Deficiency of the Stroke Relevant HDAC9 Gene Attenuates Atherosclerosis in Accord With Allele-Specific Effects at 7p21.1

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Background and Purpose—Recent genome-wide association studies identified the histone deacetylase 9 (HDAC9) gene region as a major risk locus for large-vessel stroke and coronary artery disease. However, the mechanisms linking variants at this locus to vascular risk are poorly understood. In this study, we investigated the candidacy and directionality of HDAC9 in atherosclerosis and analyzed associations between risk alleles at 7p21.1 and plaque characteristics.

Methods—Allele-dependent expression of HDAC9 was analyzed in human peripheral blood mononuclear cells of healthy donors. Effects of HDAC9 deficiency on atherosclerotic plaques were investigated in 18- and 28-week-old ApoE−/− mice by histology and immunohistochemistry. We further performed detailed plaque phenotyping and genotyping of rs2107595, the lead single-nucleotide polymorphism for large-vessel stroke, in carotid endarterectomy samples of 1858 subjects from the Athero-Express study.

Results—Gene expression studies in peripheral blood mononuclear cells revealed increased mRNA levels of HDAC9 but not of neighboring genes (TWIST1/FERD3L) in risk allele carriers of rs2107595. Compared with HDAC9+/+ApoE−/− mice, HDAC9−/−ApoE−/− mice exhibited markedly reduced lesion sizes throughout atherosclerotic aortas and significantly less advanced lesions. The proportion of Mac3-positive macrophages was higher in plaques from HDAC9−/−ApoE−/− mice, but this was largely because of a lower proportion of advanced lesions. Analysis of human atherosclerotic plaques revealed no association between rs2107595 and specific plaque characteristics.

Conclusions—Our results suggest that HDAC9 represents the disease-relevant gene at the stroke and coronary artery disease risk locus on 7p21.1, and that risk alleles in this region mediate their effects through increased HDAC9 expression. Targeted inhibition of HDAC9 might be a viable strategy to prevent atherosclerosis. (Stroke. 2015;46:00-00.)

Key Words: atherosclerosis ■ HDAC9 protein, human ■ stroke
Hence, HDAC9 represents a strong candidate gene for atherosclerosis. However, there are no expression data supporting the candidacy of HDAC9 over other genes at 7p21.1. In addition, the relationship between risk variants in this region and plaque morphology remains to be determined. We thus investigated samples from healthy subjects and patients undergoing carotid endarterectomy.15 We further tested the hypothesis that deficiency of HDAC9 attenuates atherosclerosis in mice.

Methods

Allele Carrier Status and mRNA Expression

DNA and RNA were isolated from peripheral blood mononuclear cells of healthy donors (43 men; 34 women; age 65.5±20.8 [mean±SD] years). Carrier status at rs2107595 was determined by direct sequencing. RNA was isolated using PAXgene Blood RNA Kit (PreAnalytiX GmbH) according to the manufacturer’s protocol. For each sample, 800 ng of deoxyribonuclease-treated RNA was converted into cDNA (Omniscript RT Kit, Qiagen) using Oligo dT(15) primer. For quantitative real-time polymerase chain reaction, cDNAs were diluted with water 1:9 and analyzed using TaqMan Gene Expression Assays (ABI Bioscience) for human HDAC9, TWIST1, FERD3L, and RPLP0. Measurements were performed in triplicates. Results for target genes were normalized to the reference gene RPLP0 and to a calibrator sample. All subjects provided written informed consent.

Atherosclerotic Plaque Analysis in Mice

All animal protocols were approved by the government of Upper Bavaria. HDAC9−/− mice from E. Olson14 were provided by Laurent Bavaria. HDAC9−/− mice from E. Olson14 were provided by Laurent

Statistical Analysis

For the human mRNA expression data and the marine plaque analysis, GraphPad Prism 5 (GraphPad Software) and SigmaPlot 12.5 (Systat Software Inc) were used for statistical analysis. Groupwise comparisons (n>2) were performed using a nonparametric Kruskal–Wallis test followed by a Mann–Whitney test. Pairwise comparisons were performed using a Mann–Whitney test. Differences were considered statistically significant at P<0.05. Data are expressed as mean±SEM.

rs2107595 and Human Atherosclerotic Plaques

Human carotid atherosclerotic plaques (n=1858) were obtained from the Athero-Express study, an ongoing, longitudinal, multicenter study collecting carotid atherosclerotic plaques from patients with significant (>70%) stenosis who undergo carotid endarterectomy.15,16 The medical ethics committee of the participating centers approved the study, and written informed consent was obtained from all patients. Immunohistochemical plaque phenotyping was performed as described elsewhere.13,16 In brief, carotid plaques were divided into segments of 5-mm thickness. The culprit lesion was defined and used for immunohistochemical staining. Calcification (hematoxylin and eosin) and collagen content (picrosirius red) were semiqualitatively defined as no or minor versus moderate/heavy staining as previously described.13,16 Atheroma size was defined as less than or more than 10% fat content (hematoxylin and eosin and picrosirius red). We quantitatively scored macrophages (CD68) and smooth muscle cells (α-actin) as percentage plaque area. We also determined the presence of intraplaque hemorrhage (hematoxylin and eosin) and counted the number of vessels per 3 to 4 hotspots (CD34).

rs2107595 was imputed using phased data from HapMap 2 (release 22, b36) as a reference. Quality control was performed according to standard procedures. Detailed description of genotyping, quality control, and imputation is provided in the online-only Data Supplement.

rs2107595 Risk Allele Carriers Show Increased HDAC9 mRNA Expression in Peripheral Blood Mononuclear Cells

rs2107595, the lead single-nucleotide polymorphism in METASTROKE,8 is situated in the intergenic region between
HDAC9 and TWIST1/FERD3L and colocalizes with both deoxyribonuclease I hypersensitivity clusters and histone modification hotspots (UCSC Genome Browser, genome build hg18; Figure 1A), suggesting a possible involvement of this variant in gene regulation. We thus measured mRNA expression levels of HDAC9, TWIST1, and FERD3L in peripheral blood mononuclear cells from healthy donors. HDAC9 mRNA levels were significantly increased in homozygous and heterozygous carriers of the risk allele (rs2107595A) with a gene dosage effect (Figure 1B). In contrast, mRNA levels of the adjacent genes TWIST1 and FERD3L did not correlate with allele carrier status at rs2107595 (Figure 1 in the online-only Data Supplement), suggesting that variants at this locus regulate HDAC9 expression and that the effects of this locus on stroke risk might be mediated through increased HDAC9 expression.

HDAC9 Deficiency Reduces Atherosclerotic Lesion Size in Mice
To test the hypothesis that HDAC9 deficiency attenuates atherogenesis and to refine its influence on plaque composition, we crossed HDAC9−/− mice with ApoE−/− mice and fed them a normal chow diet. HDAC9−/−ApoE−/− mice showed a significant reduction of atherosclerotic lesion size at both 18 and 28 weeks in aortic valve sections (Figure 2A and 2B) and in aortic arch sections (Figure 2C and 2D). A significant reduction in plaque area was also found in the brachiocephalic artery and aortic curvature (Figure IIA in the online-only Data Supplement). The number of plaques in the aortic arch was significantly reduced (Figure IIB in the online-only Data Supplement). Next, we categorized atherosclerotic lesion stage using the Virmani classification. We distinguished intimal xanthomas consisting mainly of foam cells from advanced atheromas characterized by fibrous cap and acellular areas. In brachiocephalic artery plaques (Figure 3A and 3B), only 2 of 8 HDAC9−/−ApoE−/− mice showed acellular areas compared with 12 of 13 HDAC9+/+ApoE−/− mice (P < 0.01; Figure 3C). The reduction in acellular cores corresponded to the increased number of nuclei per lesion area in HDAC9−/−ApoE−/− mice (Figure 3A and 3B), Relative mRNA expression of the adjacent genes of rs2107595. HDAC9 mRNA levels are significantly increased in homozygous and heterozygous carriers of the risk allele (A) with a gene dosage effect (GG n=51, GA n=22, AA n=4). Results represent means±SEM. *P<0.05, **P<0.01 (Kruskal–Wallis test P=0.003, followed by Mann–Whitney test).

rs2107595 Has No Discernable Effect on Plaque Morphology in Humans
To explore potential associations between risk alleles at HDAC9 and specific advanced plaque characteristics, we genotyped 1858 subjects with available detailed plaque phenotyping from Athero-Express. Of these subjects, 1439 passed quality control. Their demographic and clinical characteristics are presented in Table I in the online-only Data Supplement. There was no significant association between rs2107595 and specific plaque characteristics in the overall sample (Table)

Discussion
This study provides converging evidence that HDAC9 represents the disease-relevant gene at the stroke and CAD risk locus on 7p21.1 and that risk alleles at this locus mediate their effects through increased HDAC9 expression.

Our finding of increased HDAC9 mRNA expression in risk allele carriers of rs2107595, the lead single-nucleotide polymorphism in METASTROKE, is consistent with recent data from the ENCODE project showing that the majority of disease-associated variants are located in regulatory regions and control genes within a distance of ±500 kb. rs2107595 resides inside a deoxyribonuclease I hypersensitivity cluster and histone modification hotspot. In fact, rs2107595A is predicted...
to disrupt a consensus sequence for an E2F3-binding site (TRANSFAC database, data not shown), a transcription factor forming a repressor complex with Rb-proteins, thus raising the possibility that this variant is implicated in gene regulation. However, the exact mechanism by which genetic variation at this locus regulates HDAC9 expression remains

Figure 2. Histone deacetylase 9 (HDAC9) deficiency reduces atherosclerotic plaque size in ApoE-deficient mice. A, Representative images showing Oil-Red-O and hematoxylin-stained cryosections from the proximal aorta of HDAC9+/+ApoE−/− and HDAC9−/−ApoE−/− mice at 18 and 28 weeks. Scale bars, 250 μm. B, Absolute plaque areas at different distances from the aortic valves of 18-week-old mice (top) and relative plaque area (in relation to the area inside of the external elastic lamina; bottom). C, Representative images showing dissected aortic arches of 28-week-old mice. D, Total plaque area in the aortic arch of 28-week-old mice. Values represent mean plaque area for each mouse; n=8 to 13 for each genotype and time point; *P<0.05; **P<0.01 vs wild-type (Mann–Whitney test).

Figure 3. Histone deacetylase 9 (HDAC9) deficiency affects composition of atherosclerotic plaques in the brachiocephalic artery (BCA) of 28-week-old mice. A, Overview of aortic arch. Orange rectangle indicates region of interest for plaque analysis in the BCA. B, Representative images of hematoxylin and eosin–stained plaques are shown. C, Acellular core per lesion area in the BCA. D, Nuclei per lesion area in the BCA. n=8 to 13 for each genotype; scale bars, 100 μm; *P<0.05; **P<0.01 vs wild-type (Mann–Whitney test). CCA indicates common carotid artery; Curv., aortic curvature; and SCA, subclavian artery.
to be determined. TWIST1 and FERD3L, the only other genes nearby, exhibited no allele-dependent mRNA expression and are thus less likely to be implicated in locus-specific effects. However, because our expression analyses were restricted to peripheral blood mononuclear cells, we cannot exclude regulatory effects in other cell types.

Complementing our expression data, we found deficiency of HDAC9 to attenuate atherogenesis in ApoE−/− mice. The reduction of atherosclerotic lesion size reaching ≤45% in 28-week-old animals was present throughout all segments of the aortic arch (aortic valves, curvature, and branching arteries) and already significant in 18-week-old animals. Staging of lesions further revealed that HDAC9−/−ApoE−/− mice had significantly less advanced lesions compared with HDAC9+/−ApoE−/− mice. Together, these findings demonstrate a strong effect of HDAC9 expression on atherogenesis. The higher proportion of Mac3-positive macrophages in plaques from HDAC9−/−ApoE−/− mice likely reflects the lower proportion of acellular areas that are typical for advanced lesions because there was no difference of Mac3 infiltration when we restricted our analyses to intimal xanthomas. Hence, we found no obvious influence of HDAC9 deficiency on plaque morphology.

In accordance with our findings in mice, no significant association between rs2107595 and specific plaque characteristics was found in human carotid plaques from the Athero-Express study. Importantly, our power to detect an association with macrophage count, smooth muscle cell count, and vessel density was >75%. Hence, we are confident that we would have captured any prominent effect on these plaque phenotypes. However, our power to detect associations with binary plaque phenotypes was limited. Thus, we cannot exclude allele-specific effects on collagen content, atheroma size, calcification, and intraplaque hemorrhage. Interestingly, a recent meta-analysis of population-based cohorts revealed an association of rs2107595 with carotid intima media thickness measured by ultrasound. Taken together, these data suggest that HDAC9 expression promotes early atherogenesis rather than accelerating progression to vulnerable plaques. However, we cannot exclude skewing of macrophage polarization from M1 to M2 phenotype as previously reported.

The precise mechanisms by which increased HDAC9 expression promotes atherogenesis and vascular risk remain to be determined. A recent study found that targeted depletion of HDAC9 in bone marrow cells attenuates atherogenesis in LDLr−/− mice. HDAC9 was further shown to repress cholesterol efflux in macrophages. However, whether the changes in macrophages give rise to atherosclerotic lesion formation remains speculative. Given the widespread expression of HDAC9 in multiple cell types, there are several mechanisms by which increased HDAC9 expression could promote atherosclerosis. For example, HDAC9 has been shown to inhibit FOXP3 expression and the function of regulatory T cells, which protect against atherosclerosis.

Our findings imply that targeting HDAC9 may be a viable strategy to prevent atherosclerosis progression. Broad-spectrum HDAC inhibitors have multiple off-target effects and exacerbate atherosclerosis. Selective inhibitors of class Ia histone deacetylases have recently been developed but not yet sufficiently tested in vivo. Given the pleiotropic role of class Ia histone deacetylases developing inhibitors with selectivity for HDAC9 seems the most promising approach to target atherosclerotic lesion formation specifically.
In conclusion, this study provides mechanistic insights on how genetic variation in the HDAC9 gene region, a major risk locus for large-vessel stroke and CAD, promotes atherosclerosis. Moreover, our findings suggest that selective pharmacological inhibition of HDAC9 may be an effective strategy to prevent atherosclerosis and its clinical manifestations.

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Disclosures

None.

References

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SUPPLEMENTAL METHODS

Human carotid plaque studies

Genotyping
We performed two genome-wide association (GWA) experiments in 1,858 consecutive patients from Athero-Express Study. DNA was extracted from whole blood or carotid plaques following standardized protocols. The first experiment was performed in the Athero-Express Genomics Study 1 (AEGS1; n=836 patients recruited between 2002 and 2007) and was carried out by AROS Applied Biotechnology (Denmark) under study number A318 using the Affymetrix Genome-Wide Human SNP Array 5.0 (SNP5) chip (Affymetrix Inc., Santa Clara, CA, USA). The second experiment was performed in the Athero-Express Genomics Study 2 (AEGS2; n=1,022 patients recruited between 2002 and 2013). These samples were genotyped at the Genomic Analysis Center, Helmholtz Center Munich (Germany) using the Affymetrix Axiom GW CEU 1 Array (AxM).

Genotyping quality control and imputation
Quality control (QC) was performed according to standard procedures. Genotype calling was conducted using the standard settings of Affymetrix Genotyping Console 4.0 Software (GCOS4) and the proper algorithm for each chip (BRLMM-P for SNP5 and Axiom GT1 for AxM, respectively). Subsequent QC included the following steps: We first excluded samples with low average genotype calling and gender discrepancies (compared to clinical data) based on GCOS4 metrics. Next, data were filtered on 1) individual (sample) call rate > 97%, 2) SNP call rate > 97%, 3) minor allele frequencies > 3%, 4) average heterozygosity rate ± 3.0 SD, 5) relatedness (pi-hat > 0.20), 6) Hardy–Weinberg Equilibrium (HWE P-value < 1.0x10^{-6}), and 7) population stratification (based on HapMap 2, release 22, b36) by excluding samples deviating more than 6 standard deviations from the average in 5 iterations during principal component analysis and by visual inspection. All subjects were of self-reported European ancestry, which was confirmed through PCA. After QC 657 and 869 samples from AEGS1 and AEGS2 respectively, were available for imputation. A total of 403,789 markers in AEGS1 and 535,983 in AEGS2 were included before imputation. Autosomal missing genotypes were imputed in both datasets using phased data from the HapMap 2 project (release 22, b36) and BEAGLE v3.3.2 (31 October 2011).

Statistical analysis
For statistical analysis quantitative histological phenotypes were normalized through natural log (LN) transformation; outliers deviating more than ±3 SD from the LN-transformed mean were removed. Genetic analyses were corrected for age, sex, 10 principal components, year of surgery and a dummy variable representing the two genotyping batches, assuming an additive genetic model. Linear and logistic regression models were used for quantitative and binary phenotypes, respectively. IBM SPSS Statistics version 20 (release 20.0.0, IBM Corp., Armonk, NY, USA) was used for statistical analyses of baseline characteristics. PLINK v1.7 and Golden Helix SNP & Variation Suite v8.1.5 (Golden Helix, Inc., Bozeman, MT, USA) were used for genetic analyses. Our power to detect an association between rs2107595 and individual plaque phenotypes at a significance level of 0.05 [0.005] with a sample size of 1,400, an effect size of 0.182 and a minor allele frequency of 20% was >90% [>75%] for quantitative phenotypes. For binary phenotypes our power to detect an OR of 1.20 (corresponding to an effect size of 0.182) was between 20% [4%] (for collagen) and 54% [23%] (for intraplaque hemorrhage). To account for testing multiple phenotypes the level of statistical significance was set at 0.005.
**SUPPLEMENTAL TABLES**

**Table I.** Demographic characteristics of the Athero-Express Study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
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<tr>
<td>Total sample, $N$</td>
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<tr>
<td>Age in years, mean (SD)</td>
<td>68.31 (9.31)</td>
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<tr>
<td>Sex, male, %</td>
<td>67.89</td>
</tr>
<tr>
<td>Mean arterial pressure (SD), mmHg</td>
<td>106.71 (15.86)</td>
</tr>
<tr>
<td>Total cholesterol (SD), mmol/l</td>
<td>4.72 (1.24)</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>34.26</td>
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<tr>
<td>Diabetes mellitus, %</td>
<td>22.79</td>
</tr>
<tr>
<td>BMI (SD), kg/m²</td>
<td>26.29 (3.86)</td>
</tr>
<tr>
<td>Time since last cerebrovascular event, median (IQR), days</td>
<td>43.50 (18-92)</td>
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<td>Index event, %</td>
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<tr>
<td>Asymptomatic</td>
<td>28.42</td>
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<tr>
<td>Symptomatic</td>
<td>70.95</td>
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<td>Transient ischemic attack</td>
<td>44.34</td>
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<tr>
<td>Stroke</td>
<td>26.62</td>
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*SD:* standard deviation. *IQR:* interquartile range.
Table II. Association of rs2107595 with plaque phenotypes in patient subgroups from the Athero-Express Study.

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<tr>
<th>MAF</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
<th>Phenotype</th>
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<td>0.800</td>
<td>0.786</td>
<td>0.53-1.16</td>
<td>0.229</td>
<td>calcification</td>
<td>234/168</td>
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<td>0.799</td>
<td>1.024</td>
<td>0.63-1.67</td>
<td>0.926</td>
<td>collagen</td>
<td>339/63</td>
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<td>0.799</td>
<td>1.146</td>
<td>0.78-1.69</td>
<td>0.496</td>
<td>atheroma size (10%)</td>
<td>268/135</td>
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<tr>
<td>0.799</td>
<td>1.698</td>
<td>1.16-2.49</td>
<td>0.007</td>
<td>intraplaque hemorrhage</td>
<td>229/173</td>
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<td>0.799</td>
<td>1.347</td>
<td>0.97-1.88</td>
<td>0.079</td>
<td>macrophages</td>
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<td>0.801</td>
<td>0.868</td>
<td>0.70-1.07</td>
<td>0.188</td>
<td>smooth muscle cells</td>
<td>397</td>
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<td>0.791</td>
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<td>symptomatic (N=1,021)</td>
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<td>0.487</td>
<td>smooth muscle cells</td>
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<td>0.96-2.05</td>
<td>0.081</td>
<td>calcification</td>
<td>175/198</td>
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<td>0.44-1.19</td>
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<td>collagen</td>
<td>306/67</td>
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<td>0.499</td>
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<td>0.307</td>
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<td>232/140</td>
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<td>0.809</td>
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<td>0.82-1.12</td>
<td>0.559</td>
<td>vessel density</td>
<td>317</td>
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</table>

MAF: major allele frequency; odds ratios (OR) are relative to the major allele (G) on genome build 36; P-values are two-sided. Phenotype: calcification and collagen are coded as no or minor vs. moderate heavy staining, atheroma size is coded as <10% vs. >10% fat content, intraplaque hemorrhage (IPH) as no vs. yes, macrophages and smooth muscle cells are natural log (LN)-transformed percentages of plaques, and vessel density is the LN-transformed average intraplaque vessel density per 3-4 hotspots. N is the number of patients analyzed for the respective plaque phenotype. Asymptomatic: includes patients with significant stenosis (>50%) or ocular events (including amaurosis fugax or retinal infarction) but no history of transient ischemic attack (TIA) or stroke prior to carotid endarterectomy (CEA). Symptomatic: includes patients with a history of TIA or stroke prior to CEA.
SUPPLEMENTAL FIGURES

**Figure I**
Relative mRNA expression of HDAC9, TWIST1 and FERD3L in human PBMCs for different genotypes of rs2107595. HDAC9 mRNA levels are significantly elevated in homozygous and heterozygous carriers of the risk allele (A) with a gene dosage effect (GG n=51, GA n=22, AA n=4). For TWIST1 (GG n=23, GA n=3, AA n=4) and FERD3L (GG n=23, GA n=3, AA n=1) no correlation with allele carrier status was found. Results represent mean±SEM. *P<0.05, **P<0.01 (Kruskal-Wallis test followed by Mann-Whitney test).
Figure II
A. Plaque area of the main branches and the curvature of the aorta in 28-week-old mice. B, Number of plaques per aortic arch. C, Plasma cholesterol in 18 and 28-week-old male mice. Results represent mean±SEM. n = 8-13 for each genotype and time point; *P<0.05; **P<0.01 vs. wildtype (Mann-Whitney test). BCA brachiocephalic artery, CCA common carotid artery, SCA subclavian artery.
SUPPLEMENTAL REFERENCES

