Supplementary methods

Study cohorts

The studies were approved by the local ethical review boards and were carried out in accordance with the principles of the Declaration of Helsinki. All study subjects provided written informed consent. There was no overlap between subjects of the two study cohorts.

The SUMMIT

The SUrrogate markers for Micro- and Macro-vascular hard endpoints for Innovative diabetes Tools (SUMMIT) study[1] population included 1,438 subjects recruited between December 2010 and April 2013 from existing population cohorts and hospital registries at the university hospitals in Malmö (Sweden), Pisa (Italy), Dundee and Exeter (UK)—62 subjects were excluded (from the original cohort, n=1,500), due to insufficient quality of CD40/CD40L Proximity Extension Assay (PEA) results (Figure 1). Clinical characteristics of the included patients are summarized in Supplementary Table 1. Diabetes was defined by contemporary or historical evidence of hyperglycaemia (according to World Health Organization 1998 criteria; fasting plasma glucose >7.0 mmol/L or 2-hour plasma glucose >11.1 mmol/L, or both) or by current medication with insulin, sulphonylureas, metformin or other anti-diabetic drugs. To define type 2 diabetes, patients should have been diagnosed after the age of 30. Subjects diagnosed <35 years of age or treated with insulin within 12 months of diagnosis were excluded. Classification of a clinical history of cardiovascular disease included a previous diagnosis in the clinical record of non-fatal acute myocardial infarction (AMI), hospitalized unstable angina, resuscitated cardiac arrest, any coronary revascularization procedure, non-fatal stroke, transient ischemic attack confirmed by a specialist, and lower extremities arterial disease (LEAD) defined as Ankle Brachial Pressure Index (ABPI) <0.9 with intermittent claudication or prior corrective surgery, angioplasty or above ankle amputation. Exclusion criteria included renal replacement therapy, malignancy requiring active treatment, end-stage renal disease, any chronic inflammatory disease on therapy, previous bilateral carotid artery invasive interventions, or age <40 years. Additionally, subjects with atrial fibrillation were excluded. Demographics, clinical characteristics including medication, physical and laboratory examinations were obtained according to a pre-defined study protocol at all four participating centers.

The CPIP

Included in the Carotid Plaque Imaging Project (CPIP) study[2] were 198 carotid plaques collected from patients during carotid endarterectomies at the Vascular Department of Skane University Hospital (Malmö, Sweden) between 2005 and 2010. Clinical characteristics of the included patients are summarized in Supplementary Table 1. Blood samples were taken the day before endarterectomy.

Surgery indications have been described previously.[2] Briefly, symptomatic patients exhibited carotid artery stenosis (>70%) associated with ipsilateral symptoms, and asymptomatic patients exhibited carotid stenosis (>80%), but no ipsilateral symptoms 6 months prior to endarterectomy. Symptoms (i.e., amaurosis fugax, transient ischemia attack, or stroke) were evaluated by a neurologist and the stenosis degree was evaluated by ultrasound based on flow velocities as previously described.[3] Patients with atrial fibrillation were excluded.

Immediately after surgery, removed plaques were snap-frozen in liquid nitrogen. Histological analyses were performed on portions (1 mm thick) cut from the most stenotic plaque region and embedded in optimal cutting medium (Sakura Finetek Europe BV, Tokyo, Japan) and then sectioned (8 μm). Plaque homogenates were prepared from the rest of the snap-frozen plaques and used for all other plaque component quantifications as previously described.[4]

Plasma analysis

CD40 and CD40L were analysed in plaque homogenates from the CPIP cohort and in plasma from SUMMIT cohort using the PEA technique using the Proseek Multiplex CVD96x96 reagents kit (Olink Bioscience, Uppsala, Sweden) as described previously.[5]

The SUMMIT cohort: ultrasound imaging and evaluation of arterial stiffness

Atherosclerosis status was assessed via ultrasound imaging of the carotid arteries examining intima-media thickness (IMT) in the common carotid artery (CCA) and carotid bulb. Plaques were defined as focal thickenings (≥0.8 mm) of the artery wall. The length and height of each individual plaque were measured to calculate plaque area. The inter-observer variability of plaque area measurements was 8.9%±4.6%. The total plaque area represents the sum of the area all plaques identified in the left and right carotid arteries. Pulse wave velocity as an assessment for arterial stiffness was calculated using a Sphygmocor device (Atcor Medical, Sydney, Australia). Methods were performed as described previously.[6] Change in mean and maximal IMT in the CCA and bulb (in mm) at the follow-up point is expressed as the measurements taken at inclusion subtracted by measurements taken at follow-up.

The CPIP cohort: histology

Histological/immunohistochemical detection of Oil Red O, alpha smooth muscle actin, CD68,[7,8] and glycophorin A[9] was performed as previously described on frozen tissue sections. Collagen was visualized through a standard Russel-Movat pentachrome stain. For the Von Kossa calcium stain sections were fixed in 100% ethanol and then incubated in 1% silver nitrate, 5% sodium thiosulfate, and 0.1% fast nuclear red. Between incubations sections were rinsed in distilled H_2O. Stained slides were scanned and digitalized using an Aperio ScanScope digital slide scanner (Aperio Technologies Inc., Vista, CA, USA). Quantifications were performed on blinded samples, using BioPix iQ version 2.3.1 imaging software (Biopix AB, Gothenburg, Sweden). Vulnerability index was calculated as

$$\frac{\text{(CD68+) + (Glycophorin A+) + (Oil Red O+) area\%}}{\text{(Smooth muscle α-actin+) + (Collagen+) + (von Kossa+) area\%}}.$$

Paraffin-embedded sections from 22 subjects were used for immunofluorescence detection of CD40 and CD40L. Sections was deparaffinised and re-hydrated in a graded series of ethanol. Heat-induced epitope retrieval (pH 6) was performed prior to incubation with primary antibodies for 1 hour (CD40: ab13545, CD40L: ab2391; Abcam, Cambridge, UK). As a negative control an isotype control (ab27478, Abcam) of matching antibody concentration and host species was used. Sections were incubated with an Alexa 555-conjugated secondary antibody (ab150074, Abcam) for 1 hour and then mounted with a DAPI-containing mounting media (Vectashield Antifade mounting medium with DAPI, Vector Laboratories Inc., Burlingtame, CA, USA). Images were taken using a Nikon Eclipse E800 microscope (BergmanLabora, Danderyd, Sweden) with Olympus cellSens Standard 1.18 software.

The CPIP cohort: plaque component analyses on plaque homogenates

Multiplex analysis of cytokines (interleukin [IL]-6, IL-23p70, C-X-C motif chemokine ligand 1 [CXCL-1], chemokine (C-C motif) ligand [CCL]-2, -4, and -5, platelet-derived growth factor AA/BA/BB, and tumor necrosis factor-α) was performed using Luminex as previously described.[9] Matrix metalloproteinases (MMPs) -1, -2, -9, -10 and tissue inhibitor of metalloproteinases (TIMPs) -1, -2, and -3 were measured using the Mesoscale human MMP ultra-sensitive kit (Mesoscale, Gaithersburg, MD, USA) and MILLIPLEX MAP Human TIMP Magnetic Bead Panel (Milliplex, Millipore Corporation, Billerica, MA, USA), respectively, as previously described.[9] Oxidized low density lipoprotein was analysed by enzyme-linked immunosorbent assay (ELISA) as previously described.[10] Collagen and elastin were quantified using the Sircol soluble collagen assay (Biocolor, Carrickfergus, UK) and the Fastin elastin assay (Biocolor), respectively, as described previously.[4] Results were normalized to plaque wet weight and all analyses were performed according to the manufacturer's instructions.

Statistics

Shapiro-Wilk and D'Agostino-Pearson omnibus K2 tests were used to assess Gaussian distribution. Variables found to be normally distributed are shown as mean with standard deviation while non-normally distributed variables are shown as median with interquartile range. For analysis of plasma, plaque histology sections and homogenates, the Mann-Whitney U test was used to compare groups and Spearman's rank correlation was used for continuous variables. For comparisons between categorical variables, the chi-square test was used. Spearman's rank correlation was chosen (over Pearson's correlation test) due to the non-Gaussian distribution of the CD40 and CD40L variables and to account for monotonic as well as linear relationships between CD40L and the various parameters tested.

Subjects included in the SUMMIT cohort were followed up after 36 months at all four sites and information of cardiovascular events were registered. Cardiovascular events were defined as AMI (non-fatal or fatal), hospitalized unstable angina, cardiac arrest (resuscitated or fatal), any coronary revascularization procedure, stroke (non-fatal or fatal), and transient ischemic attack confirmed by a specialist, LEAD defined as ABPI <0.9 with intermittent claudication or prior corrective surgery, angioplasty, or above ankle amputation. A logistic regression model was used to test for associations with cardiovascular events and mortality during a follow-up. The model was adjusted for age, diabetes and prevalent stroke, AMI, and hypertension as covariates. The model was implemented in R using the rms package.[11] Area under the receiver operating characteristics (ROC) curve and confidence intervals were estimated by the pROC package[12] with 2,000 stratified bootstrap replicates. To further examine performances of our proposed model in decision making, sensitivities and specificities with confidence intervals were estimated by the pROC package[12] with 2,000 stratified bootstrap replicates using varying cut-off values of the predicted values from the logistic regression. Considering that the costs of false positives and false negatives may vary in clinical applications, we examined sensitivities and specificities from 10% to 90% quantile of the predicted values. A high specificity level would be preferred if less false positives were expected.

A *P*-value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 26.0

https://doi.org/10.5853/jos.2021.00178

(IBM Co., Armonk, NY, USA) and GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Adjustments for multiple comparisons were done when appropriate using the Holm-Šídák test.

Supplementary References

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