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

Ovidiu S. Cotoi, Pontus Dunér, Nayoung Ko, Bo Hedblad, Jan Nilsson, Harry Björkbacka, Alexandru Schiopu

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 incidence of CV events during a median follow-up period of 16.2 years. We found that plasma S100A8/A9 concentration is positively influenced by circulating neutrophil numbers, smoking, body mass index, glycosylated hemoglobin A1c, and low-density lipoprotein. High-density lipoprotein 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 (hazard ratio [95% confidence interval] 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 and 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. (Arterioscler Thromb Vasc Biol. 2014;34:202-210.)

Key Words: carotid intima-media thickness ■ immune system ■ inflammation ■ neutrophils

Immune system plays a central role in the development of atherosclerosis and cardiovascular disease (CVD).1 Danger-associated molecular patterns or alarmins are structurally and functionally diverse intracellular molecules that are passively released during tissue damage and cellular necrosis. Alarmins activate pattern-recognition receptors 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 and several members of this group, including heat shock proteins,3 high-mobility group box protein 1,4 and cathelicidin,5 have been shown to be proatherogenic. The S100 proteins A8 and A9 (also known as calgranulin A and B or myeloid-related proteins 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 ≈45% of all cytosolic proteins compared with only ≈1% in monocytes.6 S100A8 and S100A9 expression differs between subsets of human monocytes. Higher levels of S100A8 mRNA were detected in classical CD14++CD16– monocytes compared with their nonclassical 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

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S100A8/A9 is an endogenous ligand of toll-like receptor 4 (TLR4) and of the receptor for advanced glycation end products (RAGE). 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. Studies in S100A9 knockout mice demonstrated that S100A8/A9 is actively involved in atherosclerosis and in the vascular response to injury. 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. Serum S100A8/A9 was shown to correlate with the severity of coronary artery disease in patients with diabetes mellitus and with TNF-α, IL-1β, and IL-10 (Table 3). S100A8/A9 positively correlated with interferon γ, tumor necrosis factor (TNF) α, interleukin-1β (IL-1β), and IL-10 (Table 3). S100A8 was positively correlated with interferon γ, TNF-α, IL-1β, IL-8, IL-10, and IL-12p70. S100A9 was positively correlated with TNF-α 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).

Correlations Between S100 Proteins, CV Risk Factors, Circulating Cell Populations, and Plasma Cytokines
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 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, glycosylated hemoglobin A1c, and low-density lipoprotein cholesterol, whereas high-density lipoprotein cholesterol was negatively correlated with S100A8/A9 (Table 2). Smokers had significantly higher plasma levels of S100A8/A9 (median [interquartile range]: 1632 [1246–2280] versus 1471 [1034–1980]; P=0.003) and significantly higher blood neutrophil counts (mean±SD: 4.51±1.27 versus 3.55±1.11; P<0.001) compared with nonsmokers. S100A8/A9 did not correlate with the circulating numbers of lymphocytes, platelets, total monocytes, or any of the monocyte subpopulations considered. S100A8 and S100A9 were positively associated with nonclassical CD14+CD16+ monocytes (Table 2). All other associations tested were not significant.

Furthermore, we tested how S100 proteins correlate with the concentrations of circulating cytokines and with each other in a Spearman correlation analysis. S100A8/A9 was positively correlated with interferon γ, tumor necrosis factor (TNF) α, interleukin-1β (IL-1β), and IL-10 (Table 3). S100A8 was positively correlated with interferon γ, TNF-α, IL-1β, IL-8, IL-10, and IL-12p70. S100A9 was positively correlated with TNF-α 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 Common Carotid Artery
In a multivariate linear regression model corrected for age and sex, plasma levels of S100A8/A9 and neutrophil counts were positively associated with IMT and longitudinal IM area in the common carotid artery (Table 4, model A). In addition, carotid IMT was correlated with circulating monocyte numbers (Table 4, model A). The associations between S100A8/A9 and IM area, and, neutrophils and IM area remained significant after additional adjustment for CV risk factors (age, sex, smoking, diabetes mellitus, BMI, hypertension, low-density lipoprotein, high-density lipoprotein, and triglycerides; 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 Coronary Events and CV Death

An acute coronary event (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 in 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 because 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 hazard ratio of study participants with baseline values within the top tertile of both variables was 2.72 (confidence interval, 1.19–6.21; P = 0.018) for CE and 3.46 (confidence interval, 1.55–7.70; P = 0.002) for CV death. The corresponding hazard ratios were 1.79 (confidence interval, 1.03–3.10; P = 0.038) for CE and 2.88 (confidence interval, 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 mellitus, BMI, hypertension, and plasma lipids did not alter the associations between S100A8/A9 and CV death and between neutrophils and 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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All Cases (n=129)</th>
<th>Controls (n=535)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD), y</td>
<td>65.7 (1.2)</td>
<td>65.6 (1.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>66 (51.2)</td>
<td>200 (37.4)</td>
<td>0.005</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>34 (28.3)</td>
<td>105 (20.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>31 (24.0)</td>
<td>58 (10.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c, median (IQR), %</td>
<td>5.1 (4.8–5.4)</td>
<td>5.0 (4.6–5.2)</td>
<td>0.007</td>
</tr>
<tr>
<td>Body mass index, mean (SD), kg/m²</td>
<td>26.5 (4.0)</td>
<td>26.3 (3.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>111 (86.0)</td>
<td>423 (79.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic, mmHg</td>
<td>155 (19)</td>
<td>150 (19)</td>
<td>0.003</td>
</tr>
<tr>
<td>Diastolic, mmHg</td>
<td>90 (9)</td>
<td>88 (9)</td>
<td>0.045</td>
</tr>
<tr>
<td>LDL-C, mean (SD), mmol/L</td>
<td>4.36 (1.14)</td>
<td>4.44 (1.00)</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-C, mean (SD), mmol/L</td>
<td>1.28 (0.42)</td>
<td>1.38 (0.35)</td>
<td>0.001</td>
</tr>
<tr>
<td>TG, median (IQR)</td>
<td>1.35 (0.98–1.88)</td>
<td>1.26 (0.93–1.76)</td>
<td>NS</td>
</tr>
<tr>
<td>S100A8/A9, μg/mL</td>
<td>1.55 (1.21–2.11)</td>
<td>1.48 (1.08–2.03)</td>
<td>NS</td>
</tr>
<tr>
<td>S100A8, ng/mL</td>
<td>0.22 (0.07–0.46)</td>
<td>0.21 (0.07–0.40)</td>
<td>NS</td>
</tr>
<tr>
<td>S100A9, ng/mL</td>
<td>0.00 (0.00–0.17)</td>
<td>0.00 (0.00–0.09)</td>
<td>NS</td>
</tr>
<tr>
<td>WBC, mean (SD), ×10⁹ cells/mL whole blood</td>
<td>6.36 (1.65)</td>
<td>6.02 (1.51)</td>
<td>NS</td>
</tr>
<tr>
<td>Neutrophils, mean (SD), ×10⁹ cells/mL</td>
<td>4.02 (1.33)</td>
<td>3.73 (1.21)</td>
<td>0.024</td>
</tr>
<tr>
<td>Monocytes, mean (SD), ×10⁹ cells/mL</td>
<td>0.52 (0.22)</td>
<td>0.46 (0.23)</td>
<td>0.010</td>
</tr>
<tr>
<td>Lymphocytes, mean (SD), ×10⁹ cells/mL</td>
<td>1.81 (0.59)</td>
<td>1.83 (0.57)</td>
<td>NS</td>
</tr>
</tbody>
</table>

HbA1c indicates glycosylated hemoglobin; HDL-C, high-density lipoprotein cholesterol; IQR, interquartile range; LDL-C, low-density lipoprotein cholesterol; NS, nonsignificant; TG, triglycerides; and WBC, white blood cells.

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.
common myeloid progenitor cells stimulates the production from neutrophils. In turn, S100A8/A9 binding to RAGE on monocytes and neutrophils is correlated with atherosclerotic plaque size. 

Conversely, deficiency of the neutrophil-secreted alarmin cathelicidin has recently been shown to delay lesion progression. 

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. 

Multivariate linear regression analysis with S100 proteins as dependent variables measured in the entire study cohort (n=664). CI indicates confidence interval; CV, cardiovascular; HbA1c, glycosylated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NS, nonsignificant; and TG, triglycerides. 

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 relationships lost statistical significance when adjusting for CV risk factors (Table 5). Finally, we found a negative correlation between S100A8 and CV death in women, which was independent of traditional CV risk factors.

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 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. In addition, 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 and S100A8/A9 production.
of neutrophils and inflammatory monocytes in the bone marrow, leading to impaired regression of atherosclerotic plaques in diabetic mice. Similarly, hyperlipidemia stimulates neutrophilia through increased granulopoiesis and enhanced neutrophil release from the bone marrow. In humans, plasma S100A8/A9 correlates with insulin resistance and is increased in patients with type 2 diabetes mellitus and nondiabetic obese individuals. Weight loss leads to decreased S100A8/A9 levels alongside improved glycemic control and insulin resistance. 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. Plasma S100A8/A9 has been shown to correlate with the extent of coronary and carotid artery disease in patients with diabetes mellitus, and elevated levels of S100A8/A9 in human carotid plaques are associated with a vulnerable plaque phenotype. S100A8/A9 activates

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**Table 3. Association Between S100 Proteins and Plasma Cytokines**

<table>
<thead>
<tr>
<th>Plasma cytokines</th>
<th>S100A8/A9</th>
<th>S100A8</th>
<th>S100A9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Correlation Coefficient</strong></td>
<td><strong>P Value</strong></td>
<td><strong>Correlation Coefficient</strong></td>
<td><strong>P Value</strong></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.157</td>
<td>&lt;0.001</td>
<td>0.156</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.144</td>
<td>&lt;0.001</td>
<td>0.182</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.126</td>
<td>0.001</td>
<td>0.103</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.039</td>
<td>NS</td>
<td>0.122</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.068</td>
<td>NS</td>
<td>0.066</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.003</td>
<td>NS</td>
<td>0.055</td>
</tr>
<tr>
<td>IL-8</td>
<td>−0.005</td>
<td>NS</td>
<td>0.083</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.128</td>
<td>0.001</td>
<td>0.115</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.010</td>
<td>NS</td>
<td>0.109</td>
</tr>
<tr>
<td>IL-13</td>
<td>−0.056</td>
<td>NS</td>
<td>0.021</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S100s</th>
<th></th>
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<tbody>
<tr>
<td><strong>Correlation Coefficient</strong></td>
<td><strong>P Value</strong></td>
<td><strong>Correlation Coefficient</strong></td>
<td><strong>P Value</strong></td>
</tr>
<tr>
<td>S100A8/A9</td>
<td>0.017</td>
<td>NS</td>
<td>−0.141</td>
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<tr>
<td>S100A8</td>
<td>0.017</td>
<td>NS</td>
<td>0.489</td>
</tr>
<tr>
<td>S100A9</td>
<td>−0.141</td>
<td>&lt;0.001</td>
<td>0.489</td>
</tr>
</tbody>
</table>

IFN-γ indicates interferon-γ; IL, interleukin; NS, nonsignificant; and TNF-α, tumor necrosis factor α.

*Spearman correlation between S100 proteins and cytokine concentrations in plasma in the entire study cohort (n=664).
the vascular endothelium and increases endothelial permeability.7,28 In addition, S100A8/A9 promotes neutrophil and monocyte recruitment into the tissues by upregulating surface CD11b expression and enhancing the binding affinity of CD11b to intercellular adhesion molecule 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 patients with MI, S100A9 mRNA transcripts are increased in circulating platelets14 and S100A8/A9-positive neutrophils and macrophages infiltrate both the occluding thrombus and the infarcted myocardium.15,16 A9-positive neutrophils and macrophages infiltrate both the occluding thrombus and the infarcted myocardium.15,16

Table 5. S100 Proteins, Neutrophils, and CV Risk

<table>
<thead>
<tr>
<th>Coronary events</th>
<th>S100A8/A9</th>
<th>S100A9</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>95% CI</td>
<td>P Value</td>
</tr>
<tr>
<td>All (n=67) A*</td>
<td>1.34</td>
<td>1.06–1.71</td>
</tr>
<tr>
<td>Men (n=35) A*</td>
<td>1.25</td>
<td>0.89–1.75</td>
</tr>
<tr>
<td>Women (n=32) A*</td>
<td>1.45</td>
<td>1.04–2.03</td>
</tr>
<tr>
<td>Stroke</td>
<td>S100A8/A9</td>
<td>S100A9</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td>HR</td>
<td>95% CI</td>
<td>P Value</td>
</tr>
<tr>
<td>All (n=55) A*</td>
<td>1.06</td>
<td>0.81–1.40</td>
</tr>
<tr>
<td>Men (n=29) A*</td>
<td>1.01</td>
<td>0.69–1.47</td>
</tr>
<tr>
<td>Women (n=26) A*</td>
<td>1.13</td>
<td>0.77–1.68</td>
</tr>
<tr>
<td>CV death</td>
<td>S100A8/A9</td>
<td>S100A9</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td>HR</td>
<td>95% CI</td>
<td>P Value</td>
</tr>
<tr>
<td>All (n=32) A*</td>
<td>1.44</td>
<td>1.06–1.95</td>
</tr>
<tr>
<td>Men (n=29) A*</td>
<td>1.16</td>
<td>0.75–1.78</td>
</tr>
<tr>
<td>Women (n=41) A*</td>
<td>1.44</td>
<td>1.06–1.95</td>
</tr>
</tbody>
</table>

HR (95% CI) is expressed per 1 SD increase in the respective variable. CI indicates confidence interval; CV, cardiovascular; HR, hazard ratio; and NS, nonsignificant.

*S Cox regression analysis adjusted for age and sex (model A).
†Cox regression analysis adjusted for age, sex, diabetes mellitus, body mass index, hypertension, low-density lipoprotein, high-density lipoprotein, and triglycerides (model B).

Locally expressed in human atherosclerotic plaques,32 and hyperlipidemic apolipoprotein E–deficient RAGE–/– double knockout mice develop significantly less atherosclerosis compared with their apolipoprotein E–deficient controls.9 Binding of S100A8/A9 to RAGE triggers intracellular signaling mediated through nuclear factor-κB and amplifies myelopoiesis, vascular inflammation, and postischemic cardiac dysfunction.9,33 We found S100A8/A9 to be positively correlated with the proinflammatory cytokines interferon γ, TNF-α, and IL-1β. TNF-α and IL-1β are mainly produced by monocyte/macrophages, and interferon γ is the signature cytokine of T helper type 1 cells. All these cytokines have been shown to be proatherogenic 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 experience 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 because 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 mellitus, BMI, hypertension, and plasma lipids but became nonsignificant 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 in circulating neutrophils. 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.

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 proatherogenic role of neutrophils and S100A8/A9, our data cannot prove causality. Further mechanistic studies are required to investigate whether S100A8/A9 directly mediates the effects of neutrophils on atherogenesis and on plaque rupture. In addition, we cannot rule out the importance of monocytes/macrophages, endothelial cells, and platelets as alternative sources of S100A8/A9. However, neutrophils outweigh monocytes by >10-fold in normal blood and contain much higher amounts of S100A8/A9.

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 because compounds that block the binding of S100A8/A9 to its receptors have been developed and are already approved for clinical testing in humans.

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Disclosures
None.
References


**Significance**

Our study reveals for the first time a strong association between circulating neutrophils and the concentration of the inflammatory protein S100A8A9 in human plasma. In addition, plasma S100A8A9 is significantly increased by smoking, dyslipidemia, and hyperglycemia. S100A8A9 and the circulating neutrophils present similar associations with the extent of carotid artery disease and with the risk for future acute cardiovascular (CV) events in healthy middle-aged individuals with no previous history of CV disease. Our findings suggest that S100A8A9 might represent an important link between neutrophils, traditional CV risk factors, and CV disease and promote S100A8A9 as a potential biomarker and mediator of neutrophil involvement in CV disease. In mouse studies, S100A8A9 has previously been shown to be involved as a mediator in the pathogenesis of atherosclerosis and of post–myocardial infarction heart failure. Importantly, S100A8A9 blockers have already been developed and are approved for clinical testing, opening up exciting new therapeutic opportunities for CV disease.
Plasma S100A8/A9 Correlates With Blood Neutrophil Counts, Traditional Risk Factors, and Cardiovascular Disease in Middle-Aged Healthy Individuals
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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 described1. 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 system2. 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 lumen3. 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%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 9\(^{th}\) and 10\(^{th}\) 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 \(\chi^2\) 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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