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

The Wellcome Trust Case Control Consortium 2 (WTCCC2) Ischemic Stroke Study identified a novel association between a single nucleotide polymorphism (SNP) on chromosome 7p21.1 and ischemic stroke.1 This association was present with large artery atherosclerotic stroke but not with cardioembolic or small vessel stroke.

The most likely underlying gene is histone deacetylase 9 (HDAC9). All SNPs showing an association resided within a peak between 2 recombination hotspots and encompassed the tail end of HDAC9.1 The mechanisms underlying this association remain uncertain. HDAC9 is a member of a large family of genes that encode proteins responsible for deacetylation of histones, and therefore regulation of chromatin structure and gene transcription.2 Although known as histone deacetylases, these proteins also act on other substrates and lead to both upregulation and downregulation of genes. How HDAC9 might increase stroke risk is not clear. The specific association with large artery stroke would be consistent with a mechanism via accelerating atherosclerosis or promoting plaque instability. The HDAC9 protein inhibits myogenesis and is involved in heart development, but no specific mechanism that could predispose to pathological processes in large systemic arteries has yet been reported.3 Alternatively, it could increase risk by altering brain ischemic responses, and therefore have effects on neuronal survival; although how this would only predispose to large artery...
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 Ruchpen 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\textsuperscript{7}; 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 $\approx$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>54.9%</td>
<td>53%</td>
<td>61%</td>
<td>56.5%</td>
<td>53.3%</td>
<td>59.3%</td>
<td>56.2%</td>
<td>50.6%</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>80.6%</td>
<td>9.3%</td>
<td>27%</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>2.1%</td>
<td>8%</td>
<td>12%</td>
<td>6.1%</td>
<td>6.8%</td>
<td>15%</td>
<td>4.7%</td>
<td>10.1%</td>
</tr>
<tr>
<td>Current smoker, %</td>
<td>12.6%</td>
<td>9.4%</td>
<td>25%</td>
<td>11%</td>
<td>39%</td>
<td>15.6%</td>
<td>23%</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 tables 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 $\geq$1 cigarette per day for a year at any attended examination; cardiovascular disease was defined as coronary heart disease, stroke or transient ischemic attack, or congestive heart failure. AGES indicates Aging Gene-Environment Susceptibility-Reykjavik Study; ARIC, Atherosclerosis Risk in Communities; BMI, body mass index; CHS, Cardiovascular Health Study; CVD, cardiovascular disease; ERF, Erasmus Ruchpen Family; FHS, Framingham Heart Study; HDL, high-density lipoprotein; IMT, intima-media thickness; RS-I, Rotterdam Study I; and SHIP, Study of Health in Pomerania.
Meta-analysis
We conducted a meta-analysis of beta estimates and SEs from the 9 studies using an inverse-variance weighting approach as implemented in METAL. Before meta-analysis, we calculated a genomic inflation factor ($\lambda_c$) for each study to screen for cryptic population substructure or undiagnosed irregularities that might have inflated the test statistics. Inflation was low, with $\lambda_c$ <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 $\lambda_c$. 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. Other antibodies used for immunohistochemistry were as follows: C3D1 (PECAM1), CD45 (leucocyte 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$_2$O$_2$ (3% v/v; aqueous solution) for 5 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. Immunohistochemical 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 Axiosoplan-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 rs11984041 and 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 intact endothelia were visible (example in Figure 1A). 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 intact

Table 2. 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>PValue</th>
<th>Direction</th>
<th>N</th>
<th>N_Eff</th>
<th>PValue.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>31210</td>
<td>26187.95</td>
<td>0.006243</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>31210</td>
<td>27035.76</td>
<td>0.001833</td>
</tr>
</tbody>
</table>

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

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>PValue</th>
<th>Direction</th>
<th>N</th>
<th>N_Eff</th>
<th>PValue.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>25179</td>
<td>21616.84</td>
<td>0.002554</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>25179</td>
<td>22257.60</td>
<td>0.001395</td>
</tr>
</tbody>
</table>

SNP indicates single nucleotide polymorphism.
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.

Hypertension, % 79.3 80.0 87.5 100.0 87.5
Dyslipidemia, % 75.9 46.7 70.8 85.7 72.9
Statins, % 100.0 40.0 62.5 82.1 76
Hypertension, % 79.3 80.0 87.5 100.0 87.5
Blood pressure medication, % 82.8 80.0 79.2 92.9 84.4
History of smoking % 65.5 100.0 70.8 64.3 71.9
AHA Class V–VI, %, of the atherosclerotic arteries 82.8 73.3 62.5 NA 74.6

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)11 and in teeth, using a different antibody.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 size13; the odds ratio was 1.09 compared with 1.42 with large artery stroke in WTCCC2.1 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 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 deetylases.14 There are 18 HDACs that are encoded by different genes and grouped into 4 classes on the basis of similarity to yeast transcriptional repressors.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,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 MDA9-mediated increased VSMC proliferation, an action impeded by HDAC9 inhibition in vitro.19 HDAC inhibitors also have been shown to reduce proinflammatory cytokine expression, which has been implicated in atherosclerosis.17

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

The 2 HDAC9 SNPs we assessed for association with IMT were those most strongly associated previously with large artery stroke.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 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 symptomatic 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.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 deetylases.14 There are 18 HDACs that are encoded by different genes and grouped into 4 classes on the basis of similarity to yeast transcriptional repressors.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,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 MDA9-mediated increased VSMC proliferation, an action impeded by HDAC9 inhibition in vitro.19 HDAC inhibitors also have been shown to reduce proinflammatory cytokine expression, which has been implicated in atherosclerosis.17

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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.
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None.

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

Evidence HDAC9 genetic variant associated with ischaemic stroke increases risk via promoting carotid atherosclerosis.

CHARGE consortium membership

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