Touze E, Varenne O, Chatellier G, et al. Risk of myocardial infarction and vascular death after transient ischemic attack and ischemic stroke: a systematic review and meta-analysis. Stroke 2005;36:2748–2755.
19. Witt BJ, Ballman KV, Brown RD Jr, et al. The incidence of stroke after myocardial infarction: a meta-analysis. Am J Med 2006;119:354–359.
20. Adams HP Jr, Bendixen BH, Kappelle LJ, 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.
21. Samani NJ, Thompson JR, O'Toole L, et al. A meta-analysis of the association of the deletion allele of the angiotensin-converting enzyme gene with myocardial infarction. Circulation 1996;94:708–712.
22. Zeggini E, Weedon MN, Lindgren CM, et al. Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. Science 2007;316:1336–1341.
23. Winkelmann J, Schormair B, Lichtner P, et al. Genome-wide association study of restless legs syndrome identifies common variants in three genomic regions. Nat Genet 2007;39: 1000–1006.
24. Skol AD, Scott LJ, Abecasis GR, et al. Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. Nat Genet 2006;38:209–213.
25. Pasmant E, Laurendeau I, Heron D, et al. Characterization of a germ-line deletion, including the entire INK4/ARF locus, in a melanoma-neural system tumor family: identification of ANRIL, an antisense noncoding RNA whose expression coclusters with ARF. Cancer Res 2007;67:3963–3969.
26. Kim WY, Sharpless NE. The regulation of INK4/ARF in cancer and aging. Cell 2006;127:265–275.
27. Hannon GJ, Beach D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature 1994;371:257–261.
28. Kalinina N, Agrotis A, Antropova Y, et al. Smad expression in human atherosclerotic lesions: evidence for impaired TGF-beta/Smad signaling in smooth muscle cells of fibrofatty lesions. Arterioscler Thromb Vasc Biol 2004;24:1391–1396.
29. Chimowitz MI, Poole RM, Starling MR, et al. Frequency and severity of asymptomatic coronary disease in patients with different causes of stroke. Stroke 1997;28:941–945.
30. O'Leary DH, Polak JF, Kronmal RA, et al. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. N Engl J Med 1999;340:14–22.
31. Lorenz MW, Markus HS, Bots ML, et al. Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis. Circulation 2007;115:459–467.
32. Clarke M. Systematic review of reviews of risk factors for intracranial aneurysms. Neuroradiology 2008;50:653–664.
33. Feigin VL, Rinkel GJ, Lawes CM, et al. Risk factors for subarachnoid hemorrhage: an updated systematic review of epidemiological studies. Stroke 2005;36:2773–2780.
34. Varnava AM, Mills PG, Davies MJ. Relationship between coronary artery remodeling and plaque vulnerability. Circulation 2002;105:939–943.
35. Glagov S, Weisenberg E, Zarins CK, et al. Compensatory enlargement of human atherosclerotic coronary arteries. N Engl J Med 1987;316:1371–1375.
36. Clarke MC, Figg N, Maguire JJ, et al. Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. Nat Med 2006;12:1075–1080.
37. Samani NJ, Raitakari OT, Sipila K, et al. Coronary artery disease-associated locus on chromosome 9p21 and early markers of atherosclerosis. Arterioscler Thromb Vasc Biol 2008;28: 1679–1683.
38. Fowkes FG, Murray GD, Butcher I, et al. Ankle brachial index combined with Framingham Risk Score to predict cardiovascular events and mortality: a meta-analysis. JAMA 2008;300:197–208.