High Levels of S100A12 Are Associated With Recent Plaque Symptomatology in Patients With Carotid Atherosclerosis

Azhar Abbas, MD; Pål Aukrust, MD, PhD; Tuva B. Dahl, MSc, PhD; Vigdis Bjerkeli, ScD; Ellen B. Lund Sagen, ScD; Annika Michelsen, MSc; David Russell, MD, PhD, FRCPE; Kirsten Krohg-Sørensen, MD, PhD; Sverre Holm, PhD; Mona Skjelland, MD, PhD; Bente Halvorsen, MSc, PhD

Background and Purpose—Atherosclerosis is a progressive chronic disease, in which inflammation plays a key role. The calcium-binding proteins calgranulins including S100A8, S100A9, and S100A12 are involved in many cellular activities and pathological processes including inflammation. We therefore hypothesized that calgranulins may be markers of plaque instability in patients with carotid atherosclerosis.

Methods—Plasma levels of S100A8/A9 and S100A10 were measured in 159 consecutive patients with high-grade carotid stenosis and in 22 healthy control subjects. The mRNA levels of calgranulins were also measured within the atherosclerotic carotid plaques, and their regulation was analyzed in vitro in monocytes.

Results—Our main findings were: (1) plasma levels of S100A12 were significantly higher in patients with carotid atherosclerosis compared with healthy control subjects with the highest levels in patients with the most recent symptoms (ie, within 2 months); (2) plasma levels of S100A8/S100A9 showed a modest increase in patients with symptoms in the previous 2 to 6 months but not in the other patients; (3) mRNA levels of S100A8, S100A9, and S100A12 showed increased expression in atherosclerotic carotid plaques from patients with the most recent symptoms compared with the remaining patients; (4) in THP-1 monocytes, activation of Toll-like receptors 2 and 4 increased mRNA levels of S100A8, S100A9, and S100A12 and interleukin-1β, interferon γ, and releasate from thrombin-activated platelets significantly enhanced the expression of S100A12.

Conclusions—Our findings support a link between calgranulins and atherogenesis and suggest that these mediators, and in particular S100A12, may be related to plaque instability. (Stroke. 2012;43:1347-1353.)

Key Words: atherosclerosis • inflammation • stroke

Cerebral embolization due to carotid atherosclerosis is a major cause of transient ischemic attacks and ischemic stroke.1 Although it is generally accepted that inflammation plays a major role in the pathogenesis of atherosclerosis,2 our understanding of the regulation of this inflammatory process as well as the identification and characterization of the different mediators are not complete. The detection of “novel” participants in this complex process could therefore potentially lead to the discovery of new biomarkers for this disorder and a new target for therapy.

Members of the S100/calgranulins family such as S100A8 (myeloid-related protein-8, calgranulin A), S100A9 (myeloid-related protein-14, calgranulin B), and S100A12 (EN-RAGE, calgranulin C) are Ca2+-binding proteins that are predominantly expressed in myeloid-derived cells such as neutrophils, monocytes, and dendritic cells.3 They have been implicated in the regulation of a variety of cellular activities including inflammation.4 In fact, these proteins are highly expressed in numerous inflammatory conditions such as inflammatory bowel disease,5 rheumatoid arthritis,6 and transplant rejection,7 and they have also recently been found in murine and human atherosclerotic lesions.8,9 Their mechanisms of action are rather complex involving intracellular and extracellular roles in modulating calcium signaling, arachidonic acid metabolism, and inflammatory activation of leukocytes through innate immune signaling pathways.6 Despite this complexity, these proteins are thought to be reliable markers of inflammation.10,11

Although there are several reports regarding the role of calgranulins in atherosclerosis,9,12 few studies have examined...
their relationship to plaque stability in patients with carotid atherosclerosis. Based on their relation to inflammation and monocyte activation, we hypothesized that plasma levels of calprotectin (S100A8/A9 heterocomplex) and S100A12 may be markers of plaque instability. In the present study, we investigated this hypothesis by analyzing the relationship between plasma levels of calprotectin S100A8/A9 and S100A12 and the patients’ symptoms as well as the morphology of plaques from patients who were treated with carotid endarterectomy or carotid artery stenting. We also examined the expression of these calgranulins in atherosclerotic carotid plaques from patients with different symptomatology as well as their regulation in THP-1 monocytes that were exposed to different stimuli with relevance to atherosclerosis.

Materials and Methods

Patients and Control Subjects
One hundred fifty-nine consecutive patients with internal carotid stenosis (\(\geq 70\%\)) treated with carotid endarterectomy (136) or carotid artery stenting (11) or conservatively (12) at Oslo University Hospital, Rikshospitalet, were consecutively recruited to take part in the study (Table). The patients were classified into 3 groups according to the temporal profile of their symptoms. Forty-eight (30.2\%) patients had a stroke, transient ischemic attack, or amaurosis fugax ipsilateral to the stenotic internal carotid artery in the previous 2 months; 45 (28.3\%) patients had symptoms in the previous 2 to 6 months; and 66 (41.5\%) patients were asymptomatic (symptoms \(\geq 6\) months ago or no relevant symptoms; Table). Asymptomatic carotid stenoses were detected during the clinical examination of patients with coronary artery disease, peripheral artery disease, or stroke/transient ischemic attack \(\geq 6\) months ago. Patients with concomitant inflammatory diseases such as infections, autoimmune disorders, malignancies, and liver or kidney diseases were excluded from the study. All patients underwent a clinical neurological examination by an experienced neurologist within 2 days before and 3 days after carotid endarterectomy/carotid artery stenting. Blood samples were also collected from 22 sex- and age-matched control subjects recruited from the same area of Norway as the patients. All control subjects were healthy individuals as assessed by disease history, clinical examination, and normal levels of C-reactive protein. The protocols were approved by the Regional Health Authorities of South-Eastern Norway. The study confirms with the principles outlined in the Declaration of Helsinki for use of human tissue or subjects. Signed informed consent for participation in the study was obtained from all individuals.

Ultrasound Examination
The degree of carotid stenosis was determined using color Duplex examinations (HDI 5000; Philips). Echogenicity of the plaques was classified according to consensus criteria.\(^{13,14}\)
Blood Sampling Protocol
Venipuncture of a forearm vein was performed within 2 days before carotid endarterectomy/carotid artery stenting with minimal stasis. Peripheral venous blood was drawn into pyrogen-free tubes with ethylene-diaminetetraacetic acid as an anticoagulant. The tubes were immediately immersed in melting ice and centrifuged within 30 minutes at 2500 g for 20 minutes to obtain platelet-poor plasma. All samples were stored at −80°C until mRNA isolation.

Tissue Sampling From Carotid Plaque
Atherosclerotic carotid plaques were removed from patients during carotid endarterectomy and snap-frozen on dry ice and stored at −80°C until mRNA isolation.

Cell Culture Experiments
The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD) was cultured for 4 days in RPMI 1640 (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) in the presence of recombinant human tumor necrosis factor α (5 ng/mL; R&D Systems, Minneapolis, MN) before further incubation with or without recombinant human interleukin-1β (5 ng/mL, R&D Systems), recombinant human interferon γ (100 ng/mL; R&D Systems), lipopolysaccharide from Escherichia coli 026:B6 (5 ng/mL; Sigma, St Louis, MO), a Toll-like receptor (TLR)2 agonist (Pam3Cys; 1 μg/mL; Sigma), or platelet releasate (see subsequently). At different time points, cell pellets were harvested and stored at −80°C. In all experiments, the vehicle of the stimulus was added as a control. The endotoxin levels in all stimulants, except for lipopolysaccharide, and culture medium vehicle of the stimulus was added as a control. The endotoxin levels were synthesized using high-capacity cDNA archive kits (Applied Science, Oslo, Norway), subjected to DNase I treatment, and stored in RNA storage solution (Ambion, Austin, TX) at −80°C until mRNA isolation.

Preparation of Platelet Releasate
Platelet-rich plasma was prepared from citrated blood by centrifugation at 270 g for 10 minutes at 22°C. Preparations of releasates from platelets were performed by adding one fourth volume of acid–citrate–dextrose to the platelet-rich plasma before centrifugation at 1500 g for 7 minutes at 22°C. The platelets were then resuspended in RPMI 1640 media (PAA Laboratories) to 10^8 platelets/mL before being stimulated with 0.1 U/mL thrombin (Sigma) for 90 minutes to induce release of platelet components to the media. The platelets were then removed by centrifugation at 10,000 g for 5 minutes at 12°C, and the supernatant, representing platelet releasate from activated platelets, was added to THP-1 cells (see previously). Hirudin (0.4 U/mL; Sigma) was added to neutralize thrombin before platelet releasate were cocultured with THP-1 cells.

Real-Time Quantitative Reverse Transcriptase–Polymerase Chain Reaction
Total RNA was extracted from THP-1 cells and carotid plaques using MagNa Pure LC RNA isolation kit III (Roche Applied Science, Oslo, Norway), subjected to DNase I treatment, and stored in RNA storage solution (Ambion, Austin, TX) at −80°C. cDNA was synthesized using high-capacity cDNA archive kits (Applied Biosystems, Foster City, CA). Gene expression was assessed using the following TaqMan assays; S100A8: Assay ID: Hs00374264_g1, S100A9: Assay ID: Hs00610058_m1, S100A12: AssayID: Hs00942835_g1, CD45: AssayID Hs00365634_g1 and CD68 AssayID: Hs00154355_m1. Quantification of mRNA was performed using the ABI Prism 7500 (Applied Biosystems). Gene expression of the reference gene β-actin was used for normalization.

Enzyme Immunoassays
Plasma levels of human calprotectin S100A8/A9 heterocomplex and human S100A12 EN-RAGE were determined by enzyme immunoassays obtained from Circulex TM (Nagano, Japan) and Hycult Biotechnology (Uden, The Netherlands), respectively. The intra- and interassay coefficients of variation were <10% for both assays.

Statistical Methods
For comparison of 2 groups of individuals, the Mann-Whitney U test was used. For comparison of >2 groups, the nonparametric Kruskal-Wallis test was used. If a significant difference was found, the Mann-Whitney U test was used to calculate the difference between each pair of groups. In the in vitro studies, Student t test or Mann-Whitney U test was used depending on the distribution of data. The χ² test was used for analyzing contingency data. Coefficients of correlation were calculated by the Pearson or Spearman rank test depending on the distribution of the data. Multiple logistic regression analysis was performed to investigate the specific influence of covariates. Probability values (2-sided) were considered significant at P<0.05. All calculations were carried out with SPSS for Windows statistical software (Version 16.0; SPSS Inc, Chicago, IL).

Results
Plasma Levels of Calprotectin (S100A8/A9) and S100A12 in Patients With Carotid Stenosis and Healthy Control Subjects
When patients were divided into 3 groups according to their latest clinical symptoms (ie, symptoms within the last 2 months, n=48; symptoms within the last 2–6 months, n=45; or asymptomatic plaques, n=66), plasma levels of S100A12 were significantly higher in patients with carotid atherosclerosis as compared with healthy control subjects (n=22), with particularly high levels in those with the most recent clinical symptoms (ie, within 2 months; Figure 1A). We found significantly increased plasma levels of calprotectin (S100A8/A9) in patients with symptoms in the previous 2 to 6 months, but not in the other clinically defined subgroups, as compared with healthy control subjects (Figure 1B). The potential pathogenical significance of this pattern is unclear. In contrast to S100A12, most of the parameters in the Table, including lipid parameters, smoking habits, and plasma level of C-reactive protein did not show the most marked changes in those patients with the most recent symptoms. In the patient group as a whole, plasma levels of calprotectin and S100A12 were positively correlated to leukocyte counts (r=0.2, P=0.02; r=0.02, P=0.03, respectively) and C-reactive protein (r=0.3, P=0.001; r=0.22, P=0.006, respectively). Plasma levels of calprotectin, but not S100A12 levels, were also positively correlated to the degree of stenosis (r=0.25, P=0.002) and were significantly higher in patients with diabetes mellitus (P=0.003) and in those with low plaque echogenicity (ie, echolucent versus echogenic/heterogeneous plaques, P=0.034). No other correlations were found between calprotectin and S100A12 and the clinical and biochemical parameters that are outlined in the Table.

Based on the anti-inflammatory effects of statins, it is not unlikely that these medications could downregulate the levels of calgranulins. In fact, Morrow et al showed that intensified
statin therapy downregulated plasma levels of S100A8/A9 as compared with more moderate statin therapy.\textsuperscript{15} However, in the present study, almost all patients were on statin medication (with no information of treatment duration), and although there was no statistical significant difference in S100A8/A9 or S100A12 between patients with and without statin (data not shown), the number of patients without statin therapy is too low for making any firm conclusion.

The Expression of S100A8, S100A9, and S100A12 mRNA Within Carotid Atherosclerosis

In a subgroup of patients, plaques removed at endarterectomy were also available. As shown in Figure 2A, the increased plasma levels of S100A12 in patients with symptoms within the last 2 months (n=34) were accompanied by markedly increased mRNA levels of S100A12 within the atherosclerotic carotid plaque when compared with mRNA levels of S100A12 in patients with symptoms within the last 3 to 6 months (n=16) and in asymptomatic patients (n=15). A similar pattern was found for mRNA levels of S100A8 and S100A9 with increased expression in atherosclerotic carotid plaques from patients with the most recent symptoms (within the last 2 months; Figure 2B–C).

Although there was no significant correlation between plasma levels of S100A8/A9 and S100A12 and CD45 (as a marker of leukocyte infiltration) and CD68 (as a marker of macrophage infiltration) expression within the carotid lesion, the expression S100A8 (r=0.28, P<0.03) and S100A9 (r=0.42, P<0.001) within the lesion was significantly correlated with CD68 expression but not with CD45 expression (data not shown). No significant correlations were found for S100A12 expression (data not shown).

Regulation of S100A8, S100A9, and S100A12 in Monocytes

Monocytes are of major importance in atherosclerotic plaque development and are also important cellular sources of granulins.\textsuperscript{16} To further study the relation between calprotectin S100A8, S100A9, S100A12, and atherosclerosis, we examined the regulation of these granulins in THP-1 monocytes in response to different stimuli with relevance to atherosclerosis. Before stimulation, the THP-1 monocytes had been preactivated by tumor necrosis factor α (5 ng/mL) for 96 hours in an attempt to mimic the inflammatory microenvironment within an atherosclerotic lesion.\textsuperscript{17} Figure 3 shows that TLR2 (Pam\textsubscript{3}Cys), and in particular TLR4 (lipopolysaccharide) activation, markedly increased mRNA levels of S100A8, S100A9, and S100A12. However, although interleukin-1β, interferon γ, and releasate from thrombin-activated platelets had no effects on mRNA levels of S100A8 and S100A9, these stimuli significantly enhanced the expression of S100A12 (Figure 3).

Discussion

Calgranulins have previously been linked to atherosclerosis.\textsuperscript{9,18} In the present study we found that in patients with carotid atherosclerosis, those with the most recent symptoms had increased mRNA levels of S100A8, S100A9, and S100A12 within the atherosclerotic plaque compared with patients who did not have recent symptoms or were asymptomatic. Patients with the most recent symptoms were also characterized by the highest plasma levels S100A12 but not of calprotectin (S100A8/S100A9). Our in vitro findings suggest that this link between plasma levels of S100A12 and unstable carotid plaques could, at least in part, be due to the ability of S100A12 to reflect a wider spectrum of inflammatory pathways compared with S100A8 and S100A9. Our findings support a link between calgranulins and atherogenesis and suggest that these mediators, and in particular S100A12, may be related to plaque instability.

Increased plasma levels of S100A8/S100A9 have previously been found to predict cardiovascular events in apparently healthy individuals.\textsuperscript{11} Moreover, Ionita et al reported enhanced expression of these calgranulins in rupture-prone carotid plaques.\textsuperscript{18} In acute coronary syndrome, S100A8/S100A9 was found to be markedly expressed at the site of coronary occlusion by invading phagocytes.\textsuperscript{19} In the present study, we extend these findings by showing increased expression of S100A8/S100A9 within carotid plaques from those patients with the most recent symptoms. Although there are several studies that
have reported increased levels of S100A8/S100A9 in atherosclerotic disease, and Goyette et al showed increased S100A12 levels in the serum of a small population of patients with coronary artery disease (n=<40) compared with healthy control subjects. In the present study, we found increased levels S100A12 in carotid plaque from patients with the most recent symptoms, and in contrast to S100A8/S100A9, a similar pattern was also found in the plasma. Plasma levels of S100A12 were therefore 1 of the few examined parameters that was associated with clinically determined plaque instability (most recent symptoms). The reason for the different plasma profile of S100A8/S100A9 compared with S100A12 is at present not clear. Our findings suggest, however, that S100A12 should be studied in larger study populations as a potential biomarker for atherosclerotic disease and plaque instability. Moreover, although the correlation of S100A8 and S100A9 with CD68 within the carotid lesion further underscores a link between S100A8/A9 and macrophage activation, the lack of such a correlation for S100A12 may suggest that S100A12 could reflect other pathways in atherogenesis than those that are mirrored by S100A8/A9.

Calgranulins such as S100A8, S100A9, and S100A12 are expressed in myeloid-derived cells, but recent studies suggest that these mediators may also be induced in nonmyeloid cells such as endothelial cells and vascular smooth muscle cells. Studies in low-density lipoprotein receptor-deficient, S100A9-deficient bone marrow chimeras suggested that S100A9 expression in nonmyeloid cells may contribute to the development of atherosclerosis. Moreover, Bowman et al found that S100A12 could mediate aortic wall remodeling and aortic aneurysm at least partly through vascular smooth muscle cell-derived secretion of S100A12.

Despite these new data on calgranulin expression in nonmyeloid cells, neutrophils, monocytes, and activated macrophages seem to be of major importance as cellular sources of calgranulins during inflammation. Based on the crucial role of monocytes/macrophages in all stages of atherogenesis, the relationship between calgranulins and activation of monocytes/macrophages appears to be of particular importance with regard to their involvement in atherosclerosis. Previously, S100A8/S100A9 has been shown to enhance inflammatory responses in macrophages through TLR4 activation. In the present study we found that the activation of TLR2 and in particular TLR4 markedly enhanced the expression of S100A8/S100A9/S100A12 in THP-1 monocytes. This may suggest an S100A12-driven inflammatory loop in monocytes/macrophages with TLR4 as a crucial factor. Although S100A8/ S100A9 did not respond to the other tested stimuli, S100A12 expression in THP-1 monocytes was significantly enhanced by activation with interleukin-1β, interferon γ, and released from thrombin-activated platelets. Previously, interleukin-6 has been found to increase S100A12 expression in THP-1 cells and the ability of S100A12 to be induced by a wide range of stimuli, reflecting several upstream or upstream inflammatory pathways, could be of importance for its potential as a biomarker in atherosclerotic disease. Previous studies have

![Figure 2](http://stroke.ahajournals.org/)

**Figure 2.** mRNA levels of S100A8 (A), S100A9 (B), and S100A12 (C) in atherosclerotic plaques collected from patients with carotid stenosis during carotid endarterectomy. Patients were classified into 3 groups according to their plaque symptomatology: patients with relevant clinical symptoms such as stroke, TIA, or amaurosis fugax ipsilateral to the stenotic internal carotid artery in the previous 2 months (n=34); patients with symptoms in the previous 3 to 6 months (n=16); and asymptomatic patients (symptoms >6 months ago or no relevant symptoms; n=19). Data are mean±SEM. *P<0.05 and **P<0.01 vs the 2 other groups of patients. TIA indicates transient ischemic attack.
suggested that calgranulins may be associated with platelets.15 We found that releasate from activated platelets enhanced S100A12 expression in THP-1 monocytes. This finding further links S100A12 to carotid plaque instability and the development of transient ischemic attack and stroke.

The present study has limitations such as a relatively small numbers of patients and the lack of longitudinal data. However, although the role of calgranulins in atherogenesis is rather complex, mediating both beneficial and harmful effects, our findings provide further support for a role for these molecules, and in particular S100A12, as biomarkers in atherogenesis. This may reflect its potential ability to mirror upstreams and interacting inflammatory pathways.

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Disclosures
None.

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A correction is needed for the article, “High Levels of S100A12 Are Associated With Recent Plaque Symptomatology in Patients With Carotid Atherosclerosis” (Stroke. 2012;43:1347–1353). Dr Krohg-Sørensen’s name was published as Kirsten Krohg Sørensen, MD, PhD. This has been corrected in the online version to Kirsten Krohg-Sørensen, MD, PhD.