Plasma S100A8/A9 Correlates with Blood Neutrophil Counts, Traditional Risk Factors and Cardiovascular Disease in Middle-Aged Healthy Individuals

Materials and methods

Study population
Study participants were part of the CV cohort of the Malmö Diet and Cancer (MDC) study. The MDC is a population-based prospective cohort of 28,449 individuals enrolled between 1991 and 1996. Between October 1991 and February 1994, every other participant was also invited to take part in a substudy focusing on the epidemiology of carotid artery disease (CV arm; n=6103). In the present study, we randomly selected 700 participants, aged 63 to 68 years (mean age 65), from the MDC-CV. Out of these 700 individuals, 24 subjects with a previous history of CVD and 12 subjects that suffered haemorrhagic stroke during follow-up were excluded from further analysis. All participants provided written informed consent and the study was approved by the ethical committee at Lund University, Sweden and conducted in accordance with the Helsinki declaration.

Risk factor assessment
Current cigarette smoking was defined as any smoking within the past year. Blood pressure was measured after resting for 10 min in the supine position. Hypertension was defined as systolic blood pressure (SBP) ≥ 140 mmHg, diastolic blood pressure (DBP) ≥ 90 mmHg or use of antihypertensive medication. Diabetes mellitus was defined as a fasting whole-blood glucose level greater than 6.0 mmol/L, a self-reported physician diagnosis of diabetes or use of antidiabetic medication.

Carotid B-mode ultrasound
Analysis of the intima-media thickness (IMT) and of the intima-media (IM) area of the right common carotid artery (CCA) was performed at baseline, as previously described. Briefly, the distal 3 cm of the right carotid bifurcation, the carotid bulb and the proximal 1 cm of the internal and external carotid arteries were scanned using an Acuson 128 CT system (Siemens AG, Erlangen, Germany) with a 7-MHz transducer. Video-recorded ultrasound images were digitized in real-time by a PC-controlled frame-grabber (Imaging Technology FG-100) with a resulting pixel size of 0.1 mm. Image analysis was performed using a digitizer (Summagraphics MM-1201) and an in-house computer software written in Microsoft Pascal under the MS-DOS operating system. All images for measurement of IMT and IM area were obtained in the longitudinal projection showing the thickest intima-media complex. Mean IMT and IM area were quantified in the far wall of the CCA, along a 1 cm section proximal to the carotid bifurcation. IM area was calculated as the difference between the total area inside the adventitia and the total area of the lumen. The axial resolution of the ultrasound system was 0.3-0.5 mm and of the computerized system 0.1 mm². The mean intra-observer variability was calculated at 8.7 ± 6.2% and the mean inter-observer variability at 9.0 ± 7.2%.

Laboratory measurements
Plasma lipids (total cholesterol, HDL cholesterol and triglycerides) were measured at the Department of Clinical Chemistry, Skane University Hospital.
concentrations of S100A8, S100A9 and S100A8/A9 were measured in plasma by commercially available ELISA kits (BMA Biomedicals, Augst, Switzerland). According to the manufacturer, the level of cross-reactivity between the S100A8/A9 heterodimer and the S100A8 and S100A9 monomers and homodimers is minimal. The detection limits for S100A8, S100A9 and S100A8/A9 were 0.69, 0.31 and 4.69 ng/mL, respectively. Cytokine concentrations in plasma were measured by a multiplex immunoassay (MesoScale Discovery, Gaithesburg, MD, USA).

Flow cytometry
Peripheral mononuclear cells were frozen upon inclusion into the MDC study, stored and thawed for analysis according to previously described protocols4. The numbers of circulating white blood cells (WBC), neutrophils, lymphocytes and mixed (monocyte-rich) cells were counted using a Sysmex K-1000 system with data unit DA 1000 (TOA Medical Electronics Co.) and expressed as million cells/µL blood. The different monocyte subsets were identified by flow cytometry using scatter properties and expression of the CD14 and CD16 surface markers. The classical CD14++CD16- monocytes, non-classical CD14+CD16++ monocytes and intermediate CD14++CD16+ monocyte sub-populations, as well as all monocytes expressing CD16 were included separately into the subsequent analysis. Cell numbers were calculated by multiplying percentages of gated monocyte populations with total blood monocyte numbers. The intra- and interassay variability of the flow cytometry measurements of monocyte sub-populations were below 10%.

End-points
We studied four different outcomes: CE, stroke, CV events and CV death. The procedure for ascertaining outcome events has been described previously5, 6. All subjects were followed from the baseline examination until first hospitalization attributable to acute coronary syndrome, stroke, death, emigration from Sweden or December 31 2008, whichever came first. CE were defined as fatal or non-fatal myocardial infarction or death due to ischaemic heart disease. CV events were defined as CE or fatal or non-fatal stroke. Events were identified through linkage of the 10-digit personal identification number of each Swedish citizen with three registries: the Swedish Hospital Discharge Register, the Swedish Cause of Death Register and the Stroke in Malmö register. Myocardial infarction was defined on the basis of the International Classification of Diseases 9th and 10th revisions (ICD9 and ICD10) codes 410 and I21, respectively. Death due to ischaemic heart disease was defined on the basis of codes 412 and 414 (ICD9) or I22, I23 and I25 (ICD10). Fatal or nonfatal stroke was defined using codes 430, 431, 434 and 436 (ICD9) and I60, I61, I63 and I64 (ICD10). CV death was defined using codes 390–459 (ICD9) and I codes (ICD10) as main cause of death in the cause of death registry. Classification of outcomes using these registries has previously been validated, and the sensitivity of the registry for detecting events such as myocardial infarction has been shown to exceed 90%5, 6. Follow-up for outcomes continued until 31 December 2008.

Statistical analysis
SPSS software (version 19, SPSS Inc, Chicago, IL, USA) was used for all statistical calculations. Differences in CV risk factor burden, S100 protein concentrations and circulating cell numbers at baseline between cases and controls and between smokers and non-smokers were assessed using Mann-Whitney tests for continuous variables and χ² tests for categorical variables, as appropriate. The values of
S100A8, S100A9, S100A8/A9 and TG were logarithmically transformed for further analysis, due to skewed distributions. We used a multivariate linear regression model to test for associations between S100 protein levels, CV risk factors and circulating cell populations and a Spearman model to assess correlations between S100 proteins, plasma cytokines and each other. The different cell populations were introduced separately in the analyses to avoid co-linearity and the beta coefficient for the continuous variables was expressed per one standard deviation (SD) increase of each factor in order to allow comparison among effects. The degree of co-variation between baseline S100 protein concentrations, blood cell numbers and CCA IMT and IM area was studied by using multivariate linear regression models adjusted for age and sex (Model A) and age, sex, smoking, diabetes, BMI, hypertension, LDL, HDL and TG (Model B), with CCA IMT and IM area as dependent variables. Kaplan-Meier survival analyses with log-rank significance tests were employed to analyze event-free survival rates by S100A8, S100A9 and S100A8/A9 and neutrophil count tertiles, for incident CV events, CE, stroke and CV death. We used Cox regression analysis adjusted for age and sex (Model A) and age, sex, smoking, diabetes, BMI, hypertension, LDL, HDL and TG (Model B) to test for associations between baseline S100 proteins and neutrophil levels and CV risk. The fit of the proportional hazards model was confirmed by plotting the incidence rates over time. Data were expressed as hazard ratios (HR) and 95% confidence intervals (CI). A two-sided value of $P<0.05$ was considered statistically significant.

References