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

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Running title: S100A8/A9 and neutrophils correlate with CVD

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Abstract

Objective: The S100 alarmins A8, A9 and A8/A9, secreted by activated neutrophils and monocytes/macrophages, are involved in the pathogenesis of various inflammatory diseases. S100A8/A9 has previously been linked to atherogenesis and cardiovascular (CV) disease. We investigated whether S100A8, A9 and A8/A9 correlate with carotid artery disease and CV risk in apparently healthy individuals.

Approach and Results: We measured baseline S100A8, A9 and A8/A9 in 664 individuals aged 63 to 68 years, with no previous history of CV disease, randomly selected from the Malmö Diet and Cancer population cohort. We examined the correlations between S100 proteins and circulating cell populations, plasma cytokines, carotid artery disease and the incidence of CV events during a median follow-up period of 16.2 years. We found that plasma S100A8/A9 concentrations are positively influenced by circulating neutrophil numbers, smoking, BMI, HbA1c and LDL. HDL was negatively associated with S100A8/A9. S100A8/A9 and the neutrophil counts were positively correlated with intima-media area in the common carotid artery, independently of age, sex and CV risk factors. S100A8/A9 and circulating neutrophils presented similar associations with the incidence of coronary events [HR (95% CI) per 1 SD: 1.28 (1.03-1.59) and 1.26 (1.04-1.53), respectively] and CV death [1.34 (1.06-1.71) and 1.59 (1.33-1.90), respectively]. These relationships were mainly supported by strong associations in women, which were independent of traditional risk factors. There were no independent relationships between S100A8, S100A9 and CV disease.

Conclusions: Our study supports the value of S100A8/A9 as a potentially important link between neutrophils, traditional CV risk factors and CV disease.

Abbreviations

BMI – Body-mass index

CCA – Common carotid artery

CE – Coronary events

CVD – cardiovascular disease

DBP – Diastolic blood pressure

HDL – High-density lipoprotein

LDL – Low-density lipoprotein

IM area – Intima-media area

IMT – Intima-media thickness

MDC-CV – Malmö Diet and Cancer study – cardiovascular cohort

RAGE – Receptor for advanced glycation products

SBP – Systolic blood pressure

TG – Triglycerides

TLR4 – Toll-like receptor 4

HbA1c – Glycosylated hemoglobin

Introduction

Innate immunity plays a central role in the development of atherosclerosis and in cardiovascular disease (CVD)[1]. Danger-associated molecular patterns (DAMPs) or alarmins are structurally and functionally diverse intracellular molecules that are passively released upon tissue damage and cellular necrosis. Alarmins activate pattern recognition receptors (PRRs) on innate immune cells and stimulate phagocytosis of cellular debris and tissue repair[2]. Alarmins can also be actively secreted from activated leukocytes and under pathological conditions amplify and maintain chronic inflammatory processes involved in the pathogenesis of autoimmune disorders and cancer[2]. The involvement of alarmins in the pathogenesis of atherosclerosis has attracted increased interest in recent years as several members of this group, including heat-shock proteins (HSPs)[3], high-mobility group box protein 1 (HMGB1)[4] and cathelicidin[5] have been shown to be pro-atherogenic. The S100 proteins A8 and A9 (also known as calgranulin A and B or myeloid-related proteins (MRP) 8 and 14) are alarmins belonging to the S100 calcium-binding protein family. S100A8 and S100A9 are constitutively expressed in neutrophils and monocytes. In neutrophils S100A8 and S100A9 represent approximately 45% of all cytosolic proteins, compared to only about 1% in monocytes[6]. S100A8 and S100A9 expression differs between subsets of human monocytes, as higher levels of S100A8 mRNA were detected in classical $CD14^{++}CD16^{-}$ monocytes compared to their non-classical $CD14^{+}CD16^{++}$ counterparts[7]. S100A8 and S100A9 are secreted from activated neutrophils and monocytes/macrophages mainly as the S100A8/A9 heterodimer (also called calprotectin), which is considered to be the main biologically active compound. However, S100A8 and S100A9 also form homodimers and previous reports suggest that these compounds may have biologically distinct functions[8].

S100A8/A9 is an endogenous ligand of toll-like receptor 4 (TLR4) and of the receptor for advanced glycation endproducts (RAGE)[9, 10]. S100A8/A9 stimulates recruitment and activation of neutrophils and monocytes and plays a pivotal role as innate immune mediator in various autoimmune and inflammatory diseases[8, 10]. Studies in S100A9 knockout mice have demonstrated that S100A8/A9 is actively involved in atherogenesis and in the vascular response to injury[11]. Importantly, hyperglycemia-induced S100A8/A9 production in neutrophils stimulates further release of neutrophils and

inflammatory monocytes from the bone marrow and impairs the regression of atherosclerotic plaques in mice[12]. Serum S100A8/A9 was shown to correlate with the severity of coronary artery disease in diabetic patients[12, 13] and to predict incident CV events in healthy postmenopausal women[14]. In myocardial infarction (MI) patients, S100A8/A9 is locally expressed in infiltrating neutrophils and monocytes and is released into the systemic circulation from the site of the myocardial injury[15, 16]. Plasma S100A8/A9 levels are highly elevated in MI compared to stable and unstable angina and S100A8/A9 correlates with the incidence of recurrent CV events in MI survivors[16, 17].

The purpose of our study was to elucidate whether plasma S100A8, S100A9 and S100A8/A9 reflect the severity of existing carotid artery disease and correlate with long-term CV risk in apparently healthy middle-aged individuals. We measured baseline concentrations of S100A8, S100A9 and S100A8/A9 in 664 subjects with no previous history of CVD and examined their correlations with circulating leukocyte populations, smoking, diabetes, BMI, blood pressure, plasma lipids, glycemic control, plasma cytokines, carotid intima-media thickness (IMT) and IM area as well as with the risk for incident CV events during a median follow-up period of 16.2 years.

Material and methods

Materials and Methods are available in the online-only Data Supplement (See end of document).

Results

Population characteristics

During a median follow-up time of 16.2 years, 129 individuals suffered at least one acute CV event (Table 1). The cases had a significantly higher prevalence of diabetes mellitus, higher HbA1c, higher blood pressure and lower HDL cholesterol compared to controls (Table 1). The numbers of circulating neutrophils and monocytes were higher in cases. There was a tendency towards higher S100A8/A9 concentrations in cases, but the difference did not reach statistical significance.

Correlations between S100 proteins, CV risk factors, circulating cell populations and plasma cytokines

In order to examine the associations between baseline concentrations of S100A8, S100A9 and S100A8/A9, CV risk factor burden and circulating cell populations we performed multivariate linear regression analyses with the S100 proteins as dependent variables. Blood neutrophil counts presented the strongest association with S100A8/A9 (Figure 1 and Table 2). Other independent positive determinants of S100A8/A9 variability were age, BMI, HbA1c and LDL cholesterol, whereas HDL cholesterol was negatively correlated with S100A8/A9 (Table 2). Smokers had significantly higher plasma levels of S100A8/A9 compared to non-smokers [median (IQR): 1632 (1246-2280) vs. 1471 (1034-1980); P=0.003] and significantly higher blood neutrophil counts (mean±SD: 4.51±1.27 vs. 3.55±1.11; P<0.001). S100A8/A9 did not correlate with the circulating numbers of lymphocytes, platelets, total monocytes or any of the monocyte sub-populations considered. S100A8 and S100A9 were positively associated with the non-classical $CD14^{+}CD16^{++}$ monocytes (Table 2). All other associations tested were not significant.

Further, we tested how the S100 proteins correlate with the concentrations of circulating cytokines and with each other in a Spearman correlation analysis. S100A8/A9 was positively correlated with IFNγ, TNFα, IL-1β and IL-10 (Table 3). S100A8 was positively correlated with IFNγ, TNFα, IL-1β, IL-1, IL-8, IL-10 and IL-12p70. S100A9 was positively correlated with TNFα and negatively with IL-1β. The levels of S100A8/A9 did not correlate with S100A8 but were negatively correlated with S100A9. There was a strong association between S100A8 and S100A9 (Table 3).

S100A8/A9 and circulating neutrophils are associated with IMT and IM area in the CCA

In a multivariate linear regression model corrected for age and sex, plasma levels of S100A8/A9 and the neutrophil counts were positively associated with IMT and longitudinal IM area in the CCA (Table 4, Model A). Additionally, carotid IMT was correlated with circulating monocyte numbers (Table 4, Model A). The associations between S100A8/A9, neutrophils and IM area remained significant after additional adjustment for CV risk factors (age, sex, smoking, diabetes, BMI, hypertension, LDL,

HDL and TG) (Table 4, Model B). S100A8, S100A9 and blood lymphocyte numbers showed no associations with carotid IMT or IM area.

S100A8/A9 and blood neutrophil counts are correlated with the incidence of CE and CV death

An acute CE occurred in 83 of the participants during follow-up and there were 55 cases of stroke. CV disease was the main cause of death for 67 individuals (Table 5). We used Kaplan-Meier survival analyses with log-rank significance tests to examine the associations between baseline S100A8, S100A9 and S100A8/A9 and the incidence of CE, stroke and CV death. Similar tests were performed for baseline numbers of circulating neutrophils, as neutrophils are the leukocyte population with the strongest correlation with S100A8/A9. We found that the incidence of CE and CV death was significantly associated with baseline S100A8/A9 levels and neutrophil counts (Figure 2). In unadjusted Cox regression analyses, CV risk was most elevated among individuals with high levels of both variables. Compared with the combined bottom tertiles, the HR of study participants with baseline values within the top tertile of both variables was 2.72 (CI 1.19-6.21; *P*=0.018) for CE and 3.46 (CI 1.55-7.70; *P*=0.002) for CV death. The corresponding HRs were 1.79 (CI 1.03-3.10; *P*=0.038) for CE and 2.88 (CI 1.53-5.43; *P*=0.001) for CV death when neutrophil tertiles were considered alone.

In Cox regression models adjusted for age and sex, S100A8/A9 and neutrophil numbers presented significant associations with CE and CV death in the entire study population (Table 5, Model A). Adjustment for diabetes, BMI, hypertension and plasma lipids did not alter the associations between S100A8/A9 and CV death and between neutrophils, CE and CV death (not shown). However, after additional adjustment for smoking, only the association between baseline neutrophil counts and CV death remained statistically significant (Table 5, Model B). The relationships between S100A8/A9, neutrophils and the risk of CE and CV death were mainly supported by strong correlations in women, which were independent of traditional CV risk factors (Table 5). Neutrophil counts were correlated with the incidence of stroke in women and the S100A8 homodimer was associated with the incidence of stroke and CV death in men, but these relationship lost statistical significance when adjusting for CV risk factors (Table 5). Finally, we found a negative correlation between S100A8 and CV death in women, valid only in the fully adjusted model.

Discussion

Our study provides important clinical evidence supporting the value of S100A8/A9 as a potential biomarker of neutrophil involvement in CVD. We show that S100A8/A9 and the blood neutrophil counts in apparently healthy middle-aged individuals without previous CV disease are associated with the extent of carotid artery disease and with the long-term risk of CE and CV death. Additionally, we demonstrate that plasma S100A8/A9 is correlated with neutrophil numbers and with smoking, obesity, dyslipidemia and glycemic control, independently of blood neutrophil counts. These findings are in line with previous experimental and clinical studies demonstrating that smoking, hyperlipidemia and hyperglycemia stimulate neutrophilia[12, 18, 19] and S100A8/A9 production[12].

Although the involvement of neutrophils in CVD has long been disregarded, recent experimental and clinical evidence has revealed that these cells may play an important role both in atherogenesis and as mediators of plaque vulnerability and rupture[20, 21]. The presence of neutrophils has been detected in mouse, primate and human atherosclerotic plaques[1, 20]. In murine models of atherosclerosis, neutrophilia induced by hyperlipidemia or by CXCR4 blockade promotes atherogenesis and is correlated with atherosclerotic plaque size[18, 22]. Conversely, deficiency of the neutrophil-secreted alarmin cathelicidin has recently been shown to delay lesion progression[5]. The suggested mechanisms of neutrophil involvement in atherogenesis include endothelial activation, inflammatory monocyte recruitment, generation of reactive oxygen species, LDL oxidation, apoptosis and secretion of matrix-degrading proteases[5, 20, 21, 23]. Here, we show that neutrophils are the only leukocyte population that significantly correlates with plasma S100A8/A9 in humans and that blood neutrophil numbers are the strongest independent determinants of S100A8/A9 concentration. Nagareddy *et al.* have recently demonstrated that hyperglycemia triggers S100A8/A9 release from neutrophils[12]. In turn, S100A8/A9 binding to RAGE on common myeloid progenitor cells stimulates the production of neutrophils and inflammatory monocytes in the bone marrow, leading to impaired regression of atherosclerotic plaques in diabetic mice[12]. Similarly, hyperlipidemia stimulates neutrophilia through increased granulopoesis and enhanced neutrophil release from the bone marrow[18]. In humans, plasma S100A8/A9 correlates with insulin resistance and is increased in type 2 diabetics and in non-diabetic obese

individuals[24, 25]. Weight loss leads to decreased S100A8/A9 levels alongside improved glycemic control and insulin resistance[25]. Importantly, we show that BMI, glycemic control and plasma lipids significantly influence S100A8/A9 variability independently of neutrophil numbers, suggesting that these traditional CV risk factors might stimulate neutrophil and monocyte activation and S100A8/A9 release.

In recent years there has been increased interest in the role of S100A8/A9 in CVD[6]. Plasma S1008A/A9 has been shown to correlate with the extent of coronary and carotid artery disease in diabetic patients[12, 13] and elevated levels of S100A8/A9 in human carotid plaques are associated with a vulnerable plaque phenotype[26]. S100A8/A9 activates the vascular endothelium and increases endothelial permeability[27, 28]. Additionally, S100A8/A9 promotes neutrophil and monocyte recruitment into the tissues by up-regulating surface CD11b expression and by enhancing the binding affinity of CD11b to ICAM-1, integrins and fibrinogen[6, 8]. In hyperlipidemic mice, the absence of S100A8/A9 is associated with reduced macrophage recruitment and delayed atherosclerosis[11]. In MI patients, S100A9 mRNA transcripts are increased in circulating platelets[14] and S100A8/A9-positive neutrophils and macrophages infiltrate both the occluding thrombus and the infarcted myocardium[15, 16]. S100A8/A9 is released early into the systemic circulation during the acute coronary event and correlates with markers of myocardial injury and neutrophil counts[15, 16]. In MI survivors, elevated S100A8/A9 levels at 30 days post-event are associated with a higher risk for recurrent CV events within the next 30-day period[17]. The inflammatory activity of extracellular S100A8/A9 is mediated by the receptors TLR4 and RAGE present on various cell types[9, 29]. TLR4-mediated signaling is considered to be pro-atherogenic, as atherosclerosis progression is delayed in $ApoE^{-/-}$ or $LDLR^{-/-}$ mice deficient in TLR4 or its adaptor protein MyD88[30, 31]. S100A8/A9-induced TLR4 activation on macrophages triggers a positive autocrine feedback loop leading to increased production of inflammatory cytokines, chemokines and reactive oxygen species[10]. RAGE is locally expressed in human atherosclerotic plaques[32] and hyperlipidemic $ApoE^{-/-}RAGE^{-/-}$ double knockout mice develop significantly less atherosclerosis compared with their $ApoE^{-/-}$ controls[9]. Binding of S100A8/A9 to RAGE triggers intracellular signaling mediated through NF-kB and amplifies myelopoesis, vascular inflammation and post-ischemic cardiac dysfunction[9, 33]. We found S100A8/A9 to be positively correlated with the pro-inflammatory cytokines IFNγ, TNFα and IL-1β. TNFα and IL-1β are mainly

produced by monocyte/macrophages and IFNγ is the signature cytokine of Th1 T cells. All these cytokines have been shown to be pro-atherogenic and to play important roles in CVD[1].

We and others have previously reported important correlations between distinct populations of monocytes/macrophages and lymphocytes in apparently healthy individuals and the incidence of acute CV events[1, 34-37]. In the present study we reveal a strong association between circulating neutrophils and CV death, independently of the traditional risk factors for CVD. These data are in line with a previously published study demonstrating that healthy individuals with high baseline neutrophil counts are at increased risk to suffer an ischemic coronary event and to die during the first day after the event[38]. Measurement of S100A8/A9 contributes to further risk stratification of individuals with neutrophil values within the top tertile, as in our study CV risk was highest in participants with elevated values of both variables. The relationships between S100A8/A9 and CV death and between neutrophils and CE in the entire study population were independent of diabetes, BMI, hypertension and plasma lipids but became non-significant after additional adjustment for smoking. Within our cohort, smokers had significantly increased S100A8/A9 and neutrophil levels. Smoking-induced enhanced neutrophilia in apparently healthy individuals has previously been reported and smoking cessation led to rapid decrease of circulating neutrophils[19]. These findings suggest that the increased levels of blood neutrophils and S100A8/A9 in smokers might contribute to the elevated incidence of acute CV events in this particular risk category. In women, the associations between S100A8/A9, neutrophils and CV risk are stronger than in men and are independent of other CV risk factors. Similar findings have previously been reported by Healy *et al.* in a population of apparently healthy postmenopausal women, with a median follow-up time of 2.9 years[14].

Our study has several limitations that should be taken into account. Despite revealing similar association patterns between S100A8/A9, neutrophils and CVD, in line with previous experimental studies demonstrating a pro-atherogenic role of neutrophils and S100A8A/9, our data cannot prove causality. Further mechanistic studies are required to investigate whether S100A8/A9 directly mediates the effects of neutrophils on atherogenesis and plaque rupture. Additionally, we cannot rule out the importance of monocytes/macrophages, endothelial cells and platelets as alternative sources of

S100A8/A9. However, neutrophils outweigh monocytes by more than ten fold in normal blood and contain much higher amounts of S100A8/A9. We did not find any associations between plasma S100A8/A9 and the numbers of circulating monocytes, monocyte sub-populations or platelets at baseline, suggesting that most of circulating S100A8/A9 is neutrophil-derived.

In conclusion, our findings suggest that S100A8/A9 might represent an important link between circulating neutrophils, traditional CV risk factors and CVD and promote S100A8/A9 as a potential biomarker and mediator of neutrophil involvement in CVD. We demonstrate that blood neutrophil counts and plasma S100A8/A9 present similar association patterns with the extent of carotid artery disease and with the risk of CE and CV mortality in middle-aged individuals with no previous history of CVD. S100A8/A9 is of particular interest as a possible therapeutic target for the prevention of acute CV events, as compounds that block the binding of S100A8/A9 to its receptors have been developed and are already approved for clinical testing in humans[39, 40].

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Significance

Our study reveals for the first time a strong association between circulating neutrophils and the concentration of the inflammatory protein S100A8/A9 in human plasma. Additionally, plasma S100A8/A9 is significantly increased by smoking, dyslipidemia and hyperglycemia. S100A8/A9 and the circulating neutrophils present similar associations with the extent of carotid artery disease and with the risk for future acute CV events in healthy middle-aged individuals with no previous history of CV disease. Our findings suggest that S100A8/A9 might represent an important link between neutrophils, traditional CV risk factors and CV disease and promote S100A8/A9 as a potential biomarker and mediator of neutrophil involvement in CV disease. In mouse studies, S100A8A/A9 has previously been shown to be involved as a mediator in the pathogenesis of atherosclerosis and of post-MI heart failure. Importantly, S100A8/A9 blockers have already been developed and are approved for clinical testing, opening up for exciting new therapeutic opportunities for CV disease.

Figures

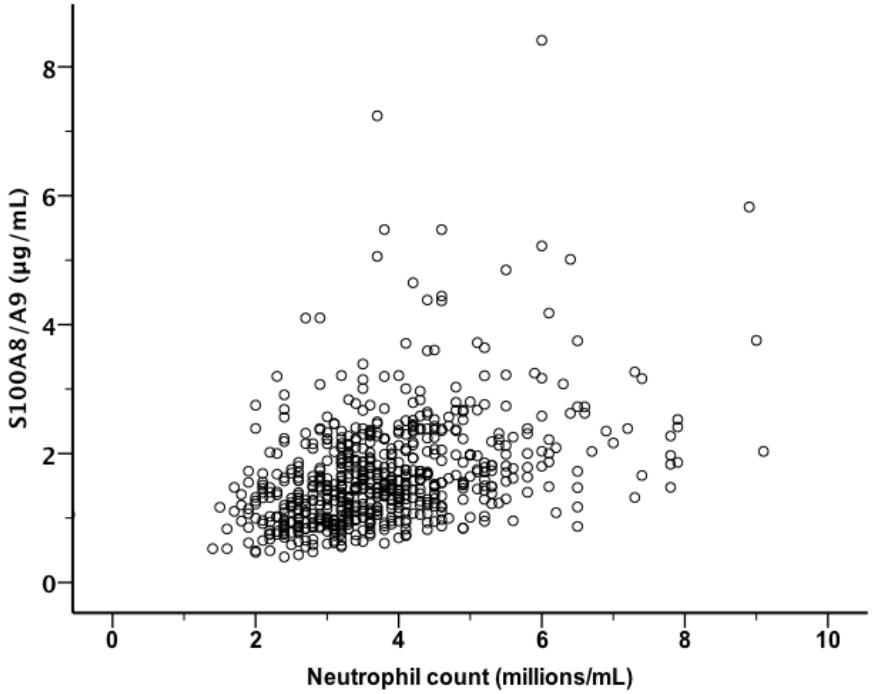

Figure 1. Correlation between S100A8/A9 and blood neutrophil counts

Scatterplot demonstrating the association between plasma S100A8/A9 and circulating neutrophil numbers. The correlation coefficient and the *P* value are calculated using the Spearman test.

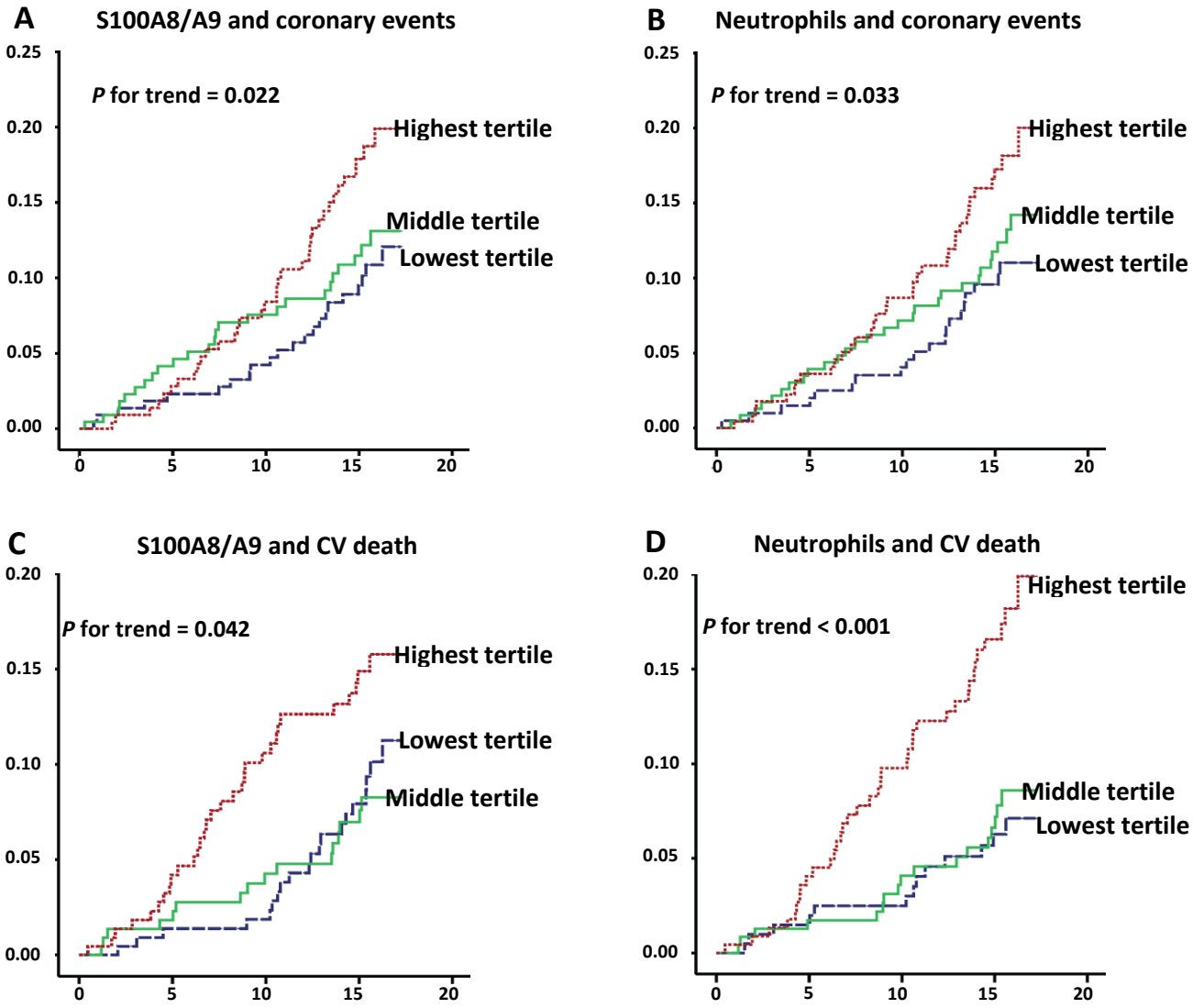

Figure 2. Associations between S100A8/A9, neutrophils and CV risk

Kaplan Meier one-minus event-free survival plots of the associations between tertiles of S100A8/A9, blood neutrophil counts and the incidence of coronary events and CV death during follow-up. The *P* values for trend are calculated using the log-rank test.

Table 1. Baseline characteristics of the study population

Characteristic	All cases (n=129)	Controls (n=535)	*P*
Age, mean (SD), y	65.7 (1.2)	65.6 (1.1)	n.s.
Gender (% male)	66 (51.2)	200 (37.4)	0.005
Current smoking, No. (%)	34 (28.3)	105 (20.5)	n.s.
Diabetes mellitus, No. (%)	31 (24.0)	58 (10.8)	<0.001
HbA1c, median (IQR)	5.1 (4.8-5.4)	5.0 (4.6-5.2)	0.007
Body mass index, mean (SD), kg/m^2	26.5 (4.0)	26.3 (3.9)	n.s.
Hypertension, No (%)	111 (86.0)	423 (79.1)	n.s.
Blood pressure, mean (SD), mm Hg			
Systolic	155 (19)	150 (19)	0.003
Diastolic	90 (9)	88 (9)	0.045
Lipids			
LDL-C, mean (SD), mmol/L	4.36 (1.14)	4.44 (1.00)	n.s.
HDL-C, mean (SD), mmol/L	1.28 (0.42)	1.38 (0.35)	0.001
TG, median (IQR)	1.35 (0.98-1.88)	1.26 (0.93-1.76)	n.s.

S100s, median (IQR)			
S100A8/A9, μg/mL	1.55 (1.21-2.11)	1.48 (1.08-2.03)	n.s.
S100A8, ng/mL	0.22 (0.07-0.46)	0.21 (0.07-0.40)	n.s.
S100A9, ng/mL	0.00 (0.00 – 0.17)	0.00 (0.00-0.09)	n.s.
Cell numbers,			
mean (SD), x10^9 cells/mL whole blood			
WBC	6.36 (1.65)	6.02 (1.51)	n.s.
Neutrophils	4.02 (1.33)	3.73 (1.21)	0.024
Monocytes	0.52 (0.22)	0.46 (0.23)	0.010
Lymphocytes	1.81 (0.59)	1.83 (0.57)	n.s.

Abbreviations: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SD, standard deviation; TG, triglycerides; WBC, white blood cells.

Table 2. Associations between CV risk factors, circulating cell populations and S100 proteins

	S100A8/A9			S100A8			S100A9		
	Beta coefficient	95% CI	*P*	Beta coefficient	95% CI	*P*	Beta coefficient	95% CI	*P*
Age	0.055	0.026-0.084	<0.001	-0.098	-0.206-0.009	n.s.	0.128	-0.144-0.400	n.s.
Male sex	0.005	-0.070-0.079	n.s.	0.326	0.055-0.597	0.019	0.693	-0.013-1.400	n.s.
Current smoking	-0.010	-0.094-0.074	n.s.	0.054	-0.256-0.365	n.s.	0.095	-0.759-0.950	n.s.
Diabetes mellitus	-0.091	-0.212-0.030	n.s.	0.069	-0.370-0.507	n.s.	0.504	-0.708-1.715	n.s.
HbA1c	0.062	0.020-0.105	0.004	-0.058	0.473- -0.215	n.s.	-0.226	-0.655-0.203	n.s.
Body mass index	0.083	0.049-0.118	<0.001	0.002	-0.121-0.125	n.s.	-0.177	-0.504-0.150	n.s.
Blood pressure									
Systolic	0.042	-0.002-0.086	n.s.	-0.130	-0.288-0.028	n.s.	-0.095	-0.547-0.357	n.s.
Diastolic	0.002	-0.042-0.046	n.s.	0.098	-0.059-0.255	n.s.	-0.076	-0.535-0.384	n.s.
Lipids									
LDL-C	0.036	0.004-0.068	0.026	-0.081	-0.195-0.032	n.s.	-0.132	-0.423-0.160	n.s.
HDL-C	-0.048	-0.089- -0.007	0.023	-0.091	-0.246-0.065	n.s.	-0.204	-0.591-0.184	n.s.
TG	0.003	-0.045-0.050	n.s.	-0.074	-0.244-0.096	n.s.	-0.426	-0.873-0.021	n.s.

Cell numbers*									
Neutrophils	0.196	0.160-0.232	<0.001	-0.075	-0.210-0.059	n.s.	-0.136	-0.483-0.211	n.s.
Lymphocytes	-0.022	-0.058-0.013	n.s.	-0.108	-0.235-0.020	n.s.	0.100	-0.213-0.413	n.s.
Platelets	-0.016	-0.052-0.020	n.s.	-0.069	-0.197-0.058	n.s.	-0.018	-0.332-0.297	n.s.
Monocytes	-0.004	-0.038-0.031	n.s.	0.083	-0.039-0.205	n.s.	0.183	-0.143-0.509	n.s.
$CD14^{++}CD16^{-}$ monocytes	0.014	-0.022-0.049	n.s.	-0.006	-0.122-0.110	n.s.	-0.173	-0.512-0.165	n.s.
$CD14^{+}CD16^{++}$ monocytes	0.010	-0.025-0.045	n.s.	0.125	0.013-0.238	0.029	0.375	0.094-0.656	0.009
$CD14^{++}CD16^{+}$ monocytes	0.001	-0.034-0.037	n.s.	-0.012	-0.129-0.106	n.s.	0.051	-0.316-0.417	n.s.

Multivariate linear regression analysis with the S100 proteins as dependent variables, measured in the entire study cohort (n=664). * The different cell populations were entered as cell numbers/μL whole blood and were introduced separately in the analyses to avoid co-linearity. The beta coefficient for the continuous variables is expressed per 1 SD increase in the respective factor. Abbreviations: HbA1c, glycosylated haemoglobin; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; SD, standard deviation.

Table 3. Association between S100 proteins and plasma cytokines

		S100A8/A9		S100A8		S100A9	
		Correlation coefficient*	*P*	Correlation coefficient*	*P*	Correlation coefficient*	*P*
Plasma cytokines	IFNγ	0.157	<0.001	0.156	<0.001	-0.058	n.s.
	TNFα	0.144	<0.001	0.182	<0.001	0.102	0.009
	IL-1β	0.126	0.001	0.103	0.008	-0.110	0.005
	IL-2	0.039	n.s.	0.122	0.002	-0.012	n.s.
	IL-4	0.068	n.s.	0.066	n.s.	-0.019	n.s.
	IL-5	0.003	n.s.	0.055	n.s.	-0.003	n.s.
	IL-8	-0.005	n.s.	0.083	0.034	0.061	n.s.
	IL-10	0.128	0.001	0.115	0.003	-0.062	n.s.
	IL-12p70	0.010	n.s.	0.109	0.005	0.016	n.s.
	IL-13	-0.056	n.s.	0.021	n.s.	0.061	n.s.
S100s	S100A8/A9			0.017	n.s.	-0.141	<0.001
	S100A8	0.017	n.s.			0.489	<0.001
	S100A9	-0.141	<0.001	0.489	<0.001		

* Spearman correlation between S100 proteins and cytokine concentrations in plasma, in the entire study cohort (n=664). Abbreviations: IL, interleukin; IFNγ, interferon γ; TNFα, tumor necrosis factor α.

Table 4. Correlation between S100 proteins and carotid artery disease

		Common carotid IMT			Common carotid IM area		
		Beta coefficient[§]	95% CI	*P*	Beta coefficient[§]	95% CI	*P*
S100A8/A9	A[†]	0.018	0.004-0.031	0.009	0.811	0.419-1.203	<0.001
	B[‡]	0.011	-0.003-0.026	n.s.	0.499	0.085-0.913	0.018
S100A8	A[†]	-0.010	-0.024-0.004	n.s.	-0.099	-0.513-0.315	n.s.
	B[‡]	-0.011	-0.025-0.004	n.s.	-0.127	-0.539-0.284	n.s.
S100A9	A[†]	-0.011	-0.033-0.010	n.s.	-0.302	-0.978-0.373	n.s.
	B[‡]	-0.016	-0.038-0.006	n.s.	-0.444	-1.107-0.220	n.s.
Neutrophils *	A[†]	0.019	0.006-0.032	0.004	0.746	0.358-1.133	<0.001
	B[‡]	0.015	0.000-0.030	0.043	0.459	0.033-0.884	0.035
Monocytes *	A[†]	0.014	0.001-0.027	0.036	0.299	-0.097-0.696	n.s.
	B[‡]	0.009	-0.005-0.023	n.s.	0.101	-0.300-0.502	n.s.
Lymphocytes *	A[†]	0.003	-0.010-0.017	n.s.	0.082	-0.318-0.482	n.s.
	B[‡]	0.000	-0.014-0.015	n.s.	-0.215	-0.634-0.204	n.s.

Multivariate linear regression analysis with common carotid IMT and area as dependent variables, measured in the entire study cohort (n=664). * The different cell populations were introduced separately in the analysis to avoid colinearity. [†] Multivariate linear regression adjusted for age and sex (Model A) and [‡] age, sex, smoking, diabetes, BMI, hypertension, LDL, HDL, TG (Model B). [§] Expressed per 1 SD of the respective variable. Abbreviations: IMT, intima-media thickness; IM area, intima-media area..

Table 5. S100 proteins, neutrophils and CV risk

		S100A8/A9			S100A8			S100A9			Neutrophil counts		
		HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Coronary events													
All (n=83)	A*	1.28	1.03-1.59	0.026	0.84	0.65-1.09	n.s.	0.98	0.64-1.50	n.s.	1.26	1.04-1.53	0.020
	B†	1.24	0.98-1.57	n.s.	0.85	0.65-1.12	n.s.	1.01	0.62-1.65	n.s.	1.26	1.00-1.55	0.047
Men (n=42)	A*	1.14	0.86-1.55	n.s.	0.92	0.65-1.32	n.s.	0.95	0.52-1.76	n.s.	1.04	0.78-1.40	n.s.
	B†	0.99	0.68-1.41	n.s.	0.95	0.65-1.39	n.s.	0.995	0.49-2.04	n.s.	0.95	0.67-1.35	n.s.
Women (n=41)	A*	1.44	1.06-1.95	0.020	0.77	0.53-1.11	n.s.	1.02	0.56-1.85	n.s.	1.51	1.16-1.96	0.002
	B†	1.60	1.09-2.37	0.018	0.69	0.44-1.09	n.s.	1.02	0.43-2.40	n.s.	1.48	1.02-2.14	0.040
Stroke													
All (n=55)	A*	1.06	0.81-1.40	n.s.	1.20	0.92-1.56	n.s.	1.01	0.73-1.62	n.s.	1.22	0.96-1.56	n.s.
	B†	1.056	0.78-1.43	n.s.	1.05	0.78-1.42	n.s.	0.91	0.58-1.45	n.s.	1.15	0.88-1.51	n.s.
Men (n=29)	A*	1.01	0.69-1.47	n.s.	1.54	1.10-2.15	0.012	1.31	0.78-2.19	n.s.	1.05	0.73-1.49	n.s.
	B†	1.16	0.75-1.78	n.s.	1.06	0.68-1.65	n.s.	0.72	0.32-1.61	n.s.	1.06	0.70-1.59	n.s.
Women (n=26)	A*	1.13	0.77-1.68	n.s.	0.85	0.55-1.31	n.s.	0.82	0.42-1.61	n.s.	1.46	1.04-2.06	0.030
	B†	0.98	0.62-1.55	n.s.	0.95	0.61-1.47	n.s.	0.76	0.36-1.63	n.s.	1.21	0.77-1.89	n.s.

CV death													
All (n=67)	A*	1.34	1.06-1.71	0.015	1.11	0.87-1.41	n.s.	1.07	0.70-1.63	n.s.	1.59	1.33-1.90	<0.001
	B†	1.35	1.04-1.75	0.026	0.95	0.72-1.26	n.s.	0.92	0.53-1.59	n.s.	1.63	1.33-1.99	<0.001
Men (n=35)	A*	1.25	0.89-1.75	n.s.	1.47	1.08-2.00	0.014	1.23	0.73-2.08	n.s.	1.34	1.02-1.75	0.034
	B†	0.98	0.66-1.45	n.s.	1.38	0.95-1.98	n.s.	1.42	0.60-3.34	n.s.	1.08	0.75-1.54	n.s.
Women (n=32)	A*	1.45	1.04-2.03	0.030	0.76	0.50-1.15	n.s.	0.81	0.37-1.77	n.s.	1.92	1.50-2.47	<0.001
	B†	1.76	1.16-2.71	0.008	0.55	0.32-0.95	0.033	0.07	0.004-1.33	n.s.	2.35	1.59-3.48	<0.001

* Cox regression analysis adjusted for age and sex (Model A) and † age, sex, diabetes, BMI, hypertension, LDL, HDL, TG (Model B). HR (95% CI) is expressed per 1 SD increase in the respective variable.

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

Materials and methods – On-line supplement

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) $\geq$ 140 mmHg, diastolic blood pressure (DBP) $\geq$ 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[1]. 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[2]. 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[3]. The axial resolution of the ultrasound system was 0.3-0.5 mm and of the computerized system 0.1 mm[2]. The mean intra-observer variability was calculated at 8.7 $\pm$ 6.2% and the mean inter-observer variability at 9.0 $\pm$ 7.2%[1].

Laboratory measurements

Plasma lipids (total cholesterol, HDL cholesterol and triglycerides) were measured at the Department of Clinical Chemistry, Skane University Hospital. Baseline

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 protocols[4]. 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 previously[5, 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 (IDC9) 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 χ^2 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.

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