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Identification of Carotid 'Vulnerable Plaque' by Contrast-enhanced Ultrasonography: Correlation with Plaque Histology, Symptoms and Cerebral Computed Tomography

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Abstract *Introduction:* Indication to carotid revascularisation is commonly determined by percent of stenosis as well as neurological symptoms and clinical conditions. High plaque embolic potential is defined as 'vulnerability'; however, its characterisation is not universally used for carotid revascularisation. We investigated the role of contrast-enhanced ultrasonography (CEUS) to identify carotid vulnerable plaque.
Methods: Patients undergoing carotid endarterectomy were preoperatively evaluated by cerebral computed tomography (CT) scan and CEUS. Contrast microbubbles detected within the plaque indicated neovascularisation and were quantified by decibel enhancement (dB-E). Plaques were histologically evaluated for five features: (microvessel density, fibrous cap thickness, extension of calcification, inflammatory infiltrate and lipid core) and blindly scored 1–5 to assess plaque vulnerability. Analysis of variance (ANOVA), Fisher's and Student's *t*-test were used to correlate patients' characteristics, histological features and dB-E.
Results: In 22 patients, dB-E (range 2–7.8, mean 4.85 $\pm$ 1.9 SD) was significantly greater in symptomatic (7.40 $\pm$ 0.5) vs. asymptomatic (3.5 $\pm$ 1.4) patients ($p = 0.002$). A higher dB-E was significantly associated with thinner fibrous cap (<200 μm, 5.96 $\pm$ 1.5 vs. 3 $\pm$ 1, $p = 0.01$) and greater inflammatory infiltrate (3.2 $\pm$ 0.9 vs. 6.4 $\pm$ 1.2, $p = 0.03$). Plaques with vulnerability score of 5 had significantly higher dB-E compared with those with vulnerability

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score of 1 (7.6 ± 0.2 vs. 2.5 ± 0.6, respectively, $p = 0.001$). Preoperative ipsilateral embolic lesions at CT were correlated with higher dB-E (5.96 ± 1.5 vs. 3.0 ± 1.0, $p = 0.01$).
Conclusion: CEUS with dB-E is indicative of the extent of plaque neovascularisation. It can be used therefore as a marker for vulnerable plaque.

Introduction

Carotid artery stenosis is one of the leading causes of stroke and the benefit of carotid surgical revascularisation in both symptomatic and asymptomatic patients has been shown by a number of trials.[1–4] The percent of carotid stenosis is presently the main parameter in the indication to revascularisation; however, other plaque characteristics, namely its composition and morphology, could play a significant role in the development of stroke, and should therefore be considered in the decision-making process.[5]

Neurological symptoms are associated with a number of histological patterns, such as higher immature microvessel density, subsequent intraplaque haemorrhage and fibrous cap rupture.[6–8] Angiogenesis is also related to an increased inflammatory infiltrate and plaque instability.[9,10] Thus, plaque characterisation could be an important adjunct for stroke risk stratification in patients with asymptomatic carotid artery stenosis.

Carotid contrast-enhanced ultrasonography (CEUS), a relatively new technique for the evaluation of plaque structure, allows detection of the plaque microvessels to identify the carotid 'vulnerable plaque', associated with high embolic potential.[11–13] As this field has been scarcely investigated in the literature, we performed a study to correlate CEUS findings with neurological symptoms, cerebral ischaemic lesions and plaque histology to identify the 'vulnerable plaque', namely its embolic potential.

Materials and Methods

Patients

A series of consecutive patients with either symptomatic or asymptomatic carotid artery stenosis ≥70% were submitted to carotid endarterectomy (CEA), according to ESVS and Society of Vascular Surgeons (SVS) recommendations. Symptomatic carotid stenosis, defined as ipsilateral cerebral ischaemic events (major or minor stroke or transient ischaemic attack (TIA) or amaurosis fugax) that occurred in the last 6 months, were evaluated by independent in-hospital neurologists.[14,15] Epidemiological data, neurological symptoms, vascular risk factors (hypertension, coronary-artery disease, chronic-obstructive pulmonary disease, dyslipidaemia, diabetes mellitus, current smoking and chronic-renal failure) and current therapy (acetylsalicylic acid (ASA), anticoagulant and hydroxymethyl glutaryl coenzyme A reductase inhibitor) were recorded in a database software. Emergency procedures for acute ischaemic symptoms were not included in the study.

Preoperative cerebral computed tomography (CT) scan was performed in all cases to identify possible ischaemic lesions. CEUS plaque evaluation was performed in the days immediately before surgery. All patients gave their informed consent for the study before CEA, which was performed in a standard fashion through a laterocervical approach.

CT scan

Cerebral CT was performed in all patients using a multislice (16-slice) GE Light Speed scanner (General Electric, Milwaukee, WI, USA). According to Mofidi et al.,[16] the presence of cerebral ischaemic lesion ipsilateral to the side of the carotid plaque was considered a positive CT evaluation. Cerebral ischaemic lesions were defined as areas of low attenuation, involving both grey and white matter or focal areas of encephalomalacia. Cerebral CT evaluation was performed blindly by two different operators within 2 weeks before surgery.

CEUS

Carotid ultrasonography was performed with an ultrasound machine (Philips IU22, Amsterdam, the Netherlands) by a blinded expert operator (with more than 100 carotid ultrasound examinations performed per year), within 2 weeks before surgery. A contrast-specific software operating at low mechanical index was used. The power modulation mode (IU22) was used. The second-generation echo-contrast agent, SonoVue® (Bracco, Milan, Italy), was employed. SonoVue® is based on microbubbles stabilised by phospholipids and filled with sulphur hexafluoride with a median diameter of 2.5 μm. The low solubility and the high resistance of the shell to the mechanical effect of the ultrasound (US) beam allow this contrast agent to act for a long period of time (10 min). The contrast solution is prepared immediately before administration by adding 5 ml of sterile saline solution (0.9% NaCl) to the vial and by vigorously shaking it for at least 20 s. SonoVue® was administered as a bolus at a dose of 5 ml and was injected intravenously through a 20-gauge intravenous catheter, followed by a 10-ml-flush of saline water (0.9% NaCl). A linear probe with a transmission frequency of 10—12 MHz was used by a blinded expert operator. Common, internal and external carotid arteries were visualised in the longitudinal and the transverse planes. Images of plaques in B, Colour and Power modes were digitally stored. CEUS evaluation was performed as follows. After presetting real-time, contrast-enhanced imaging modality with coded pulse-inversion technique, image settings were adjusted for maximising the contrast signal. The mechanical index was preset to 0.13 and the frame rate to 12 per s, to reduce microbubble destruction, with image depth at 3—5 cm, according to the patients' anatomical necks characteristics. The longitudinal carotid plaques images in real-time visualisation with the presence of microbubbles in carotid lumen and in the plaques' microvessels were digitally stored for later outline-blinded software analysis. Image storing was performed for at least

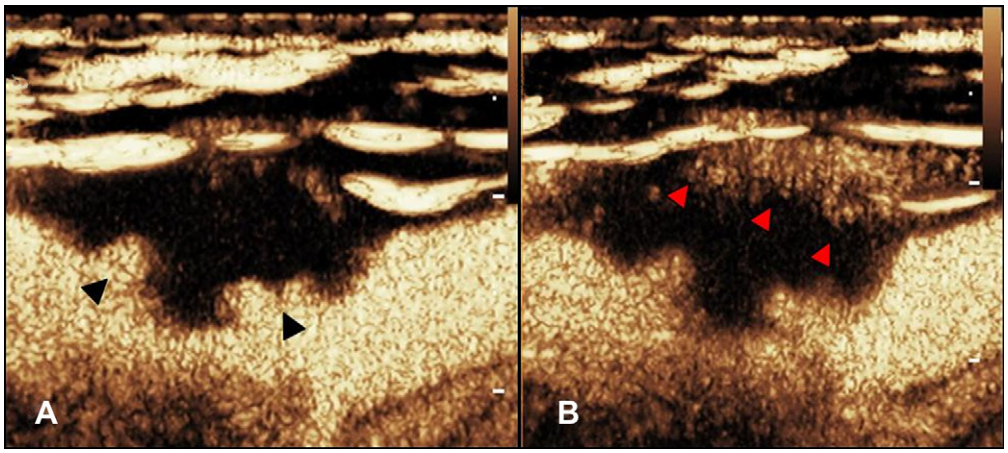

Figure 1 Image sequences of carotid plaque before (A) and after (B) CEUS microvessel detection. Panel B shows the area within the plaque filled with neovessels (red arrows). In panel A, only the border of the plaque is visible (black arrows) showing two ulcers.

10 min, from the moment of injection to the time of decreasing microbubbles concentration in carotid lumen. QLab® (Philips Healthcare, India) software was used for the outline plaques vascularisation analysis, obtaining the maximum level of dB-Enhanced (dB-E) at the peak of microbubbles' concentration for each plaque analysed. The stored images sequences were evaluated identifying the carotid plaque, delineating the region of interest and eventually performing the software analysis of microbubbles concentration within the plaques and recording dB-E values (Fig. 1).

Histological analysis

Carotid plaques were removed in full during surgery to preserve the plaque structure. After 24 h of decalcification, samples were cut in serial sections and the area with the highest percentage of stenosis was identified and defined as the area of interest for further analysis. Plaque tissue samples were fixed in formalin buffered 10% and embedded in paraffin; 5-μm-thick haematoxylin and eosin-stained sections were observed under a light microscope (LM, Olympus CX42). Carotid atherosclerotic lesions were defined according to the American Heart Association (AHA) classification,[17] and grouped as type I—VI ranging from the initial lesion to the complicated lesion. An experienced pathologist performed all histopathological analysis.

Vulnerability

Based on histological studies of culprit plaques, vulnerable plaques are generally felt to have three histologic hallmarks compared with stable plaques[18–21]: a larger lipid core (>40% of total lesion area), a thinner fibrous cap and many inflammatory cells. In addition to the above triad, previous studies have noted two other morphological features that define a vulnerable plaque: calcified lesions[22] and neoangiogenesis.[23]

We define vulnerability of the plaque following these last assumptions. Extension of calcification and the lipid core was evaluated on entire circumferential arterial sections dividing the field into four equal parts. The extension was scored in numerical values ranging from 0 to 4 (0 = no calcification or core at all, 1 = 1/4, 2 = 2/4, 3 = 3/4 and 4 = 4/4). Fibrous cap thickness was measured with a score of 0 (>200 μm) or 1 (<200 μm), and by evaluating intraplaque haemorrhage with a value of 0 if absent or 1 if present. Cellular inflammation infiltrate was characterised with the following immunohistochemical markers: CD68 as a macrophage marker, Tryptase (serine proteinase stored in the mast cell secretory granules) as a mast cell marker and CD3 as a lymphocyte marker. Microvessel density count was obtained with CD34 as a marker of immature and mature endothelial cells (Ecs). Table 1 summarises this histological features in score

Table 1 Histological considerations to evaluate the vulnerability of the carotid plaque.

Histological features	Parameters	Evaluation	Score
Calcification extension	Sections	≤2/4	0
		>2/4	1
Lipid core extension	Sections	≤2/4	0
		>2/4	1
Thin fibrous cap	Sections	≥200 μm	0
		>200 μm	1
Inflammation score		Score of the markers	
CD68	0 to 3+	<4+	0
CD3	0 to 3+	≥4+	1
Tryptase	0 to 3+		
Neoangiogenesis CD34	Microvessel density	<50 mm^2	0
		≥50 mm^2	1

number to evaluate vulnerable plaques. Anti-alpha smooth muscle actin (alpha-SMA) antibody was used to provide a general view of the plaque architecture.

Immunohistochemical assay

The carotid samples were immediately fixed in 10% neutral, buffered, pH-6.9 formalin. Macrophotographs of the sections in its integrity were taken. Formalin-fixed paraffin embedded 5-μm-thick tissue carotid sections were dewaxed and rehydrated through graded steps of ethanol absolute (Xilol 30 min, 100% 10 min, 95% 5 min, 70% 5 min). Antigen retrieval was performed with a heat-mediated method at 1 atm, 120 °C for 20 min in a citrate buffer solution (pH 6) and cooling for 20 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 in absolute methanol for 10 min at room temperature (rt), in the dark. Antigen–antibody reaction was developed with the NovoLink Polymer Detection Kit (Novocastra, Newcastle, UK). Sections were incubated overnight in a wet chamber at 4 °C with monoclonal antibodies against CD3 (1:100, rabbit monoclonal antibody, SP7 clone, Neomarker, Fremont, CA, USA), CD68 (1:200, monoclonal mouse anti-human, PG-M1 clone; Dako A/S, Copenhagen, Denmark), Tryptase (1:50, monoclonal mouse anti-human mast cell tryptase, AA1 clone; Dako A/S, Copenhagen, Denmark), CD34 (1:80, monoclonal mouse anti-human, Q-BEnd-10 clone; Dako A/S, Copenhagen, Denmark) and ACML (1:9000, monoclonal anti-alpha smooth muscle actin, 1A4 clone, Sigma, Saint Louis, Missouri, USA); then, sections were incubated with NovoLink™ Polymer for 30 min at rt, and subsequently with diaminobenzidine (NovoLink™ DAB Substrate Buffer) for 1 min. Cell nuclei were stained with Mayer's haematoxylin (Sigma Chemicals). Negative controls were done without the primary antibody against CD3, CD68, Tryptase, CD34 or ACML. Finally, samples were dehydrated with graded steps of ethanol absolute and mounted onto glass slides using Canada Balsam (Sigma–Aldrich C1795). The sections were observed under a light microscope (Leitz Wetzlak, Germany 12 V max, 100W) connected with a charge-coupled device (CCD) camera Olympus CX42. Images were acquired using Image-Pro Plus software (Media Cybernetics http://www.mediacy.com) and processed with ImageJ free software (http://rsbweb.nih.gov/ij/).

Table 2 Epidemiology and preoperative clinical characteristics.

	Mean ± SD	
Age, years	72.3 ± 8.5	
	N	%
Male gender	15	68
Neurological symptoms	7	32
Transient ischaemic attack	4	18
Stroke	3	14
Ischaemic lesion at CT ipsilateral to carotid stenosis	9	41
Hypertension	21	95
Dislipidemia	9	41
Diabetes	8	36
Smoke	5	23
Chronic-obstructive pulmonary disease	7	32
Chronic-renal failure (GFR < 60 ml/min)	5	23
Coronary-artery disease	6	27
ASA	22	100
Anticoagulant	0	0
Hydroxymethyl glutaryl coenzyme A reductase inhibitor user	10	46

CT: computed tomography; ASA: acetylsalicylic acid; GFR: glomerular filtration rate.

Inflammatory infiltrate evaluation

An average of 5 fields per plaque transverse section was evaluated for each section; the area evaluated for each image was 0.12 mm^2 at a magnification of 10×. The positivity for each antibody was arbitrarily semiquantitatively scored as intense (3+), moderate (2+), low (1+) or absent (0). To quantify the inflammation infiltrate, we summed the score

Table 3 Comparative evaluation of plaque dB-Enhancement and patient characteristics.

	N (%)	dB-E	*p* value
Age			
>80 years	4 (18)	4.0 ± 2.0	0.38
<80 years	18 (82)	5.5 ± 2.1	
Gender			
Male	15 (68)	4.5 ± 2.2	0.67
Female	7 (32)	3.7 ± 1.7	
Neurological symptoms			
Yes	7 (32)	7.4 ± 0.5	0.006
No	15 (68)	3.5 ± 1.4	
Dislipidemia			
Yes	13 (59)	4.2 ± 0.5	1.00
No	9 (4)	4.2 ± 2.5	
Diabetes			
Yes	8 (36)	3.6 ± 1.1	1.00
No	14 (64)	4.8 ± 2.5	
Smoke			
Yes	5 (23)	4.0 ± 1.4	0.83
No	17 (77)	4.1 ± 2.1	
Chronic-obstructive pulmonary disease			
Yes	7 (32)	4.3 ± 2.5	1.00
No	15 (68)	4.2 ± 2.0	
Chronic-renal failure (GFR < 60 ml/min)			
Yes	3 (14)	3.0 ± 1.4	0.34
No	19 (86)	4.5 ± 2.0	
Coronary-artery disease			
Yes	6 (28)	3.5 ± 0.7	0.67
No	16 (72)	4.4 ± 2.2	
Hydroxymethyl glutaryl coenzyme A reductase inhibitor user			
Yes	10 (46)	3.6 ± 1.0	0.34
No	12 (54)	4.9 ± 2.7	

dB-E: dB-Enhancement; GFR: glomerular filtration rate.

Table 4 Evaluation of clinical and histological characteristics and dB-Enhancement in asymptomatic and symptomatic patients.

	Asymptomatic patients			Symptomatic patients		
	N (%)	dB-E	*p* value	N (%)	dB-E	*p* value
Epidemiology						
Age						
>80 years	2 (13)	4.0 ± 1.1	0.76	2 (29)	6.8 ± 0.6	0.4
<80 years	13 (87)	3.5 ± 1.5		5 (71)	7.5 ± 0.6	
Gender						
Male	9 (60)	4.2 ± 0.5	0.74	6 (86)	7.3 ± 0.5	–
Female	6 (40)	3.7 ± 1.7		1 (14)	7.4	
Dislipidemia						
Yes	8 (53)	4.2 ± 0.5	0.28	1 (14)	7.0	–
No	7 (47)	3.0 ± 1.7		6 (86)	7.3 ± 0.6	
Diabetes						
Yes	7 (47)	3.5 ± 1.9	1.0	1 (14)	7.0	–
No	8 (53)	3.7 ± 1.5		6 (86)	7.3 ± 0.6	
Smoke						
Yes	3 (20)	4.0 ± 1.4	0.83	2 (29)	6.9 ± 0.5	0.52
No	12 (80)	3.4 ± 1.5		5 (71)	7.5 ± 0.4	
Chronic-obstructive pulmonary disease						
Yes	5 (33)	3.7 ± 1.4	0.71	2 (29)	6.9 ± 0.5	0.73
No	10 (67)	3.0 ± 1.5		5 (71)	7.5 ± 0.4	
Chronic-renal failure (GFR <60 ml/min)						
Yes	3 (20)	3.0 ± 1.4	0.56	2 (29)	7.1 ± 0.5	1.0
No	12 (80)	3.7 ± 1.5		5 (71)	7.3 ± 0.4	
Coronary-artery disease						
Yes	3 (20)	3.5 ± 0.7	0.95	3 (43)	7.4 ± 0.2	1.0
No	12 (80)	3.5 ± 1.6		4 (57)	7.3 ± 0.3	
Hydroxymethyl glutaryl coenzyme A reductase inhibitor user						
Yes	7 (47)	3.6 ± 1.2	0.76	3 (43)	6.9 ± 0.6	0.51
No	8 (53)	3.3 ± 2.0		4 (57)	7.2 ± 0.5	
CT ischaemic lesion ipsilateral to carotid stenosis						
Yes	3 (20)	4.5 ± 0.7	0.31	3 (43)	7.2 ± 0.3	0.85
No	12 (80)	3.3 ± 1.5		4 (57)	7.4 ± 0.7	
Histology						
Vessel density						
$< 50/mm^2$	12 (80)	3.0 ± 0.7	0.30	1 (14)	6.8	–
$\geq 50/mm^2$	3 (20)	4.0 ± 1.5		6 (86)	7.4 ± 0.6	
% of area occupied						
<5 by vessels	11 (73)	4.1 ± 0.9	1.0	2 (29)	7.2 ± 0.5	0.58
≥5 by vessels	4 (27)	4.2 ± 1.8		5 (71)	7.4 ± 0.5	
Vessels diameter						
<5 μm	13 (87)	3.8 ± 2.0	0.42	2 (29)	7.2 ± 0.5	0.58
≥5 μm	2 (13)	4.5 ± 1.1		5 (71)	7.4 ± 0.5	

Table 4 (*continued*)

	Asymptomatic patients			Symptomatic patients		
	N (%)	dB-E	*p* value	N (%)	dB-E	*p* value
Extension of calcification						
III	1 (6)	3.8 ± 1.0	0.75	1 (14)	7.6	–
II	6 (40)	3.5 ± 2.8		4 (57)	7.1 ± 0.5	
I	6 (40)	4.0 ± 0.9		2 (29)	7.3 ± 0.5	
0	2 (13)	4.0 ± 0.8		2 (29)	6.8 ± 0.6	
				–		
Extension of the lipid core						
III	3 (20)	4.5 ± 0.7	0.60	1 (14)	7.5	–
II	8 (53)	4.0 ± 2.0		4 (57)	7.3 ± 0.6	
I	4 (27)	3.9 ± 0.7		2 (29)	7.4 ± 0.5	
Fibrous cap						
<200 μm	8 (53)	4.9 ± 1.0	0.09	5 (71)	7.3 ± 0.4	1.0
≥200 μm	7 (47)	3.1 ± 1.1		2 (29)	7.1 ± 0.5	
Intraplaque haemorrhage						
Yes	5 (33)	4.5 ± 0.8	0.56	4 (57)	7.2 ± 0.3	0.85
No	10 (67)	3.6 ± 1.5		3 (43)	7.4 ± 0.7	
Inflammatory infiltrate						
Yes	4 (27)	5.4 ± 1.2	0.10	6 (85)	7.4 ± 0.6	–
No	11 (73)	3.2 ± 1.8		1 (15)	6.8	

positivity for CD3, CD68 and Tryptase. For a sample with an inflammation score lower than 4+ and higher than or equal to 4+, we attributed, respectively, a value of 0 (low) or 1 (high) inflammatory infiltrate.

Neoangiogenesis evaluation

Neoangiogenesis was expressed as microvessel density, identified by CD34-positive immunostaining. The 5 images were converted to stack, transformed in montage, converted from colour images to 8-bit greyscale and scale was set (1600 px mm^{-1}). The total area in square millimetre was evaluated for each stack, and then we set the lower and upper threshold values to select the stained area for CD34. For each stack, we count the total number of microvessels, their diameter and the percentage of area occupied by vessels for each photograph. The mean microvessels density per section was expressed as a function of the total area analysed in the 5 photographs (number of microvessels per mm^2).

Statistical analysis

Continuous data are expressed as mean ± standard deviation (SD), and categorical variables by relative and absolute frequencies. Analysis of differences between groups was performed with Fisher's exact test for categorical variables and with unpaired Student's *t*-test for continuous variables. To compare continuous variables in more than two groups, one-way analysis of variance (ANOVA) with *posthoc* test analysis was performed. The value of $p < 0.05$ was considered significant. Statistical tests were performed using Statistical Package for Social Sciences (SPSS® 13.0) for Windows® computer software (SPSS, Chicago, IL, USA).

Results

Patients' characteristics and CEUS

From September 2009 to January 2010, 22 consecutive patients were included into the study. Patients' demographic characteristics, vascular risk factors, clinical presentations and current therapy are summarised in Table 2. All the 22 patients underwent CT scan and CEUS evaluation within 2 weeks prior to surgery. CEAs were achieved with no complications (death, stroke, TIA, myocardial infarction or cranial nerve injury), and all carotid specimens were suitable for histology and immunohistochemical analysis. No patient was excluded from the study due to technical difficulties in CEUS performance, sample plaque processing or other kinds of complications.

The comparative evaluation of plaque through dB-E and patients' demographic characteristics, current therapy, vascular risk factors and clinical presentations are reported in Table 3. No statistically significant differences were identified for demographic characteristics, vascular risk factors or current therapy. As shown in Table 3, patients with ischaemic neurological symptoms (stroke, TIA or amaurosis fugax) had significant higher carotid dB-E compared with asymptomatic ones (7.40 ± 0.5 dB-E vs. 3.5 ± 1.4 dB-E, respectively, $p = 0.006$). In asymptomatic and symptomatic patients, there was no significant correlation between single characteristics and dB-E values, as detailed in Table 4.

CT scan and CEUS

The cerebral CT scan evaluation revealed the presence of at least one focal low attenuation in 9 (41%) patients. There were five (55%) patients with a single capsular ipsilateral lesion, two (22.5%) patients with bilateral multiple areas of low attenuation indicative of many ischaemic lesions and two (22.5%) patients with an encephalomalacic lesion. The evaluation of clinical characteristics differences between positive cerebral CT scan patients and negative ones are reported in Table 5; the presence of an ipsilateral embolic lesion on preoperative CT scans was significantly correlated with higher dB-E on CEUS compared with patients with negative cerebral CT scan (5.96 $\pm$ 1.5 dB-E vs. 3.0 $\pm$ 1.0 dB-E, respectively, $p = 0.01$).

Histology and immunohistochemical assay and CEUS

The extension of calcification was stratified histologically in five classes: class IV in none, III in two (9%) cases, II in 10 (46%) cases, I in 8 (36%) cases and 0 in 2 (9%) cases. Extension of the lipid core was scored as class IV in no case, III in four (18%) cases, II in 12 (54%) cases, I in 6 (27%) cases and 0 in NOE case. The presence of fibrous cap $<$200 μm

Table 5 Comparative evaluation of clinical characteristics between patients with positive and negative cerebral CT scan.

	Total patients (%)	Positive cerebral CT (%)	Negative cerebral CT (%)	*p* value
Age		73.4 $\pm$ 10.1	71.7 $\pm$ 7.0	0.65
Gender				
Male	15 (68)	7 (32)	8 (36)	0.64
Female	7 (32)	2 (9)	5 (23)	
Neurological symptoms				
Symptomatic	7 (32)	6 (27)	1 (5)	0.007
Asymptomatic	15 (68)	3 (14)	12 (54)	
Dislipidemia				
Yes	13 (59)	6 (27)	7 (32)	0.63
No	9 (41)	3 (14)	6 (27)	
Diabetes				
Yes	8 (36)	4 (18)	4 (18)	0.60
No	14 (64)	5 (23)	9 (41)	
Smoke				
Yes	5 (23)	3 (14)	2 (9)	0.60
No	17 (77)	6 (27)	11 (50)	
Chronic-obstructive pulmonary disease				
Yes	7 (32)	2 (9)	5 (23)	0.60
No	15 (68)	7 (32)	8 (36)	
Chronic-renal failure (GFR $<$ 60 ml/min)				
Yes	3 (14)	1 (5)	2 (9)	1.0
No	19 (86)	8 (41)	11 (45)	
Coronary-artery disease				
Yes	6 (28)	3 (14)	3 (14)	0.65
No	16 (72)	6 (27)	10 (45)	
ASA				
Yes	10 (46)	5 (23)	5 (23)	0.67
No	12 (54)	4 (27)	8 (27)	
Hydroxymethyl glutaryl coenzyme A reductase inhibitor user				
Yes	10 (46)	5 (23)	5 (23)	0.67
No	12 (54)	4 (23)	8 (31)	
dB-E		5.96 $\pm$ 1.5	3.0 $\pm$ 1.0	0.01

GFR: glomerular filtration rate; ASA: acetylsalicylic acid. The presence of cerebral ischaemic lesion ipsilateral to the side of carotid plaque was considered a positive CT evaluation.

was identified in 13 (59%) specimens and the presence of intraplaque haemorrhage in 9 (41%) specimens. The correlation between histological findings and the levels of dB-E at CEUS investigation is reported in Table 6. According to the AHA classification, 5 (23%) specimens were classified 5a, 5 (23%) class 5b and 12 (55%) class 6; no significant differences were identified between AHA class plaques and clinical and CEUS characteristics.

The immunohistochemical analysis showed the presence of inflammation infiltrate in 10 (45%) specimens. The presence of inflammation infiltrate in the carotid plaque specimens was related to higher dB-E at CEUS evaluation, compared with specimens with no inflammation (7.4 ± 1.2 dB-E vs. 3.2 ± 0.9 dB-E, respectively, $p = 0.03$).

The neovascularisation examination identified a vessel density of 57.2 ± 70.0 nm m^{-2}; the mean area occupied by the vessel lumen was 6.2 ± 6.7%. The majority of neovessels identified had a mean diameter of 25 μm (80.4% of vessels); in five cases, the vessels reached a diameter of 660 μm. The presence of elevated vessel density (≥50 mm^2) was identified in nine (41%) specimens; a high percentage of area was occupied (≥5%) by neovessels in nine (41%) cases. Table 7 reports the comparative evaluation of histological plaques' vascularisation and dB-E values at CEUS investigation.

The immunohistochemical analysis also revealed different patterns of CD34 staining. Microvessel morphology ranged from small regular vessel, flattened, irregular lobulate-shaped vessel or big angiomatoid vessel (Fig. 3). Microvessels were identified in the medial border of the lipid core, within the cap, within the lipid core, within the intima and in shoulder.

Table 6 Comparative evaluation of plaque characteristics and dB-Enhancement levels.

Plaque characteristics	N (%)	dB-E	*p* value
Extension of calcification			
IV	0 (0)		0.54
III	2 (9)	4.5 ± 1.8	
II	10 (46)	4.0 ± 2.8	
I	8 (36)	4.7 ± 1.7	
0	2 (9)	7.8 ± 1.1	
Extension of the lipid core			
IV	0 (0)		0.52
III	4 (18)	3.0 ± 1.1	
II	12 (54)	5.3 ± 2.3	
I	6 (27)	4.7 ± 0.3	
0	0 (0)		
Fibrous cap			
<200 μm	13 (59)	5.96 ± 1.5	0.01
≥200 μm	9 (41)	3.0 ± 1.0	
Intraplaque haemorrhage			
Yes	9 (41)	3.5 ± 2.9	0.36
No	13 (59)	4.7 ± 0.9	
Inflammation infiltrate			
Yes	10 (45)	7.4 ± 1.2	0.03
No	12 (55)	3.2 ± 0.9	

Table 7 Comparative evaluation of histological carotid plaques vascularisation elements and dB-Enhancement levels (dB-E).

Plaques vascularisation	N (%)	dB-E	*p* value
Vessel density			
<50/mm^2	13 (59)	2.5 ± 0.7	0.04
≥50/mm^2	9 (41)	5.5 ± 1.2	
% of area occupied			
< 5	13 (59)	4.3 ± 1.5	0.85
≥ 5	9 (41)	4.6 ± 2.5	
Vessels diameter			
<5 μm	15 (68)	4.2 ± 2.2	0.69
≥5 μm	7 (32)	5.0 ± 1.4	

The percentage of number of vessels per region was 59% in the shoulder, 26% in the cap and 15% in the core.

The carotid vulnerability scored 1 in 6 (27%) patients with dB-E 2.5 ± 0.6; scored 2 in 10 (45%) patients with dB-E 4.6 ± 0.8; scored 3 in none; scored 4 in 3 (14%) with dB-E 7.1 ± 0.5 and scored 5 in three (14%) with dB-E 7.6 ± 0.2. The dB-E levels were significantly different between groups ($p = 0.001$) as shown in Fig. 2 and Table 8.

Discussion

With this study, we have observed a direct correlation between CEUS characteristics of the carotid plaque and cerebral symptoms. Histological indicators of plaque vulnerability such as elevated number of microvessels, high vessel total area, thin fibrous cap and inflammatory infiltrate are associated with higher levels of dB-E at CEUS. Similarly, clinical symptoms and CT scan signs of cerebral infarction are correlated with higher levels of dB-E at CEUS evaluation. Specifically, patients with positive cerebral CT scan had significantly higher plaque dB-E than patients with negative CT; thus, CEUS may help in the identification of plaques with higher embolic potential. Few data have been published in the literature so far in this regard.

The relationship between histological aspects of carotid plaque vascularisation and cerebral symptoms has been previously described by McCarty et al.,[7] who identified a higher number of microvessels in symptomatic plaques. Dunmore et al.[6] similarly showed this correspondence and identified a relationship between cerebral symptoms and the carotid plaque vessel morphology; irregular dysmorphic vessels were found almost exclusively in plaques from symptomatic patients. Others have observed a significantly higher microvessel counts in patients with CT evidence of ipsilateral cerebral infarction.[16] While all these authors confirm the role of carotid plaque histological features in association with plaque's vulnerability and in the development of cerebral ischaemic events, the role of CEUS in the identification of carotid plaque vascularisation has been only partially investigated. Coli et al.[24] and Saha et al.[11] compared semiquantitatively CEUS with histological characteristics. Their findings revealed that contrast enhancement within the plaque is correlated with higher number of

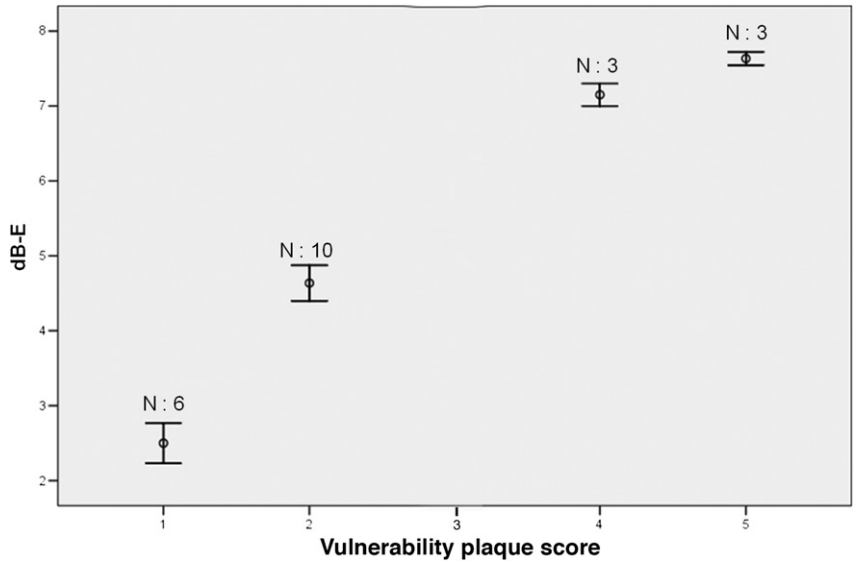

Figure 2 dB-Enhancement evaluation of plaque vulnerability score. Differences between groups are statistically significant at ANOVA analysis ($p = 0.001$).

microvessels, similar to the results of other studies.[25,26] Symptomatic patients had higher contrast enhancement in CEUS evaluation in other studies.[25,26] Therefore, CEUS has been proposed as a method to identify preoperatively vulnerable plaques. With the present study, we were able to combine all those previous findings with CT results, thus defining the strict correlation among CEUS appearance, histological features and clinical significance. Moreover, an objective and reproducible CEUS measurement was adopted. In this sense, CEUS could be helpful in identifying asymptomatic plaque with higher embolic potential.

The present study reveals that plaques with elevated number of microvessels and high vessel total area show a significantly higher contrast enhancement at CEUS software evaluation. A significantly higher dB-E was found in plaques with thin fibrous cap and elevated inflammatory infiltrate. These histological findings are not directly visible at CEUS evaluation; but, inflammation and thin fibrous cap are supposedly involved in the same process of plaque evolution towards vulnerability, similar to the new vessels growth. Kockx et al.[10] and Moreno et al.[27] suggested that neovascularisation supplies a plaque with leucocytes and lipoproteins and promotes plaque expansion and vulnerability. Leucocytes subsequently play a direct role with metalloproteinases and cytokine release in the development of the thin fibrous cap.[28]

The main limitation of our study is the relatively scarce number of patients enrolled, due to the high volume of analysis requested by the protocol (CEUS examination, immunohistochemical and morphological analysis and post-processing evaluation); however, a meaningful association was found within the data collected. Another limitation could be the origin of the ischaemic CT lesions, which may be due to causes different from the carotid plaque; however, we feel that in the absence of other recognisable sources of embolism, the presence of an ipsilateral significant carotid plaque can be reasonably considered as the cause of the lesion. We believe that CEUS, although relatively new and with no actual standardisation for plaque examination and evaluation, may become a suitable standard technique for the identification of vulnerable plaques. The previous studies report different numbers of bolus of the contrast injection and different periods of image storing. Plaque contrast enhancement was obtained in

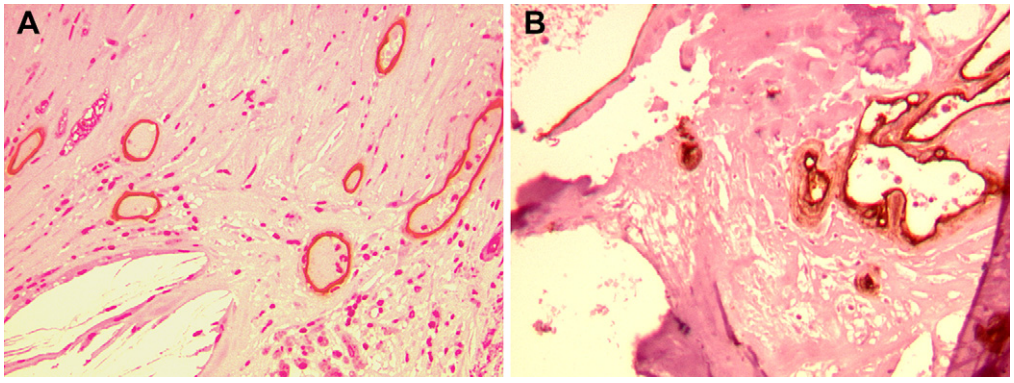

Figure 3 CD34 immunostaining of intraplaque microvessels. A) Small, regularly-shaped microvascular pattern; B) Cavernous, angiomatoid microvascular pattern.

Table 8 "Plaque vulnerability" scores and dB-E values expressed as men and ±SD. In the present table are also expressed symptomatic and asymptomatic patients' data subset.

Vulnerability score	N (%)	dB-E	*p* value	N (%)	dB-E	*p* value	N (%)	dB-E	*p* value
	Total patients			Symptomatic patients			Asymptomatic patients		
Score 1	6 (27)	2.5 ± 0.6	0.001	0 (0)	—	0.3	6 (40)	2.5 ± 0.6	—
Score 2	10 (45)	4.6 ± 0.8		2 (29)	5.4 ± 0.6		8 (53)	4.5 ± 0.8	
Score 3	0 (0)			0 (0)	—		0 (0)	—	
Score 4	3 (14)	7.1 ± 0.5		2 (29)	7.2 ± 0.2		1 (6)	6.8	
Score 5	3 (14)	7.6 ± 0.2		3 (42)	7.6 ± 0.2		0 (0)	—	

many cases by operator semiquantitative evaluation, but Xiong et al. report a software analysis of plaque dB-enhancement. Although no data for comparing software and operator semiquantitative evaluation are presently available in the literature, the software plaque analysis performed in our study could become an objective method to investigate plaque vascularisation at CEUS, which could help in indication to revascularisation, particularly in asymptomatic patients.

Conclusion

CEUS with dB-E is a strong indicator of the extent of plaque neovascularisation, with high correlation with ipsilateral cerebral ischaemic events (stroke or TIA). This technique could be therefore used to identify vulnerable plaques characterised by structural elements with high embolic potential.

Conflict of Interest/Funding

None.

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