Evidence HDAC9 Genetic Variant Associated With Ischemic Stroke Increases Risk via Promoting Carotid Atherosclerosis

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Background and Purpose—A novel association between a single nucleotide polymorphism on chromosome 7p21.1 and large-vessel ischemic stroke was recently identified. The most likely underlying gene is histone deacetylase 9 (HDAC9). The mechanism by which HDAC9 increases stroke risk is not clear; both vascular and neuronal mechanisms have been proposed.

Methods—We determined whether the lead single nucleotide polymorphisms were associated with asymptomatic carotid plaque (N=25 179) and carotid intima-media thickness (N=31 210) detected by carotid ultrasound in a meta-analysis of population-based and community cohorts. Immunohistochemistry was used to determine whether HDAC9 was expressed in healthy human cerebral and systemic arteries. In the Tampere Vascular Study, we determined whether HDAC9 mRNA expression was altered in carotid (N=29), abdominal aortic (N=15), and femoral (N=24) atherosclerotic plaques compared with control (left internal thoracic, N=28) arteries.

Results—Both single nucleotide polymorphisms (rs11984041 and rs2107595) were associated with common carotid intima-media thickness (rs2107595; P=0.0018) and with presence of carotid plaque (rs2107595; P=0.0022). In both cerebral and systemic arteries, HDAC9 labeling was seen in nuclei and cytoplasm of vascular smooth muscle cells, and in endothelial cells. HDAC9 expression was upregulated in carotid plaques compared with left internal thoracic controls (P=0.00000103). It was also upregulated in aortic and femoral plaques compared with controls, with mRNA expression increased in carotid compared with femoral plaques (P=0.0038).

Conclusions—Our results are consistent with the 7p21.1 association acting via promoting atherosclerosis, and consistent with alterations in HDAC9 expression mediating this increased risk. Further studies in experimental models are required to confirm this link. (Stroke. 2013;44:1220-1225.)

Key Words: atherosclerosis ■ carotid stenosis ■ expression experiments ■ genetics ■ intima-media thickness

Received November 19, 2012; accepted January 29, 2013.
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*Membership of Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium is listed in online-only Data Supplement.
The online-only Data Supplement is available with this article at http://stroke.ahajournals.orglookup/suppl/doi:10.1161/STROKEAHA.111.000217/-/DC1.
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Strok* is available at http://stroke.ahajournals.org

DOI: 10.1161/STROKEAHA.111.000217

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stroke is unclear.\textsuperscript{4} HDAC inhibitors have been suggested as a treatment for ischemic stroke.\textsuperscript{4}

We performed a series of experiments to explore the mechanisms underlying the 7p21.1 association with large artery stroke. First, we determined whether the SNP associated with large artery stroke was also associated with asymptomatic carotid plaque and carotid intima-media thickness (IMT) measured in community populations. Duplex ultrasound imaging can noninvasively visualize atherosclerotic plaques themselves and diffuse thickening of the arterial wall (thickened IMT), which is an independent predictor of stroke.\textsuperscript{5} Second, we determined whether HDAC9 was expressed in cerebral and systemic large arteries. Finally, we determined whether mRNA expression of HDAC9 was altered in atherosclerotic plaque using data from the Tampere Vascular Study.\textsuperscript{6}

### Methods

**Associations With Carotid Plaque and IMT**

Associations with carotid plaque and common carotid artery (CCA) IMT were examined in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium that brings together 5 population-based studies, and 4 additional community-based studies that had collaborated with the CHARGE consortium in a previous genome-wide association study (GWAS) of these phenotypes.\textsuperscript{7} All individuals have GWAS data; these were used to perform a look-up of the SNPs.

Measurements of CCA-IMT were available on 31,210 participants from 9 studies, and of carotid artery plaque on 25,179 participants from 7 studies. The individual studies were as follows: Aging Gene-Environment Susceptibility-Reykjavik Study (AGES), Atherosclerosis Risk in Communities (ARIC) Study, Cardiovascular Health Study (CHS), Framingham Heart Study (FHS), the Rotterdam Study I (RS-I), Old Order Amish (Amish) Study, Erasmus Rucphen Family (ERF) Study, SardiNIA Study, and Study of Health in Pomerania (SHIP). For all studies in the meta-analyses, each participant provided written informed consent, and the local institutional review board approved the study. Studies contributing to this meta-analysis have been described in detail previously; details are summarized in Table 1.

Each study evaluated the carotid arteries using B-mode ultrasonography and previously described reading protocols.\textsuperscript{7} Data were used from the baseline examination, or the first examination in which carotid ultrasonography was obtained. CCA-IMT was typically summarized as the mean of the maximum of several measurements. For most studies, this was an average of multiple measurements of both the left and right arteries. All studies measured the far wall, and in addition, several included the near wall. We also examined atherosclerotic thickening of the carotid artery wall, defined in 7 of the 9 studies by either the presence of plaque (ARIC, AGES, ERF, CHS, RS-I, SHIP) or the proxy measure of stenosis >25\% (FHS).

**Genotyping and Imputation**

The 9 studies used commercial genotyping platforms available from Illumina and Affymetrix. Each study performed genotyping quality control checks and imputed ≈2.5 million polymorphic autosomal SNPs described in the HapMap Utah residents with ancestry from northern and western Europe population for each participant using available imputation methods. Details of individual study genotyping, imputation, and quality control procedures have been previously published.

### Statistical Analysis Within Studies

Each study independently implemented a predefined GWAS analysis plan. For the continuous measures of CCA-IMT, we evaluated cross-sectional associations of log(IMT) and genomewide variation using linear regression models (or linear mixed effects models, in Amish, FHS, and ERF to account for family relatedness). For each of the 2.5 million SNPs, each study fit additive genetic models relating genotype dosage (0–2 copies of the variant allele) with the study trait. For the dichotomous outcome of plaque, each study used logistic regression models (or general estimating equations clustering on family to account for familial correlations in FHS and ERF). In our primary analyses, all studies adjusted for age and sex. Some studies made additional adjustments, including study site (ARIC and CHS), familial structure (Amish, FHS, and ERF), or for whether the DNA had been whole genome amplified (FHS). Full details have been previously published.\textsuperscript{7}

### Table 1. Details of the Individual Cohorts in the CHARGE Collaboration

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AGES (N = 3073)</th>
<th>Amish (N = 1054)</th>
<th>ARIC (N = 7767)</th>
<th>CHS (N = 3261)</th>
<th>ERF (N = 1809)</th>
<th>FHS (N = 3004)</th>
<th>RS-I (N = 4699)</th>
<th>SardiNIA (N = 4235)</th>
<th>SHIP (N = 2309)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>76.4 (5.4)</td>
<td>48.1 (15.9)</td>
<td>54.3 (5.7)</td>
<td>72.3 (5.4)</td>
<td>48.5 (14.5)</td>
<td>58.5 (9.7)</td>
<td>68.9 (8.7)</td>
<td>43.5 (17.5)</td>
<td>61.8 (9.5)</td>
</tr>
<tr>
<td>Women, %</td>
<td>57.7%</td>
<td>53%</td>
<td>53%</td>
<td>61%</td>
<td>56.5%</td>
<td>53.3%</td>
<td>59.3%</td>
<td>56.2%</td>
<td>48.6%</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>80.6%</td>
<td>27%</td>
<td>51%</td>
<td>51%</td>
<td>51.4%</td>
<td>40.5%</td>
<td>55.9%</td>
<td>29.1%</td>
<td>72.4%</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>11.6%</td>
<td>8%</td>
<td>12%</td>
<td>6.1%</td>
<td>8.6%</td>
<td>10%</td>
<td>4.8%</td>
<td>10.1%</td>
<td></td>
</tr>
<tr>
<td>Current smoker, %</td>
<td>12.6%</td>
<td>9.4%</td>
<td>25%</td>
<td>11%</td>
<td>39.4%</td>
<td>15.6%</td>
<td>23.4%</td>
<td>20.2%</td>
<td>19.2%</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>217.9 (44.5)</td>
<td>211.3 (48.1)</td>
<td>214.7 (40.5)</td>
<td>213.0 (38.9)</td>
<td>214.4 (42.6)</td>
<td>205.9 (39.7)</td>
<td>256.0 (46.8)</td>
<td>208.6 (42.1)</td>
<td>234.3 (47.9)</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>61.0 (17.1)</td>
<td>55.7 (14.8)</td>
<td>50.7 (16.8)</td>
<td>55.3 (15.8)</td>
<td>49.5 (14.1)</td>
<td>51.1 (16.1)</td>
<td>51.8 (13.9)</td>
<td>64.4 (14.9)</td>
<td>55.3 (17.8)</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td>107.0 (59.0)</td>
<td>74.9 (47.1)</td>
<td>136.0 (89.5)</td>
<td>140.4 (76.4)</td>
<td>118.6 (68.1)</td>
<td>142.3 (138.6)</td>
<td>N/A</td>
<td>87.2 (61.4)</td>
<td>177.6 (134.8)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.1 (4.5)</td>
<td>26.9 (4.7)</td>
<td>26.9 (4.7)</td>
<td>26.3 (4.5)</td>
<td>26.8 (4.7)</td>
<td>27.9 (5.1)</td>
<td>26.3 (3.7)</td>
<td>25.3 (4.7)</td>
<td>28.5 (4.6)</td>
</tr>
<tr>
<td>Prevalent CVD</td>
<td>21.9%</td>
<td>6.9%</td>
<td>5%</td>
<td>0%</td>
<td>3.1%</td>
<td>10.4%</td>
<td>30.8%</td>
<td>1.7%</td>
<td>8.4%</td>
</tr>
<tr>
<td>IMT common carotid</td>
<td>0.97 (0.1)</td>
<td>0.74 (0.2)</td>
<td>0.77 (0.2)</td>
<td>1.03 (0.2)</td>
<td>0.82 (0.2)</td>
<td>0.74 (0.2)</td>
<td>1.02 (0.2)</td>
<td>0.54 (0.1)</td>
<td>0.93 (0.2)</td>
</tr>
</tbody>
</table>

Numbers in table are mean (SD) or percentage. N in the column headers indicates number of participants with common carotid IMT available. Diabetes mellitus was defined as fasting blood glucose >125 mg/dL, a random blood glucose of >200 mg/dL, or use of insulin or oral hypoglycemic agents; hypertension was defined as blood pressure >140/90 mm Hg or on antihypertensive medication; current cigarette smoking was defined as self-reported cigarette smoking of ≥1 cigarette per day for a year at any examination; cardiovascular disease was defined as coronary heart disease, stroke or transient ischemic attack, or congestive heart failure. AGES indicates Aging Gene-Environment Susceptibility-Reykjavik Study; ARIC, Atherosclerosis Risk in Communities; BMI, body mass index; CHS, Cardiovascular Health Study; CVD, cardiovascular disease; ERF, Erasmus Rucphen Family; FHS, Framingham Heart Study; HDL, high-density lipoprotein; IMT, intima-media thickness; RS-I, Rotterdam Study I; and SHIP, Study of Health in Pomerania.
**Table 3. CHARGE Results of the Association Analyses for the 2 Lead SNPs Tested for CCA-IMT**

<table>
<thead>
<tr>
<th>CCA-IMT SNP</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Freq1.Z</th>
<th>Effect</th>
<th>SE</th>
<th>P Value</th>
<th>Direction</th>
<th>N</th>
<th>N_Eff</th>
<th>P Value.Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs11984041</td>
<td>t</td>
<td>c</td>
<td>0.088</td>
<td>0.0077</td>
<td>0.0027</td>
<td>0.00391</td>
<td>++++++++</td>
<td>31 210</td>
<td>26187.95 0.006243</td>
<td></td>
</tr>
<tr>
<td>rs2107595</td>
<td>a</td>
<td>g</td>
<td>0.146</td>
<td>0.0065</td>
<td>0.0021</td>
<td>0.00184</td>
<td>++++++++</td>
<td>31 210</td>
<td>27035.76 0.001833</td>
<td></td>
</tr>
</tbody>
</table>

CCA-IMT indicates common carotid artery intima-media thickness; and SNP, single nucleotide polymorphism.

**Meta-analysis**

We conducted a meta-analysis of beta estimates and SEs from the 9 studies using an inverse-variance weighting approach as implemented in METAL. Before meta-analysis, we calculated a genomic inflation factor ($\lambda$) for each study to screen for cryptic population substructure or undiagnosed irregularities that might have inflated the test statistics. Inflation was low, with $\lambda <1.09$ in all studies. We applied genomic control to each study whose genomic inflation factor was $>1.00$ by multiplying all of the SEs by the square root of the study-specific $\lambda$. For IMT, we express the association of each SNP and log(IMT) as the regression slope($\beta$), its SE($\beta$), and a corresponding $P$ value.

For the presence of plaque, we calculated a meta-analysis odds ratio, which represents the increase or decrease in the odds of plaque for each additional copy of the coded allele of the SNP.

We performed a look-up of 2 SNPs at 7p21.1. The WTCCC2 study found the strongest association with rs11984041, whereas recent GWAS meta-analyses in 12,389 ischemic stroke individuals and 62,004 controls found the strongest association with rs2107595, which is in linkage disequilibrium with rs11984041. To account for the 2 SNPs, we applied a Bonferroni correction and predefined a significance level of 0.025.

**Immunohistochemistry Studies of HDAC9 in Normal Arteries**

Expression of HDAC9 was examined by immunohistochemistry in human large arteries derived from surgical or post-mortem material: aorta (n=7), internal carotid (n=5), middle cerebral (n=5), and coronary arteries (n=5). Tissues were used with ethical approval via the UK National Research Ethics Service.

Anti-HDAC9 antibodies were rabbit polyclonal, 18970, and 59718 (both Abcam, Cambridge, United Kingdom); 18970 is raised against the peptide EVPVGLEPISPLDLRT (corresponding to residues 12–27 of human HDAC9 isoform 1) present in human HDAC9 isoforms 1,3,5,6,7,CRA_g, CRA_i, CRA_j, and 59718 is raised against a peptide corresponding to amino acids 541 to 590 at the C-terminus of human HDAC9 isoform 6 that is found in human HDAC9 isoforms 3,6,7,8,9,10, CRA_h, CRA_i, CRA_j.

Other antibodies used for immunohistochemistry were as follows: CD31(PECAM1), CD45 (leukocyte common antigen; clones 2B11 and PD7/26), and CD68 (clone PG-M1); all mouse monoclonals from Dako, Ely, United Kingdom; and smooth muscle $\alpha$-actin (mouse monoclonal, clone 1A4) and smooth muscle myosin (mouse monoclonal, clone h-SMV) from Sigma-Aldrich, Poole, United Kingdom.

Paraffin wax–embedded sections (6 $\mu$m) were processed for standard immunohistochemical labeling. Endogenous peroxidase activity was quenched by $H_2O_2$ (3% v/v, aqueous solution) for 8 minutes. After high-pressure heat-induced antigen retrieval (30 s, 125°C, in pH 7.8 Tris-citrate buffer), sections were exposed to primary antibodies. HDAC9 primary antibodies ab18970 and ab59718 were applied to human tissues (1:300) and to pig tissue (1:500) overnight at 4°C.

Antibody labeling was visualized using a peroxidase-conjugated secondary reagent (Envision kit, K-5007, Dako, Ely, United Kingdom) and diaminobenzidine chromogen, then counterstained with Mayer’s hematoxylin. Sections were examined on a Zeiss Axioplan-2 microscope driven by Axiovision software (version 4.7).

**Messenger RNA Expression Studies**

Carotid, femoral, and aortic atherosclerotic plaques constituting the intima and inner media were prospectively obtained between 2005 and 2009 from patients fulfilling the following inclusion criteria: (1) carotid endarterectomy attributable to asymptomatic or symptomatic >70% carotid stenosis, or (2) femoral or (3) aortic endarterectomy with aortoiliac or aortobifemoral bypass attributable to symptomatic peripheral arterial disease. Whole thickness left internal thoracic artery samples were used as controls and obtained during coronary artery bypass surgery. All open vascular surgical procedures were performed at the Division of Vascular Surgery and Heart Center, Tampere University Hospital. The study was approved by the local ethics committee; all patients gave informed consent.

Fresh tissue samples were immediately soaked in RNAlater solution (Ambion Inc) and homogenized using an Ultra-Turrax T80 homogenizer (IKA). RNA was extracted with the Trizol reagent (Invitrogen) and miRNEasy Mini-Kit (Qiagen) with the RNase-Free DNase Set (Qiagen) according to manufacturer instructions. The RNA isolation protocol was validated by analyzing the integrity of the RNA with the RNA 6000 Nano Chip Kit (Agilent).

The expression levels were analyzed with an Illumina HumanHT-12 v3 Expression BeadChip (Illumina). In brief, 300–500 ng of RNA was reverse transcribed in cRNA and biotin-UTP labeled using the IlluminaTotalPrep RNA Amplification Kit (Ambion), and 1500 ng of cRNA was then hybridized to the Illumina HumanHT-12 v3 Expression BeadChip.

The BeadChips were scanned with the Illumina Scan system. After background subtraction, raw intensity data were exported using the Illumina Genome Studio software. Further data processing was conducted by means of R language and appropriate Bioconductor modules. Data were log2-transformed, and robust multichip average and robust spline normalization (rma_rsn) were used.

**Results**

**Associations With Carotid Plaque and IMT**

Both SNPs (rs11984041 and rs2107595) were associated with both CCA-IMT (Table 2) and with presence of carotid plaque (Table 3). The strongest associations were seen for SNPs rs2107595; CCA-IMT $P=0.0018$; carotid plaque $P=0.0022$.

**Immunohistochemistry Studies of HDAC9 in Normal Arteries**

In all arterial beds, strong HDAC9 labeling was seen in nuclei and cytoplasm of VSMC, and in endothelial cells, where intact endothelia were visible (example in Figure 1A). In the medial layer, a high fraction of cells were labeled (80% to 90%) with

Table 3. CHARGE Results of the Association Analyses for the 2 Lead SNPs Tested for Carotid Plaque

<table>
<thead>
<tr>
<th>Carotid Plaque SNP</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Freq1.Z</th>
<th>Effect</th>
<th>SE</th>
<th>P Value</th>
<th>Direction</th>
<th>N</th>
<th>N_eff</th>
<th>P Value.Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs11984041</td>
<td>t</td>
<td>c</td>
<td>0.097</td>
<td>0.1069</td>
<td>0.0374</td>
<td>0.00425</td>
<td>++++++++</td>
<td>25 179</td>
<td>21 616.84 0.002554</td>
<td></td>
</tr>
<tr>
<td>rs2107595</td>
<td>a</td>
<td>g</td>
<td>0.159</td>
<td>0.0911</td>
<td>0.0298</td>
<td>0.00222</td>
<td>++++++++</td>
<td>25 179</td>
<td>22 257.60 0.001395</td>
<td></td>
</tr>
</tbody>
</table>
a similar distribution to the VSMC-specific marker smooth muscle myosin (Figure 1). A minority of medial cells, with fibroblast-like morphology, were negative for HDAC9 and smooth muscle myosin, consistent with normal incidence of structural fibroblasts. Similar results were obtained with the 2 different anti-HDAC9 antibodies used.

Distinct patterns of labeling were observed with primary antibodies to leukocyte common antigen (CD45) and a lysosomal marker for macrophage/monocytic cells (CD68) at similar titer. Immunolabeling was absent in adjacent negative control sections treated without primary antibody (Figures 1 and 2).

**Messenger RNA Expression Studies**

Gene expression was analyzed from 29 carotid, 15 abdominal aorta, 24 femoral plaques, and 28 atherosclerosis free left internal thoracic artery controls. Demographics and American Heart Association plaque grading for the different plaques are shown in Table 4.

HDAC9 expression was upregulated in carotid plaques compared with left internal thoracic artery controls. (P=0.00000103; fold change [FC]=3.06). It was also upregulated in aortic plaques (P=0.0038; FC=1.76) and femoral plaques (P=0.038; FC=1.57) compared with controls. HDAC9 mRNA expression was greater in carotid compared with femoral plaques (P=0.0038; FC=1.76), although there was no significant difference between carotid and aortic plaques (P=0.90; FC=1.19).

**Discussion**

Our results show the 7p21.1 locus, previously associated with large artery stroke, is associated with asymptomatic carotid plaque and carotid IMT in community populations. This is consistent with a mechanism related to acceleration of the progression of atherosclerosis. HDAC9 is the most likely gene underlying this association. Consistent with this, we demonstrated that HDAC9 is expressed in VSMC and endothelium of healthy human adult large arteries, including cerebral and systemic arteries. A similar pattern was obtained with 2 antibodies raised against 2 nonoverlapping HDAC9-specific sequences. Consistent with a role in atherosclerosis, we found increased expression of HDAC9 mRNA in carotid atherosclerotic plaques.

Although canonical HDACs are ubiquitously expressed, Class IIA HDACs (including HDAC9) have more restricted expression. Expression in heart, pancreatic islets, spinal cord, and brain of mouse embryos has been demonstrated, and...
Table 4. Demographics, Risk Factors, and AHA Plaque Class of Plaques From Different Vascular Beds

<table>
<thead>
<tr>
<th></th>
<th>Carotid Plaque</th>
<th>Aortic Plaque</th>
<th>Femoral Plaque</th>
<th>Control Arteries</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>29</td>
<td>15</td>
<td>24</td>
<td>28</td>
<td>96</td>
</tr>
<tr>
<td>Age (median), y (SD)</td>
<td>70 (9.5)</td>
<td>61.0 (10.8)</td>
<td>76.0 (9.4)</td>
<td>69.0 (9.6)</td>
<td>69.0 (10.2)</td>
</tr>
<tr>
<td>Men, %</td>
<td>62.1</td>
<td>73.3</td>
<td>70.8</td>
<td>82.1</td>
<td>71.9</td>
</tr>
<tr>
<td>Body mass index (median), kg/m² (SD)</td>
<td>25.6 (3.4)</td>
<td>25.9 (4.2)</td>
<td>26.7 (4.3)</td>
<td>28.2 (5.1)</td>
<td>27.0 (4.4)</td>
</tr>
<tr>
<td>Dyslipidemia, %</td>
<td>75.9</td>
<td>46.7</td>
<td>70.8</td>
<td>85.7</td>
<td>72.9</td>
</tr>
<tr>
<td>Statins, %</td>
<td>100.0</td>
<td>40.0</td>
<td>62.5</td>
<td>82.1</td>
<td>76</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>79.3</td>
<td>80.0</td>
<td>87.5</td>
<td>100.0</td>
<td>87.5</td>
</tr>
<tr>
<td>Blood pressure medication, %</td>
<td>82.8</td>
<td>80.0</td>
<td>79.2</td>
<td>92.9</td>
<td>84.4</td>
</tr>
<tr>
<td>History of smoking %</td>
<td>65.5</td>
<td>100.0</td>
<td>70.8</td>
<td>64.3</td>
<td>71.9</td>
</tr>
<tr>
<td>AHA Class V–VI, %, of the atherosclerotic arteries</td>
<td>82.8</td>
<td>73.3</td>
<td>62.5</td>
<td>NA</td>
<td>74.6</td>
</tr>
</tbody>
</table>

AHA indicates American Heart Association.

human tissue lysates for HDAC9 mRNA show high expression in skeletal muscle and brain. There are reports of HDAC9 protein expression using immunohistochemical labeling in cerebral medulloblastoma tumors (using one of the antibodies we used, ab59718)\textsuperscript{11} and in teeth, using a different antibody.\textsuperscript{12} We have been unable to find published data on HDAC9 expression in human blood vessels.

Since its discovery as a risk factor for stroke, a recent very large GWAS meta-analysis in 63,746 coronary artery disease cases and 130,681 controls has found an association of the 7p21.1 locus with coronary artery disease but with a much smaller effect size\textsuperscript{13}; the odds ratio was 1.09 compared with 1.42 with large artery stroke in WTCCC2.\textsuperscript{1} This suggests this locus predisposes to large artery disease in the carotid arteries to a much greater extent than to coronary artery disease. Interestingly, we found HDAC9 mRNA expression was greater in carotid compared with femoral plaques. How such a risk factor would preferentially increase risk of carotid plaque is uncertain. One possible factor is flow-dependent mechanisms dependent on local anatomy; local hemodynamic factors, and the anatomy of the carotid bifurcation, are known to be related to early atherosclerotic changes.\textsuperscript{14}

Taken together, our results are consistent with the 7p21.1 locus acting as a risk factor for atherosclerosis. Such an association with large artery stroke could be via increasing plaque development, or by mechanisms that result in plaque instability and increase the risk of subsequent thromboembolism, the major cause of stroke in large artery disease. The association with asymptomatic carotid plaque, plaques that have not yet become unstable and symptomatic, would support the former mechanism. We also found an association with carotid IMT, consistent with increased risk occurring at the earlier stages of plaque formation. Increased carotid IMT is believed to occur with both early atherosclerosis and also vascular remodeling.\textsuperscript{15}

HDACs catalyze removal of acetyl groups from ε-amino groups of lysine residues in a variety of proteins. HDACs have been studied mainly in the context of chromatin, where they serve an epigenetic function by deacetylating nucleosomal histones and altering the electrostatic properties of chromatin leading to gene repression. However, it is now recognized that HDACs deacetylate many nonhistone proteins, and are therefore also referred to as lysine deacetylases.\textsuperscript{16} There are 18 HDACs that are encoded by different genes and grouped into 4 classes on the basis of similarity to yeast transcriptional repressors.\textsuperscript{17} HDAC9 is a member of the class IIa HDACs. The class IIa HDACs interact with members of the myocyte enhancer factor-2 transcription factor family,\textsuperscript{18} which are regulators of VSMC proliferation. Given the VSMC expression of HDAC9, increased risk of large-vessel disease could be via promotion of atherosclerosis as a consequence of MDAC9-mediated increased VSMC proliferation, an action impeded by HDAC9 inhibition in vitro.\textsuperscript{19} HDAC inhibitors also have been shown to reduce proinflammatory cytokine expression, which has been implicated in atherosclerosis.\textsuperscript{17}

The antiepileptic drug sodium valproate has nonspecific HDAC inhibitory properties and has been shown to inhibit atherosclerosis in animal models.\textsuperscript{20} Intriguingly, sodium valproate therapy in man has been associated with lower stroke and myocardial infarction rates compared with other antiepileptic drugs.\textsuperscript{21} Specific inhibitors to a variety of HDACs are currently being developed and might offer potential in stroke and cardiovascular prevention.\textsuperscript{17}

The 2 HDAC9 SNPs we assessed for association with IMT were those most strongly associated previously with large artery stroke.\textsuperscript{19} They are in close linkage disequilibrium; in the 1000 genomes European ancestry individuals, linkage disequilibrium measures between the 2 SNPs are $R^2=0.568$, D-prime=0.936. Both are likely to be markers for an as-yet-unknown functional variant.

There are potential limitations to this study. Not all patients in whom IMT was measured had carotid plaque measured also. However, this would have tended to reduce power to detect association with plaque, and we found such an association. The CHARGE consortium includes several different populations, which introduces heterogeneity; therefore, we analyzed using a meta-analysis approach, and the associations we found were consistent across almost all populations (Table 2). In the mRNA expression studies, we used relatively small sample sizes, although we were still able to detect upregulation of HDAC9 in atherosclerotic plaque.

In conclusion, our results are consistent with the 7p21.1 association, which has previously been associated with symptomatic large artery stroke, acting via alterations in HDAC9 expression promoting atherosclerosis. Further studies in experimental models are now required to prove this association is indeed mediated via accelerated carotid atherosclerosis.
Acknowledgments
We thank the staff of Oxford Brain Collection and St George’s Hospital Cellular Pathology for supplying human tissue.

Sources of Funding
This study was supported in part by European Union 7th Framework Program funding for the AtheroRemo project [201668]. Tampere Vascular study has been supported by the Academy of Finland, Tampere, University Hospital Medical Fund (grant 9M048 and 9N035 for TeLeht), the Finnish Foundation of Cardiovascular Research, the Tampere Tuberculosis Foundation, and the Emil Aaltonen Foundation. Dr Hainsworth is supported by Action Medical Research and Neuroscience Research Foundation.

Disclosures
None.

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*Stroke*. 2013;44:1220-1225; originally published online February 28, 2013; doi: 10.1161/STROKEAHA.111.000217

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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/44/5/1220

Data Supplement (unedited) at:
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Evidence HDAC9 genetic variant associated with ischaemic stroke increases risk via promoting carotid atherosclerosis.

CHARGE consortium membership

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The following studies contributed to the CHARGE Consortium GWAS discovery stage:

The Age, Gene/Environment Susceptibility (AGES) Reykjavik Study was funded by US National Institutes of Health (NIH) contract N01-AG-12100, the National Institute on Aging (NIA) Intramural Research Program, Hjartavernd (the Icelandic Heart Association) and the Althingi (the Icelandic Parliament).

The Old Order Amish Studies were supported by grants and contracts from the NIH including R01 AG18728 (Amish Longevity Study), R01 HL088119 (Amish Calcification Study), U01 GM074518-04 (The Amish Pharmacogenomics of Anti-Platelet Intervention (PAPI) study) and U01 HL072515-06 (the Heredity and Phenotype Intervention (HAPI) heart study), the University of Maryland General Clinical Research Center grant M01 RR 16500, the Baltimore Veterans Administration Medical Center Geriatrics Research and Education Clinical Center and the Paul Beeson Physician Faculty Scholars in Aging Program. We thank our Amish research volunteers for their long-standing partnership in research and the research staff at the Amish Research Clinic for their hard work and dedication.

The Atherosclerosis Risk in Communities Study (ARIC) was carried out as a collaborative study supported by National Heart, Lung, and Blood Institute contracts N01-HC-55015, N01-HC-55016, N01-HC-55018, N01-HC-55019, N01-HC-55020, N01-HC-55021, N01-HC-55022, R01HL087641, R01HL59367 and R01HL086694; National Human Genome Research Institute contract U01HG004402; and US National Institutes of Health contract HHSN268200625226C. The authors thank the staff and participants of the ARIC study for
their important contributions. Study infrastructure was partly supported by grant number UL1RR025005, a component of the US National Institutes of Health and NIH Roadmap for Medical Research.

The Erasmus Rucphen Family Study was supported by grants from The Netherlands Organisation for Scientific Research, Erasmus Medical Center and the Centre for Medical Systems Biology (CMSB). We are grateful to all study participants and their relatives, the general practitioners and neurologists for their contributions and to P. Veraart for her help in genealogy, J. Vergeer for the supervision of the laboratory work and P. Snijders for his help in data collection.

Cardiovascular Health Study: This CHS research was supported by NHLBI contracts N01-HC-85239, N01-HC-85079 through N01-HC-85086; N01-HC-35129, N01 HC-15103, N01 HC-55222, N01-HC-75150, N01-HC-45133, HHSN268201200036C and NHLBI grants HL080295, HL087652, HL105756 with additional contribution from NINDS. Additional support was provided through AG-023629, AG-15928, AG-20098, and AG-027058 from the NIA. See also http://www.chs-nhlbi.org/pi.htm. DNA handling and genotyping at Cedars-Sinai Medical Center was supported in part by the National Center for Research Resources, grant UL1RR033176, and is now at the National Center for Advancing Translational Sciences, CTSI grant UL1TR000124; in addition to the National Institute of Diabetes and Digestive and Kidney Disease grant DK063491 to the Southern California Diabetes Endocrinology Research Center.

The Framingham Heart Study of the National Heart, Lung, and Blood Institute of the US National Institutes of Health and Boston University School of Medicine was supported by the National Heart, Lung, and Blood Institute's Framingham Heart Study (Contract No.
N01-HC-25195) and its contract with Affymetrix, Inc. for genotyping services (Contract No. N02-HL-6-4278) and by grants from the National Institute of Neurological Disorders and Stroke (NS17950, P.A.W.) and the National Institute of Aging (AG08122, AG16495, P.A.W. and AG033193, S.S.). A portion of this research used the Linux Cluster for Genetic Analysis (LinGA-II) funded by the Robert Dawson Evans Endowment of the Department of Medicine at Boston University School of Medicine and Boston Medical Center. Analyses reflect intellectual input and resource development from the Framingham Heart Study investigators participating in the SNP Health Association Resource (SHARe) project.

Rotterdam Study I and II (RS I and RS II): The Rotterdam GWAS was funded by the Netherlands Organisation of Scientific Research (NWO, De Nederlandse Organisatie voor Wetenschappelijk Onderzoek) Investments (number 175.010.2005.011, 911-03-012), the Research Institute for Diseases in the Elderly (014-93-015; RIDE2), the Netherlands Genomics Initiative (NGI)/Netherlands Consortium for Healthy Aging (NCHA) project number 050-060-810. This study was further supported by an NWO grant (vici, 918-76-619). The Rotterdam Study was funded by the Erasmus Medical Center and Erasmus University, Rotterdam, Netherlands Organization for the Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry for Health, Welfare and Sports, the European Commission (DG XII), and the Municipality of Rotterdam. The authors are very grateful to the participants and staff from the Rotterdam Study, the participating general practitioners and the pharmacists. We thank P. Arp, M. Jhamai, M. Moorhouse, M. Verkerk and S. Bervoets for their help in creating the GWAS database. We would like to thank T.A. Knoch, L.V. de Zeeuw, A. Abuseiris and R. de Graaf as well as their institutions the Erasmus Computing Grid, Rotterdam, The Netherlands, and especially the National German MediGRID and Services@MediGRID part of the German D-Grid, both funded by
the German Bundesministerium fuer Forschung und Technology under grants #01 AK 803 A-H and #01 IG 07015 G, for access to their grid resources.

The SardiNIA Study: This work was supported by the Intramural Research Program of the National Institute on Aging, NIH. The SardiNIA ('Progenia') team was supported by Contract NO1-AG-1-2109 from the National Institute on Aging. The efforts of G.R.A. were supported in part by contract 263-MA-410953 from the National Institute on Aging to the University of Michigan and by research grants HG005581 and HL084729 from the National Institutes of Health (to G.R.A.).

The Study of Health in Pomerania (SHIP) is part of the Community Medicine Research net of the University of Greifswald, Germany, which is funded by the Federal Ministry of Education and Research (grants number 01ZZ9603, 01ZZ0103 and 01ZZ0403), and the Ministry of Cultural Affairs as well as the Social Ministry of the Federal State of Mecklenburg-West Pomerania. Genome-wide data have been supported by the Federal Ministry of Education and Research (grant no. 03ZIK012) and a joint grant from Siemens Healthcare, Erlangen, Germany, and the Federal State of Mecklenburg-West Pomerania. The SHIP authors are grateful to the contribution of A. Teumer, A. Hoffmann and A. Petersmann in generating the SNP data. The University of Greifswald is a member of the 'Center of Knowledge Interchange' program of the Siemens AG.