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Original Paper

Spotty Carotid Plaques Are Associated with Inflammation and the Occurrence of Cerebrovascular Symptoms

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Keywords
Ultrasound in stroke · Plaque · Cerebrovascular disease

Abstract
Background: Echolucent carotid plaques have been related to an increased risk of ischemic cerebrovascular events. The aim of the present study was to evaluate whether a new objective ultrasonographic parameter, the statistical geometric feature (SGF), reflecting spottiness of carotid plaques, can be associated with cerebrovascular symptoms and with a rupture-prone plaque phenotype. ***Methods:*** The plaques of 144 patients who underwent carotid endarterectomy were included in this study. SGF and plaque area were estimated by outlining the plaque on ultrasound (US) images. The correlation coefficient for inter- and intraobserver variability was 0.69 and 0.93, respectively. The SGF values were normalized to the degree of stenosis (SGF/DS). The plaques collected at surgery 1 day after the US were analyzed histologically, and inflammatory markers and matrix metalloproteinases (MMPs) were measured. ***Results:*** Patients with ipsilateral hemispheric symptoms had higher SGF/DS compared to patients without symptoms (0.82 [0.59–1.16] vs. 0.70 [0.56–0.89], $p = 0.01$). Analysis of plaque components revealed a positive correlation between SGF/DS and the percentage of the plaque area stained for lipids, macrophages, and hemorrhage. A correlation was also found between SGF/DS and plaque expression of interleukin-6, monocyte chemoattractant protein-1, macrophage inflammatory protein-1β, vascular endothelial growth factor A, C-C motif chemokine 3 and 20, and MMP-9. An inverse correlation was found with plaque levels of osteoprotegerin. ***Conclusions:*** The present study supports the concept that spottiness is a feature of the carotid plaques rich in inflammation and can be associated with the typical phenotype of high-risk plaques.

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Introduction

Rupture of an atherosclerotic plaque with subsequent thrombus formation in the carotid artery is a frequent cause of ischemic cerebrovascular events. Besides the degree of stenosis, plaque components are very important. Rupture-prone plaques are rich in lipids, macrophages, and inflammation and are associated with the occurrence of symptoms [1, 2]. The instability of atherosclerotic plaques depends on a complex interplay between inflammation and cell death, as well as degradation and synthesis of connective tissue proteins.

Strategies of risk stratification trying to detect rupture-prone plaques should ideally rely on noninvasive diagnostic tools. In the case of carotid plaques, B-mode ultrasound (US) has been shown to be reliable in the quantification of the atherosclerotic burden [3]. Additionally, plaque composition has been related to its echogenicity. Echolucent plaques, containing increased amount of lipids and hemorrhage, are more rupture prone, whereas echogenic ones are more stable and rich in calcium and fibrous tissue [4, 5].

One of the pitfalls of the US technique is its subjectivity among observers. To partly overcome this issue, an objective computer-assisted quantification of plaque echogenicity has been developed by calculating the gray-scale median (GSM) [6]. The GSM represents a quantification of the overall gray scale of the plaque and has not been implemented in the clinical daily routine yet. Therefore, several efforts have been made in order to identify other parameters that can be assessed by US and reflect plaque composition and its local, more subtle variations.

The statistical geometric feature (SGF) is the result of an objective quantification that describes heterogeneity, namely spottiness of the plaques, by assessing the local variations of the intensity in US signals within a plaque [7]. A spotty pattern in the plaque, reflected in high SGF values, would correspond to a rather blended composition. Recent studies based on intravascular US and optical coherence tomography have shown that coronary plaques with spotty calcifications are more vulnerable when compared to plaques with plainer structure [8, 9]. The aim of the current study was to evaluate spottiness by analyzing a new objective parameter (SGF) of carotid plaques and to test its possible association with a rupture-prone plaque phenotype and the occurrence of cerebrovascular symptoms.

Material and Methods

Study Population

A total of 350 patients who underwent carotid endarterectomy (CEA) between February 2006 and December 2012 at the Vascular Center of Skåne University Hospital were identified for inclusion in this study. Indications for surgery were previously described in detail [10].

From the initial cohort of 350 patients who underwent CEA during the study period, only 144 had measurable US images and accessible histology. From the 206 that were not used, 143 was excluded as the images were taken either in other institutions or using a different scanner or not using standardized parameters (i.e., angle between transducer face and examined vessel <60°, vertical TGC, maximal dynamic range, high persistence, and high frame rate). Extensive acoustical shadowing that completely jeopardized the assessment of the plaque structure underneath was present in 32, who were then excluded from the study. Plaques with only partial acoustical shadowing (which did not jeopardize the analysis of the whole plaque) were still included, but the shadowed area was not outlined as one could not evaluate the plaque underneath. Thirty-one plaques were excluded due to bad quality either of the histology or any of the other parameters measured. This resulted in the inclusion of 144 plaques.

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Information about comorbidities, past medical history, and clinical data were obtained through standardized preoperative interviews and review of the medical records.

US and Artery Measurement System

One day before surgery, a clinical carotid US was performed by a certified sonographer using an Acuson Sequoia (Acuson, Mountain View, CA, USA) with a 7-MHz probe according to a previously published method [11]. Plaque images were taken using fixed settings on the US machine, stored on Xcelera (Philips, Amsterdam, The Netherlands) in Dicom format, and subsequently converted to bmp format.

The Artery Measurement System, a commercially available program developed at Chalmers University of Technology in collaboration with the Wallenberg Laboratory for Cardiovascular Research, Gothenburg University, Sweden, was used for the measurements of SGF, GSM, and plaque area. US images consist of pixels of varying intensities, from the darkest (level 0) to the brightest (level 255). The gray scale of the plaque on the US image can be influenced by several factors, such as the level of gain set on the US machine, the depth of the plaque, and the echogenicity of the surrounding tissues. To make SGF values comparable, the gray scale of the images needs to be standardized prior to measurement, as described previously [7]. Hence, the gray scale in each image was changed linearly, giving the blackest pixel in the lumen the value 0 and the brightest pixel in the adventitia the value 255.

The method to assess SGF has been previously described [7]. Briefly, for the assessment of SGF, the software randomly selects one pixel in the middle of the plaque. The pixels nearby are included as long as their gray scales are close to the gray scale of the randomly selected pixel. When that area is defined, the software randomly chooses another pixel to form a new area. Area, SGF, and GSM of the outlined plaque were automatically obtained. A high SGF value defines a plaque with a large amount of areas with different intensities, i.e., spottiness, while a low SGF value corresponds to less spotty plaques (Fig. 1). To take into consideration the possible influence of the complexity and size of the atherosclerotic burden, the SGF measurements were adjusted to the degree of stenosis produced by the plaque (SGF/DS).

All measurements were performed by one biomedical scientist (S.H.) with long experience in performing US investigations. Furthermore, inter- and intraobserver studies were performed by reassessing 20 plaques (S.H. and G.Ö.). Reproducibility studies were done at least 1 week after the first measurement with the biomedical scientist blinded to earlier results. The intraclass correlation coefficient for inter- and intraobserver variability was 0.69 and 0.93, respectively.

Sample Preparation and Histology

Plaques were snap-frozen in liquid nitrogen immediately after surgical removal. Plaque homogenates were prepared as previously described [12]. One-millimeter-thick fragments from the most stenotic region were taken for histology.

Stainings for lipids (Oil Red O), vascular smooth muscle cells (α-actin), and macrophages (CD68) were performed as previously reported [13]. For the staining for intraplaque hemorrhage, the primary monoclonal mouse anti-human glycophorin A antibody (DakoCytomation, Glostrup, Denmark) at a concentration of 0.79 μg/mL and secondary polyclonal biotinylated rabbit anti-mouse antibody (DakoCytomation) at a concentration of 4 μg/mL were used. The calcium amount was analyzed by von Kossa staining with the use of silver nitrate solution (VWR International LLC, Radnor, PA, USA). Measurements of the area of plaque (% area) for the different stainings were quantified blindly using Biopix iQ 2.1.8 (Gothenburg, Sweden) after scanning with ScanScope Console Version 8.2 (LRI Imaging AB, Vista, CA, USA).

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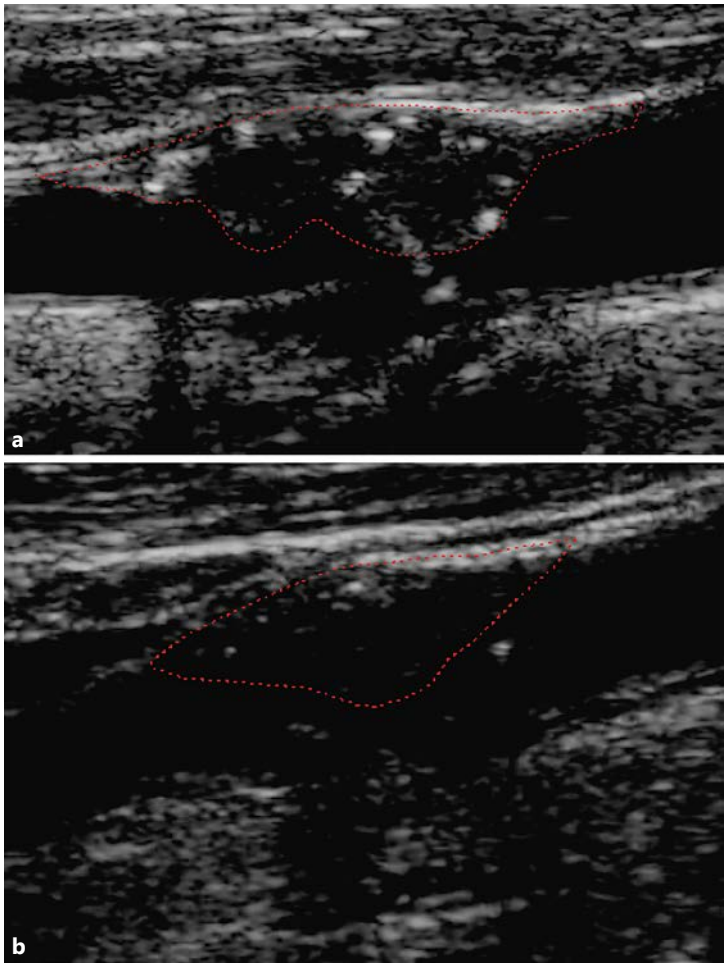

Fig. 1. The outlined plaques after a standardization procedure (see text for details). **a** Spotty plaque (SGF = 94). **b** Plainer plaque (SGF = 47). SGF, statistical geometric feature.

Inflammatory Markers and Matrix Metalloproteinase Assessment

Aliquots of 50 µL of plaque homogenate were centrifuged at 13,000 *g* for 10 min. Twenty-five microliters of the supernatant were removed and used for measuring interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), tumor necrosis factor-α, and interferon-γ. The procedure was performed according to the manufacturer's instructions (Human Cytokine/Chemokine Immunoassay, Millipore Corp., Danvers, MA, USA) and analyzed with Luminex 100 IS 2.3 (Luminex Corp., Austin, TX, USA). Matrix metalloproteinase-1 (MMP-1), MMP-3, MMP-9, and MMP-10 were analyzed in 25 µL of the supernatant using the Mesoscale human MMP ultrasensitive kit (Mesoscale, Gaithersburg, MD, USA), according to the manufacturer's instructions. Vascular endothelial growth factor A (VEGF-A), osteoprotegerin (OPG), and C-C motif chemokine 3 (CCL3) and CCL20 levels were analyzed by the Proximity Extension Assay technique using the Proseek Multiplex CVD$^{96\times96}$ reagents kit (Olink Bioscience, Uppsala, Sweden) as previously described [14]. Data analysis was performed by a preprocessing normalization procedure using Olink Wizard for GenEx (Multid Analyses, Gothenburg, Sweden). VEGF-A, CCL3, CCL4, and CCL20 levels are presented as arbitrary units. Results were normalized to the wet weight of the plaque (Table 1).

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Table 1. Clinical characteristics of the study cohort

	Total (n = 144)	Asymptomatic (n = 56)	Symptomatic (n = 88)	p value
Age, years	70 (65–76.7)	68 (64–72)	74 (66.2–79)	<0.01
Male sex	95 (66%)	37 (66%)	58 (66%)	ns
Degree of stenosis	90 (80–95)	95 (85–95)	90 (80–95)	0.03
Gray-scale median	28 (17–46)	32 (19.5–52.5)	26.5 (16.7–42.2)	ns
Plaque area, cm^2	69.1 (54.8–93.5)	69.1 (45.8–84.7)	69.4 (56.9–100.2)	ns
SGF/DS	0.78 (0.59–1.04)	0.70 (0.56–0.89)	0.82 (0.59–1.16)	0.01
Current smoker	42 (29%)	16 (29%)	26 (29%)	ns
Hypertension[1]	107 (74%)	45 (80%)	62 (70%)	ns
Diabetes	52 (36%)	19 (34%)	33 (37%)	ns
Body mass index	26.3 (24–29.4)	26.5 (23.7–29.4)	26.1 (24–29.2)	ns
Statin use	129 (90%)	51 (91%)	78 (89%)	ns
Total cholesterol, mmol/L	4.2 (3.5–5)	3.9 (3.4–4.8)	4.3 (3.7–5.2)	ns
HDL, mmol/L	1.1 (0.9–1.3)	0.9 (0.8–1.4)	1.1 (0.9–1.3)	ns
LDL, mmol/L	2.4 (1.8–3.2)	2.1 (1.7–3.1)	2.5 (2–3.7)	0.04
Triglycerides, mmol/L	1.3 (1–1.8)	1.3 (1–2)	1.3 (1–1.7)	ns
hsCRP, mg/L	4.4 (2.5–7)	3.5 (1.8–7.7)	3.7 (2–7)	ns
HbA1c, mmol/mol	50 (43–60)	50 (43–60)	51 (42–63)	ns

Categorical variables are expressed as crude numbers (%), continuous variables as median (interquartile range). The χ^2 test was used for comparison of categorical variables, the Mann-Whitney test for continuous variables. HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; ns, not significant; SGF/DS, statistical geometric feature values normalized to the degree of stenosis. [1] Systolic pressure >140 mm Hg or use of antihypertensive drugs.

Statistical Analysis

Continuous variables are presented as median (interquartile range), while categorical variables are presented as percentages. Mann-Whitney U test and Spearman rank correlation were used since all continuous variables were nonnormally distributed. Simple and multiple linear regressions were used to explore the relationship between two or more continuous variables, while logistic regression was used in case of dichotomous variables. A p value <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA).

Results

The carotid plaques of 144 patients (70 [65–76.7] years, 95 males) who underwent CEA between February 2006 and December 2012 were included in this study. Eighty-eight plaques (61%) were associated with ipsilateral hemispheric symptoms (19 amaurosis fugax, 32 transient ischemic attacks, 37 strokes), while 56 (39%) were not associated with symptoms.

Patients with symptomatic plaques were older (74 [66.2–79] vs. 68 [64–72] years, p < 0.01) and had a significantly lower degree of stenosis (90% [80–95] vs. 95% [85–95], p = 0.03) than patients who did not suffer of ischemic symptoms preoperatively (Table 1). Additionally, serum low-density lipoprotein levels were higher in symptomatic patients (2.5 [2–3.7] vs. 2.1 [1.7–3.1] mmol/L, p = 0.04).

As shown in Figure 2, patients with ipsilateral hemispheric symptoms had higher SGF/DS compared to asymptomatic patients (0.82 [0.59–1.16] vs. 0.70 [0.56–0.89], p = 0.01). This

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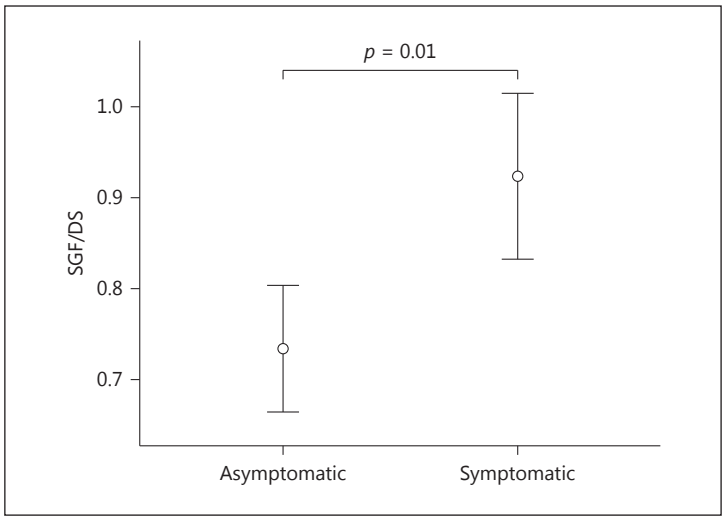

Fig. 2. Error bars of the statistical geometric feature values normalized to the degree of stenosis (SGF/DS) in patients with (symptomatic) or without cerebrovascular symptoms (asymptomatic).

finding could be confirmed after regression analysis adjusting for age, gender, diabetes, hypertension, smoking habits, statin use, and serum low-density lipoprotein levels ($p < 0.01$).

Analysis of plaque components (Table 2) revealed a positive correlation between SGF/DS and the percentage of the plaque area stained for macrophages ($r = 0.207$, $p = 0.02$), lipids ($r = 0.213$, $p = 0.02$), and hemorrhage ($r = 0.276$, $p < 0.01$).

Asymptomatic plaques did not show a significant difference when compared to symptomatic plaques in terms of GSM (32 [19.5–52.5] vs. 26.5 [16.7–42.2], $p = 0.18$) and plaque area (69.1 [45.8–84.7] vs. 69.4 [56.9–100.2] cm^2, $p = 0.11$).

Staining for vascular smooth muscle cells ($r = -0.146$, $p = 0.13$) and calcium as measured by von Kossa staining ($r = -0.170$, $p = 0.09$) did not show any significant correlation with SGF/DS.

A positive correlation was found between SGF/DS and plaque levels of IL-6 ($r = 0.302$, $p < 0.01$), MCP-1 ($r = 0.241$, $p = 0.01$), MIP-1β ($r = 0.313$, $p < 0.01$), VEGF-A ($r = 0.233$, $p = 0.02$), CCL3 ($r = 0.357$, $p < 0.01$), CCL20 ($r = 0.239$, $p = 0.01$), and MMP-9 ($r = 0.227$, $p = 0.02$). An inverse correlation was found between SGF/DS and plaque levels of OPG ($r = -0.443$, $p < 0.01$).

Discussion

The main result of the present study is that SGF/DS, an objective measurement for spottiness adjusted to the degree of stenosis in carotid US, positively correlates with components common in rupture-prone human carotid plaques.

The assessment of carotid plaque echogenicity on US images in a standardized way has been widely explored, mostly by the use of GSM. In particular, lesions with low GSM values, defined as echolucent, are considered more rupture prone and have been associated with an increased risk of cerebrovascular events [15]. However, a wide range of GSM threshold values has been proposed in order to distinguish echolucent from echogenic plaques. This variation may be attributable to different approaches for the standardization of the images acquired and analyzed in B-mode [16]. Additionally, the GSM represents the median value of the gray scale of the whole atherosclerotic area and may not necessarily reflect the presence of particular regional components.

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Table 2. Spearman correlation between SGF/DS and the assessed plaque components (n = 144)

	R	*p* value
Histology (% of area)		
CD68	0.207	0.02
Alpha-actin	–0.146	ns
ORO	0.213	0.02
Glycophorin A	0.276	<0.01
von Kossa	–0.170	ns
Inflammatory markers		
Interleukin-6 (pg/g)	0.302	<0.01
Interferon-γ (pg/g)	–0.130	ns
Tumor necrosis factor-α (pg/g)	–0.004	ns
MCP-1 (pg/g)	0.241	0.01
MIP-1β (pg/g)	0.313	<0.01
VEGF-A (AU/g wet weight plaque)	0.233	0.02
OPG (AU/g wet weight plaque)	–0.443	<0.01
CCL3 (AU/g wet weight plaque)	0.357	<0.01
CCL20 (AU/g wet weight plaque)	0.239	0.01
MMPs (pg/g)		
MMP-1	0.108	ns
MMP-3	–0.124	ns
MMP-9	0.227	0.02
MMP-10	–0.110	ns

AU, arbitrary unit; CCL, C-C motif chemokine; MCP-1, monocyte chemoattractant protein-1; MIP-1β, macrophage inflammatory protein-1β; MMP, matrix metalloproteinase; ns, not significant; OPG, osteoprotegerin; ORO, Oil Red O; SGF/DS, statistical geometric feature values normalized to the degree of stenosis; VEGF-A, vascular endothelial growth factor A.

In the current study cohort, the GSM did not differentiate carotid plaques that have caused ischemic symptoms from asymptomatic ones. These findings were confirmed also after correction for the degree of stenosis and plaque area.

The SGF has been proposed in order to describe heterogeneity (i.e., spottiness) of plaque in an objective way [7]. By assessing the local variations of the intensity in US signals within a plaque, the SGF may add more information on the underlying plaque composition.

In contrast to the GSM, the SGF/DS ratio was different in plaques associated with ischemic symptoms and in asymptomatic ones. This could be the result of the variability in composition of different parts of the atherosclerotic lesions, resulting in variable US signals. The SGF/DS ratio takes into account such variability, possibly allowing a more detailed characterization of the plaque composition and, indirectly, of its stability.

The aim of the current study was to evaluate whether SGF correlates with a particular type of plaque composition in specimen collected by CEA. Plaque composition has been associated with the risk of ischemic events. The majority of studies focusing on the composition of atherosclerotic plaques have been based on histology. Novel quantitative techniques for biological components are now available, allowing us to study atherosclerosis beyond the histological approach. Moreover, to overcome the limitations of histology, biochemical quantitative analyses of whole plaque homogenates have been performed. In the current study, histological and biochemical analysis showed consistent results in terms of increased inflammatory response in patients with spotty plaques. In particular, the SGF/DS correlated positively with intraplaque levels of MCP-1, MIP-1β, and IL-6, as well as with an increased presence of macro-

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phages, intraplaque hemorrhage, and lipids. These findings suggest that the SGF/DS can be associated with the typical phenotype of high-risk plaques. The positive correlation between the SGF/DS and plaque levels of MMP-9 and VEGF-A is also in line with the previously mentioned findings. In fact, MMP-9 activity has been shown to be increased in unstable plaques [17], while VEGF-A contributes to plaque vulnerability at the advanced stage of atherosclerosis [18].

Circulating levels of OPG have been related to the increase in cardiovascular risk in patients with diabetes [19]. More recently, OPG (intraplaque and circulating) was associated with carotid plaque stability as expressed by the reduced incidence of plaque-related cerebrovascular events [20]. In the current study, a strong negative correlation between plaque expression of OPG and SGF/DS was detected. OPG is a marker of intraplaque stabilization [20]. Consistent data regarding the expression of macro- and microcalcifications could be detected in this study.

A nonsignificant inverse correlation could be found between the percentage of the plaque area stained for vascular smooth muscle cells and SGF/DS. Vascular smooth muscle cells are important for maintaining plaque stability [21]. The occurrence of ischemic symptoms could be the result of a reduction of the stabilizing effect of vascular smooth muscle cells, leading to loss or weakening of the protective cap with subsequent plaque rupture [22, 23].

CCL3 and CCL20 are cytokines belonging to the CC chemokine family that is involved in the recruitment and activation of polymorphonuclear leukocytes during the acute inflammatory state. It has been proved that they burst the inflammatory reaction that characterizes the advanced stages of atherosclerosis [24]. However, their functional roles in atherogenesis remain undefined. In the current study, a positive correlation was found between SGF/DS and the plaque expression of CCL3 and CCL20, also pointing to the proinflammatory pattern of spotty plaques.

Limitations

The present study has certain limitations. Its observational design does not allow conclusions on causality. Regarding the limitations related to the assessment of plaque inflammation based on histological analyses of tissue sections, the combination with biochemical analysis on plaque homogenates allows us to consider our results to be representative of the lesion at large. Moreover, since the biological characteristics of the removed plaque are the result of the continuous repairing processes that take place in atherosclerotic lesions, plaque spottiness could reflect an ongoing repairing process rather than mechanisms involved in the development of plaque vulnerability.

Since the SGF is evaluated in the longitudinal image of the plaque, the issues of further regional differences remain. Additionally, the composition of atherosclerotic lesions changes with time. Therefore, it would be interesting to study spottiness in a longitudinal perspective, as well as the effects of drugs, such as statins, on the SGF. Furthermore, the partial overlap in values of SGF/DS in the symptomatic and asymptomatic groups raises the question of whether it can effectively distinguish carotid plaques that are at high risk of ischemic events. To answer this query, a prospective study is required. Finally, in this study only advanced plaques were analyzed, making our results not necessarily applicable to earlier, smaller atherosclerotic lesions.

Conclusions

The findings of the present study support the concept that spottiness is a feature of the carotid plaques rich in inflammation. Whether early detection of spottiness, and thereby signs of inflammation within the plaque, could be used to identify patients at risk of cerebral

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ischemic events needs to be studied. The further use of this feature in risk stratification models needs to be tested in longitudinal studies including patients with less advanced atherosclerotic lesions.

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Statement of Ethics

Informed consent was given by all patients, and the study was approved by the local ethics committee (Regional Ethics Committee, Lund, 472/2005).

Disclosure Statement

The authors declare no conflicts of interest.

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