Supplementary Methods

Cohort

We obtained blood sample from randomly selected 395 carotid endarterectomy patients included in the Carotid Plaque Imaging Project (CPIP) biobank (Lund University, Malmö, Sweden). The criteria for inclusion were symptomatic carotid plaques (stroke, transient ischemic attack, or amaurosis fugax) with a degree of stenosis >70% or asymptomatic plaques with a degree of stenosis >80%. The degree of stenosis was assessed by duplex ultrasound and all patients were evaluated by a neurologist prior to surgery.[1,2] Clinical information and blood samples were collected from each participant prior to surgery. Forty-five carotid plaques were randomly collected for histological analyses. All patients were followed up for cardiovascular events (CVE, i.e., myocardial infarction, unstable angina, ischemic stroke, transient ischemic attack, amaurosis fugax, vascular interventions, and deaths caused by cardiovascular disease) using data from the Swedish National Inpatient Health Register. Among these, information on all key covariates was available for a subset of 345 patients (Supplementary Figure 3). The median follow-up time for CVE was 51 months (interquartile range 26–93). All patients gave written informed consent. The study was approved by the local ethical review board at Lund University (472/2005; September 8, 2005) and followed the declaration of Helsinki.

Histology

Eight-micrometer thick tissue sections from the most stenotic region of the plaque were stained for lipids (Oil Red O), intraplaque hemorrhage (glycophorin A), macrophages (CD68, cluster of differentiation 68), smooth muscle cells (alpha-actin), and collagens (Movat pentachrome) as previously described.[3] Plaque regions displaying positive staining for various markers were quantified using the Biopix iQ 2.1.8 software (Gothenburg, Sweden). This tool enabled precise measurement of areas within the plaque that were positively stained for the smooth muscle cell marker. The percentage of the positively stained region relative to the entire plaque was then determined.

Genotyping and human haptoglobin gene polymorphisms imputation

All patients underwent genotyping. Participants in this study were genotyped utilizing the Illumina Infinium OmniExpress-24 BeadChip array (San Diego, CA, USA), with amplified DNA extracted from whole blood samples. The relationships among participants were estimated by employing the KING (Kinship-based INference for Genome-wide association studies; version 2.3.0),[4] which analyzed the genotyped data. The human haptoglobin (Hp) genetic structural variations were imputed using the Beagle software (version 11Mar19.69c).[5]

Hp genetic structural variations have been previously demonstrated to be accurately imputed.[6] Utilizing a phased reference panel consisting of Hp structural data generated from droplet digital polymerase chain reaction (located at chr16:72,090,310–72,093,744, hg19) as the ground truth, the imputation method employed flanking single nucleotide polymorphisms (SNPs) (chr16:71,088,193–73,097,663) for subjects included in the 1000 Genomes Project and Beagle software to impute Hp alleles such as Hp1S, Hp1F, Hp2FF, Hp2FS, and Hp2SS, where F and S represent the haptoglobin protein running slower (S) or faster (F) in the protein gel.[5] In this study, we used a phased reference panel restricted to 274 unrelated subjects of European origin and executed Beagle with default parameters and additional settings (nsamples=15, niterations=15, maxwindow=2,000) on our genotype data, i.e., SNPs at chr16:71,088,193–73,097,663 with Hardy-Weinberg equilibrium (HWE) *P*-value no less than 0.001 and genotyping success rate no less than 0.90. The counts of the obtained Hp1 alleles Hp1S, Hp1F, and Hp2 (Hp2FS and Hp2SS) were used in subsequent analyses.

Statistical analysis

Data are presented as mean±standard deviation or median (interquartile range) depending on their distribution. The Mann-Whitney U test was used for continuous variables, and the chi-square test was used for categorical variables. Associations between Hp and postoperative CVE were examined using Kaplan-Meier estimate and Cox proportional hazard regression on all patients, as well as on symptomatic and asymptomatic patients, respectively. Hazard ratios were reported from three Cox regression models: (1) unadjusted, (2) age and sex adjusted, and (3) age, sex, and low-density lipoprotein (LDL) cholesterol adjusted. The interaction between Hp1S and the presence of symptoms (symptomatic and asymptomatic patients) in predicting postoperative CVE was examined accounting for age, sex, and LDL.

Mann-Whitney U test was conducted to examine difference in histological components (Oil Red O, glycophorin A, CD68, and Movat pentachrome) between plaques from Hp1S carriers and non-carriers. A permutation test was also performed by shuffling Hp1s carriers and non-Hp1S carriers 1,000 times.

Statistical analyses were implemented using IBM SPSS Statistics Version 26 (IBM Corp., Armonk, NY, USA). Statistical significance was defined as $P<0.05$.

Supplementary References

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