

# Contrast-Enhanced Ultrasound Imaging of Intraplaque Neovascularization in Carotid Arteries

## Correlation With Histology and Plaque Echogenicity

Stefano Coli, MD,\* Marco Magnoni, MD,\* Giuseppe Sangiorgi, MD, FESC,†  
Massimiliano M. Marrocco-Trischitta, MD,\* Giulio Melisurgo, MD,\* Alessandro Mauriello, MD,‡  
Luigi Spagnoli, MD,‡ Roberto Chiesa, MD,\* Domenico Cianflone, MD, FESC,\*  
Attilio Maseri, MD, FACC\*

*Milan and Rome, Italy*

- Objectives** This study was designed to evaluate contrast-enhanced ultrasound imaging of carotid atherosclerosis as a clinical tool to study intraplaque neovascularization.
- Background** Plaque neovascularization is associated with plaque vulnerability and symptomatic disease; therefore, imaging of neovascularization in carotid atherosclerosis may represent a useful tool for clinical risk stratification and monitoring the efficacy of antiatherosclerotic therapies.
- Methods** Thirty-two patients with 52 carotid plaques were studied by standard and contrast-enhanced ultrasound imaging. In 17 of these patients who underwent endarterectomy, the surgical specimen was available for histological determination of microvessel density by CD31/CD34 double staining. Plaque echogenicity and degree of stenosis at standard ultrasound imaging were evaluated for each lesion. Contrast-agent enhancement within the plaque was categorized as absent/peripheral (grade 1) and extensive/internal (grade 2).
- Results** In the surgical subgroup, plaques with higher contrast-agent enhancement showed a greater neovascularization at histology (grade 2 vs. grade 1 contrast-agent enhancement: median vasa vasorum density: 3.24/mm<sup>2</sup> vs. 1.82/mm<sup>2</sup>, respectively,  $p = 0.005$ ). In the whole series of 52 lesions, echolucent plaques showed a higher degree of contrast-agent enhancement ( $p < 0.001$ ). Stenosis degree was not associated with neovascularization at histology or with the grade of contrast-agent enhancement.
- Conclusions** Carotid plaque contrast-agent enhancement with sonographic agents correlates with histological density of neovessels and is associated with plaque echolucency, a well-accepted marker of high risk lesions, but it is unrelated to the degree of stenosis. Contrast-enhanced carotid ultrasound imaging may provide valuable information for plaque risk stratification and for assessing the response to antiatherosclerotic therapies, beyond that provided by standard ultrasound imaging. (J Am Coll Cardiol 2008;52:223–30) © 2008 by the American College of Cardiology Foundation

Vasa vasorum are physiological structures that provide nourishment to the vessel wall and play an important role in both early and advanced stages of atherosclerosis. They are normally present in the adventitia of most muscular and conduit arteries and extend into the outer layer of the media in larger vessels. The concept that vasa vasorum are involved in the pathophysiology of atherosclerosis dates back to the

work of Köester (in 1876) (1), Winternitz (in 1938) (2), and was revived by Barger and colleagues (in 1984) (3), who clearly showed in post-mortem samples that coronary atherosclerotic segments presented a rich vascular network extending from the adventitia to the full thickness of media and intima.

In animal models, hyperplasia of vasa vasorum is an early event of hypercholesterolemia-induced atherosclerosis and appears to precede endothelial dysfunction (4). Accordingly, human pathological studies show neovascularization already in type II and, more prominently, in type III atherosclerotic plaques (according to the American Heart Association classification) (5). Experimental studies also indicate that neovascularization is necessary for plaque development, as angiogenesis inhibitors can reduce plaque growth (6).

From the \*Department of Cardiothoracic and Vascular Diseases, University Vita-Salute, San Raffaele Scientific Institute, Milan, Italy; and the †Department of Cardiovascular Diseases and the ‡Department of Pathology, University of Rome Tor Vergata, Rome, Italy. Dr. Coli has received consultancy honorarium from GE Healthcare.

Manuscript received December 12, 2007; revised manuscript received February 8, 2008, accepted February 12, 2008.

**Abbreviations  
and Acronyms****ICA** = internal carotid  
artery**IMT** = intima media  
thickness

Plaque neovessels originate mainly from the adventitia, less often from the main vessel lumen (7), and extension of vasa vasorum to the full thickness of the media and intima of atherosclerotic segments represents pathological neovascularization.

Several pathological studies showed that a more extensive plaque neovascularization is associated with features of plaque vulnerability and with clinically symptomatic disease (8–10).

The mechanisms by which vasa vasorum contribute to the development of plaque instability may be their role in leukocyte recruitment and plaque hemorrhage. Endothelial cells in plaque neovessels express more adhesion molecules than those in the main arterial lumen, which favor leukocyte recruitment (11). Moreover, these microvessels are immature and fragile and thus prone to rupture and hemorrhage, which promote plaque instability and represent an important source of free cholesterol from red blood cells membranes, with consequent macrophage infiltration and necrotic core enlargement (12).

These findings stimulate the search for techniques suitable for the study of human plaque neovascularization *in vivo*. Particularly, direct imaging of vasa vasorum may allow the assessment of the response to antiatherosclerotic therapies and may improve carotid plaque risk stratification. Magnetic resonance imaging with gadolinium infusion has been employed in humans to evaluate carotid plaque vasa vasorum, with a good agreement with histologic findings (13). More recently, contrast-enhanced ultrasound imaging has been proposed for imaging of carotid vasa vasorum (14), but no comprehensive studies have yet been published. This approach takes advantage of the high spatial and temporal resolution of vascular ultrasound imaging and of the properties of contrast-agent microbubbles, which behave as pure intravascular tracers (15). Therefore, we compared direct visualization of neovascularization of carotid plaques by contrast-enhanced ultrasound imaging with histological findings subsequently obtained in surgical carotid specimens. We also correlated plaque neovascularization, assessed by contrast-enhanced ultrasound imaging, with standard ultrasound imaging plaque characterization in terms of echogenicity and degree of stenosis, including a larger series of carotid lesions not subjected to endarterectomy.

**Methods**

**Patient population and study protocol.** Between April and October of 2005, we enrolled 32 patients (27 males, age  $69.9 \pm 8.1$  years) in the study. The patients were selected from those admitted to the coronary care unit or to the vascular surgery unit, who had a clinical indication to standard carotid ultrasound imaging. The examination was requested either because they were referred for carotid endarterectomy or screened for carotid atherosclerosis after

admission for ischemic heart disease. Inclusion criteria were at least 1 carotid atherosclerotic stenosis  $>30\%$ , age  $>18$  years, and ability to provide an informed consent. Exclusion criteria were hypersensitivity to albumin, blood-derived products, or to the ultrasound imaging contrast agent; severe pulmonary hypertension; possible pregnancy; previous carotid surgery or angioplasty; and poor quality of the standard ultrasound imaging study.

Carotid color Doppler ultrasound was recorded in all study patients, and then contrast-enhanced ultrasound imaging was performed by 2 of the researchers (S.C., M.M.). In the enrolled patients who had a clinical indication for carotid surgery, the surgical specimens were also collected. The protocol was approved by our local ethical committee and all patients provided an informed consent.

**Standard and contrast-enhanced carotid ultrasound imaging.** Imaging was performed with a GE-Vivid 7 ultrasound machine (GE Healthcare, Chalfont St. Giles, United Kingdom), using a 7L probe, for both standard and contrast-enhanced studies. First, the carotid bifurcation was imaged bilaterally by B-mode ultrasound, color Doppler, and pulsed-wave Doppler, and the examination was digitally stored for later review. Special care was taken to image and record all distinct plaques seen at each side.

The patients were then submitted to contrast-enhanced ultrasound imaging, with special attention to the previously identified lesions. The preset real-time, contrast-specific imaging modality (pulse inversion) was switched on and image settings adjusted to maximize contrast signal visualization. A low mechanical index was employed (0.08 to 0.10). Perfluoropropane-filled albumin microspheres (Optison, GE Healthcare) were used as a contrast agent. A vial of Optison was diluted with saline up to 10 ml (Optison 3 ml + saline 7 ml) and then a 2-ml bolus was injected and repeated as needed. Good images could be obtained for about 2 min after each bolus. For a single examination, no more than 2 vials of Optison were used. The studies were digitally stored for later analysis. The patients were observed for 30 min before returning to their wards.

Standard and contrast-enhanced images were reviewed offline by 2 readers (S.C., M.M.).

Each visible plaque was classified in terms of echogenicity at standard imaging, according to a widely used classification scheme (16): class I: uniformly echolucent, class II: predominantly echolucent, class III: predominantly echogenic, class IV: uniformly echogenic, and class V: extensive calcification with acoustic shadowing. The degree of stenosis determined by a plaque was measured according to current guidelines (17).

In contrast-enhanced images, all the plaques appear dark and hypoechoic (because of tissue signal suppression), and the adventitia still appears as a bright echogenic line. Moving bright spots within the plaque or on its adventitial side were considered to represent the contrast agent's bubble signal coming from plaque neovascularization. On the contrary, fixed echogenic spots were considered to be strong tissue acoustic

reflectors. For each plaque contrast-agent enhancement (neovascularization) was categorized as follows: grade 1: no bubbles within the plaque or bubbles confined to plaque adventitial side and/or shoulder; grade 2: bubbles reaching plaque core and/or extensive contrast-agent enhancement throughout the plaque. This scale reflects the fact that plaque neovascularization should derive mainly from the adventitial vasa vasorum network and, therefore, progressively grow from the external layers toward the plaque core. In case of disagreement between readers, a consensus was reached.

**Histologic sampling and immunohistochemical studies.** Carotid endarterectomy was performed in each patient with the eversion technique that consists in transecting the internal carotid artery (ICA) at its origin at the carotid bulb, turning inside out (i.e., everting) the ICA over the plaque, and removing the latter en bloc with no longitudinal transection. After fixation in 10% buffered formalin and decalcification, if necessary, the specimens were sliced transversely every 5 mm after, then embedded in paraffin and stained with Movat's pentachrome stains. For each plaque, 3 to 5 sections were examined according to the extension of the plaque itself.

For each histologic sample, the cross-sectional image was acquired by a CCD Nikon digital camera DS-Qi1 (Nikon Instruments Inc., Melville, New York) connected to P4 IBM computer S50 (IBM, Armonk, New York). For each segment, the following histologic variables were evaluated: 1) lumen area (L); 2) internal elastic lamina (IEL) area; and 3) cross-sectional plaque area calculated as [IEL - L]. Histologic areas were calculated using the program Scion Image (Scion Corporation, Frederick, Maryland) manually tracing the perimeter of the different vessel components.

The immunohistochemical study was performed on serial 5- $\mu$ m thick sections cut from paraffin blocks in order to characterize and quantitate the vascularization present within the plaque using a double staining with CD31 (1:100 dilution, purchased from Dako, Glostrup, Denmark) and CD34 (1:4,000 dilution, Dako) antibodies cocktail with an overnight incubation at 4°C, with diaminobenzidine as the final chromogen.

First-order vasa vasorum (running longitudinally along the entire length of the carotid artery) and second-order vasa vasorum (running transversely around the vessel wall of the artery) were identified on the histologic specimen. The number of first- and second-order vasa vasorum were counted at a magnification of 20 $\times$  and related to the cross-sectional plaque area based on the method previously described (18). Two pathologists (A.M., G.S.) who were blinded to the clinical findings graded all histocytological components of the plaques. Intraobserver agreement was 95%. For the entire vessel circumference, either an average of 20 fields per section were counted or a number of microscopic fields until the standard error of the mean was <5% (19). The total number of vasa vasorum per square millimeter was used for correlation with imaging data.

**Statistical analysis.** Continuous data are presented as mean  $\pm$  standard deviation or as median with minimum (min)

and maximum (max) if more appropriate (non-normal distribution). Comparison of microvessel densities between groups was done with the Mann-Whitney *U* test. The degree of stenosis was categorized as <50%, 50% to 69%,  $\geq$ 70%, and chi-square analysis was used. The association between plaque echogenicity and contrast-agent enhancement was evaluated by chi-square analysis. The Fisher exact test was used when appropriate. Statistical analysis was performed with SPSS 11.0 software (SPSS Inc., Chicago, Illinois).

Multivariate logistic regression analysis was also performed in order to adjust for degree of stenosis. The correlation of the plaques within patient was taken into account using the generalized estimation equation method. This method was run with proc GENMOD of SAS version 8.2 (SAS Institute, Cary, North Carolina).

## Results

**Standard carotid ultrasound imaging.** Clinical characteristics of the patient population are reported in Table 1. Among the 32 patients enrolled, 16 had 2 to 3 separate plaques, as a result of multiple bilateral and/or ipsilateral disease; therefore, a total of 52 plaques were studied in the whole group (Table 2). Plaques were evenly distributed among different echogenicity classes (I: 14%, class II: 21%, class III: 17%, class IV: 23%, class V: 25%). The majority of lesions determined a stenosis between 50% and 69%. Six patients had a recent (within 6 months) neurological event consistent with carotid artery distribution; 7 plaques were considered symptomatic because 1 patient had bilateral cerebrovascular events.

**Contrast-enhanced carotid ultrasound imaging.** The examination was well tolerated in all patients, and good quality images were available for all the plaques identified by standard ultrasound imaging. After contrast agent injection, the main arterial lumen was well opacified with contrast agent for about 2 min. In nearly one-third of the cases,

**Table 1** Clinical Profile of the Whole Study Population and of the Surgical Subgroup

	Patient Population (n = 32)	Surgical Subgroup (n = 17)
<b>Clinical characteristics</b>		
Mean age $\pm$ SD (yrs)	69.9 $\pm$ 8.1	68.05 $\pm$ 8.25
Male gender, n (%)	27 (84)	14 (82)
Symptomatic patients, n (%)*	6 (19)	3 (17)
<b>Cardiovascular risk factors</b>		
Family history, n (%)	13 (40)	7 (41)
Overweight/obesity, n (%)	12 (37)	7 (41)
Dyslipidemia, n (%)	18 (56)	7 (41)
Hypertension, n (%)	24 (75)	12 (71)
Diabetes mellitus, n (%)	11 (34)	5 (29)
Active cigarette smoking, n (%)	9 (28)	5 (29)
History of CAD, n (%)	14 (44)	6 (35)
History of PAD, n (%)	12 (38)	6 (35)
Previous† TIA/stroke, n (%)	4 (13)	3 (18)

\*Neurological symptoms within 6 months; †>6 months.

CAD = coronary artery disease; PAD = peripheral artery disease; TIA = transient ischemic attack.

**Table 2** Main Features of All Studied Carotid Plaques and of Surgical Lesions Only

	All Plaques (n = 52)	Surgical Lesions (n = 17)
Plaque characteristics, n (%)		
Stenosis <50%	13 (25)	0 (0)
Stenosis 50%–69%	24 (46)	9 (53)
Stenosis ≥70%	15 (29)	8 (47)
Plaque echogenicity, n (%)		
Class I	7 (14)	1 (6)
Class II	11 (21)	6 (35)
Class III	9 (17)	3 (18)
Class IV	12 (23)	2 (12)
Class V	13 (25)	5 (29)
Degree of plaque neovascularization, n (%)		
Grade 1	35 (68)	11 (64)
Grade 2	17 (32)	6 (36)

contrast-agent enhancement was extensive and/or reached the inner regions of the plaque (grade 2 enhancement). In 2 cases, contrast-enhanced ultrasound imaging revealed carotid plaque ulcerations that were not detected by previous standard ultrasound imaging. The examination was well tolerated and no adverse reactions were observed.

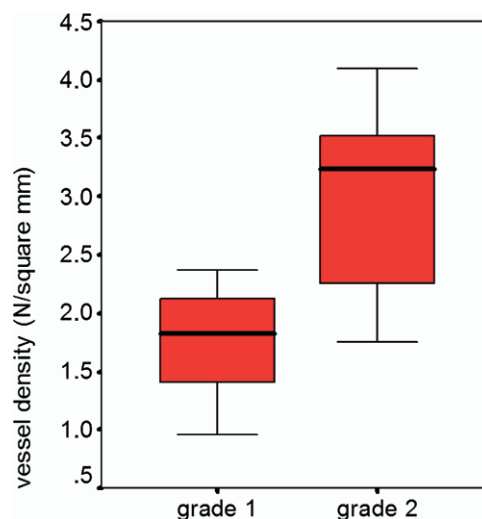
**Correlation between histology and contrast-enhanced carotid ultrasound imaging.** In 17 patients, a surgical carotid endarterectomy was performed according to the referring physician prescription, and therefore, histological analysis was performed. Details of this subgroup of patients are presented in Table 1. Surgical specimens were available for the plaque determining the narrowest stenosis (Table 2); the corresponding plaque ultrasound imaging characteristics were matched with histology. The median number of vasa vasorum per square millimeter was 2.13 (min: 0.96, max: 4.09). Plaques with more intense contrast-agent enhancement showed a significantly higher degree of neovascularization at histology (median vasa vasorum density: 3.24/mm<sup>2</sup>, min: 1.75/mm<sup>2</sup>, max: 4.09/mm<sup>2</sup>, in plaques with grade 2 contrast-agent enhancement vs. density: 1.82/mm<sup>2</sup>, min: 0.96/mm<sup>2</sup>, max: 2.36/mm<sup>2</sup>, in plaques with grade 1 contrast-agent enhancement,  $p = 0.005$ ) (Fig. 1). There was no difference in vasa vasorum density between plaques determining a stenosis <70% or ≥70% (median vasa vasorum density: 2.13/mm<sup>2</sup>, min: 1.18/mm<sup>2</sup>, max: 4.09/mm<sup>2</sup>, in plaques <70% vs. density: 2.06/mm<sup>2</sup>, min: 0.96/mm<sup>2</sup>, max: 3.38/mm<sup>2</sup>, in plaques ≥70%,  $p = 0.50$ ). An example of the correlation between imaging and histology is provided in Figures 2 and 3.

**Correlation between standard and contrast-enhanced ultrasound imaging.** Echogenicity by standard ultrasound imaging and contrast-agent enhancement were correlated for each of the 52 identified plaques. More echolucent plaques had a significantly higher degree of contrast-agent enhancement compared with more echogenic ones ( $p = 0.001$  by chi-square analysis for trend, see Fig. 4). No

significant association was found between the degree of stenosis and contrast-agent enhancement (the prevalence of grade 2 contrast-agent enhancement in plaques determining a stenosis <50%, 50% to 69%, and ≥70% was 27%, 41%, and 27%, respectively,  $p = 0.56$ ). When plaques were categorized as echolucent (class I to II) or echogenic (class III to V), a positive association between echolucency and more intense neovascularization was still present ( $p < 0.001$  by chi-square analysis, odds ratio: 11.6, 95% confidence interval: 3.0 to 45.4) and this association remained significant even after correcting for degree of stenosis and accounting for correlation of plaques within patients ( $p < 0.001$ ). In the surgical subgroup, there was a trend for a higher prevalence of intense contrast-agent enhancement in hypoechoic lesions (62.5% of class I to II plaques had grade 2 contrast-agent enhancement vs. 37.5% of class III to V plaques), which did not reach statistical significance ( $p = 0.15$ ); moreover, there was no difference in histological microvessel density between echolucent and echogenic lesions (median vasa vasorum density: 2.21/mm<sup>2</sup>, min: 0.96/mm<sup>2</sup>, max: 3.66/mm<sup>2</sup>, in class I to II plaques vs. density: 2.13/mm<sup>2</sup>, min: 1.18/mm<sup>2</sup>, max: 4.09/mm<sup>2</sup>, in class III to V plaques,  $p = 1.00$ ). Figure 5 and Online Video 1 show an example of an hypoechoic lesion with extensive (grade 2) contrast-agent enhancement.

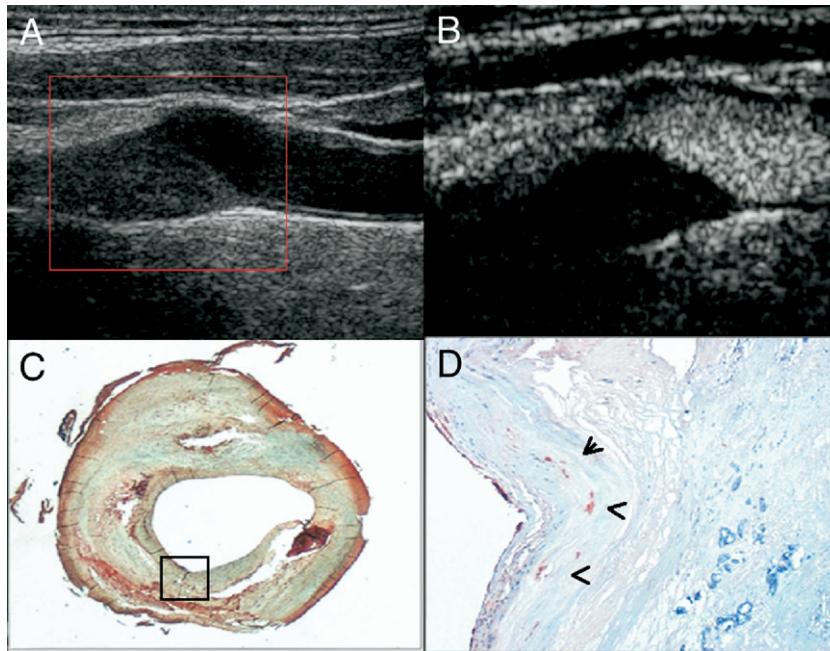
## Discussion

Our findings indicate that contrast-enhanced carotid ultrasound imaging appears a promising technique for the direct visualization of intraplaque vasa vasorum in carotid arteries. We have found a good correlation between vasa vasorum



**Figure 1** Vessel Density at Histology According to Plaque Contrast-Agent Enhancement

The density of vasa vasorum at histology (number per square millimeter) is greater in plaques with grade 2 contrast-agent enhancement at ultrasound imaging compared with those with grade 1.



**Figure 2** Plaque Without Contrast-Agent Enhancement Showing Scant Neovascularization at Histology

**Panel A** shows a large, predominantly hypoechoic plaque, at the origin of the internal carotid artery. No plaque enhancement can be detected by contrast-enhanced ultrasound imaging (**B**). **Panel C** shows the micrograph of 1 of the sections studied of the corresponding plaque (Movat's pentachrome staining,  $\times 0.5$ ). **Panel D** represents the inset in panel C. In the region nearby the fibrous cap, only a few newly formed small vessels CD31/34 positive are present (arrow,  $\times 10$ ).

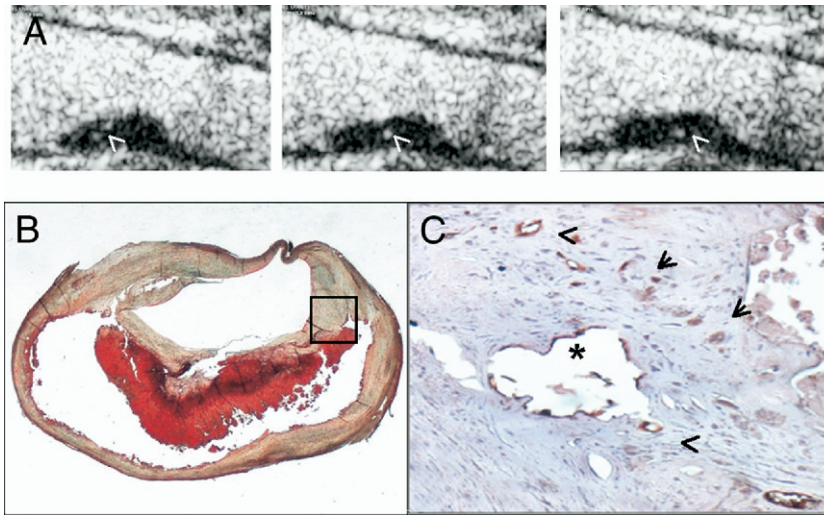
density at histology and the degree of contrast-agent enhancement by ultrasound imaging. In a larger group of plaques, including nonsurgical ones, we have also observed that neovascularization assessed by contrast-agent enhancement is greater in more echolucent lesions, but is unrelated to the degree of stenosis. As plaque echolucency is a marker of high-risk lesions (20), this finding is in agreement with the concept that more vulnerable plaques have a higher degree of neovascularization (8–10). To our knowledge, this is the first published extensive report making a direct comparison between quantitative histology and contrast-enhanced ultrasound imaging on a series of surgical specimens and describing the correlation between plaque echogenicity, degree of stenosis, and contrast-agent enhancement.

Sonographic contrast agents have already been used in carotid imaging to enhance blood flow signal from the main arterial lumen and improve vessel wall delineation for the measurement of intima media thickness (IMT) (21) and degree of stenosis (22). Rajaram *et al.* (23) described for the first time the presence of contrast-agent enhancement in carotid plaques as an unexpected finding during IMT measurement studies and ascribed this phenomenon to plaque neovascularization. Indeed, contrast-enhanced ultrasound imaging has already been used to study tissue perfusion in the myocardium and other organs (24) and to study tumor angiogenesis (25), supporting the concept that microbubbles seen within the plaque are showing local neovascularization. Contrast-agent microbubbles are pure intravascular tracers;

therefore, they can only reach plaque tissue by vascular channels. According to pathological studies, the large majority of these vessels do not originate from the main arterial lumen, but from the adventitia, where a network of vasa vasorum physiologically exists (7). Very recently, 2 papers have been published on vasa vasorum imaging by contrast-enhanced ultrasound imaging. Vicenzini *et al.* (26) described the visualization of contrast-agent microbubbles within carotid plaques as a marker of vascularization, even though no systematic histological validation was performed. Shah *et al.* (27) reported a good correlation between carotid contrast-enhanced ultrasound imaging of intraplaque neovascularization and a semiquantitative histological score on surgical specimens. Our study is the first one to report a good correlation between quantitative histological neovessel density and contrast-agent enhancement. The strength of this association may also be underestimated, because microvessel density at pathology may be loosely correlated to functional estimates of blood flow by imaging, particularly for immature and irregular neovessels such as those observed in atherosclerotic lesions or neoplastic tissue (28).

In animal models of neoplastic tissue, direct comparison between histology and contrast-enhanced ultrasound imaging with careful image superimposition has shown that contrast-enhanced ultrasound imaging correctly identified highly vascularized areas and that vessels between 32 and 99  $\mu\text{m}$  could be identified (29).

It is highly unlikely that our findings can be explained on the basis of imaging artifacts. Only moving bright spots



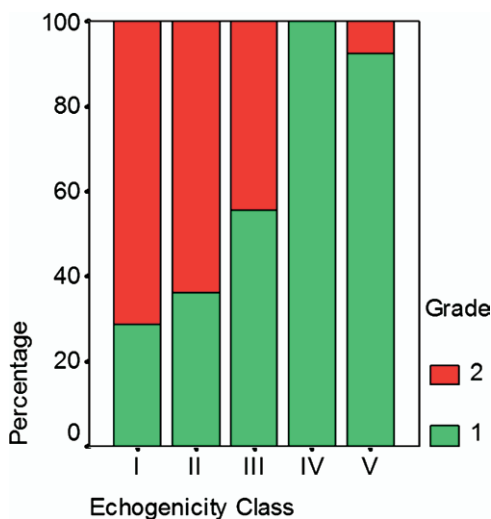
**Figure 3** Plaque With Grade 2 Contrast-Agent Enhancement Showing Abundant Neovascularization at Histology

**Panel A** shows 3 consecutive frames of contrast-enhanced ultrasound imaging of an atherosclerotic lesion in the carotid bulb, showing contrast microbubbles within the plaque (grade 2 contrast-agent enhancement). Micrograph in **panel B** shows a carotid hemorrhagic fibrocalcific plaque with an eccentric large necrotic core occupying approximately 60% of the plaque area (Movat's pentachrome staining,  $\times 0.5$ ). In the area of a thin fibrous cap (**C**, which represents the inset in **B**) nearby the atheroma, numerous CD31/CD34 positive neovessels are present. It is worth noting the presence of a large first-order vasa vasorum (\*) with a thin vessel wall surrounded by small second-order vasa (arrows,  $\times 10$ ).

within the plaques were considered to be a sign of local neovascularization, and fixed bright echoes were considered to be strong tissue reflectors. With careful adjustment of imaging settings and contrast-specific modality, it is quite easy to look for contrast-agent microbubbles within plaque tissue, because most of the plaque appears dark (tissue signal is suppressed) except for the bright line of the adventitia.

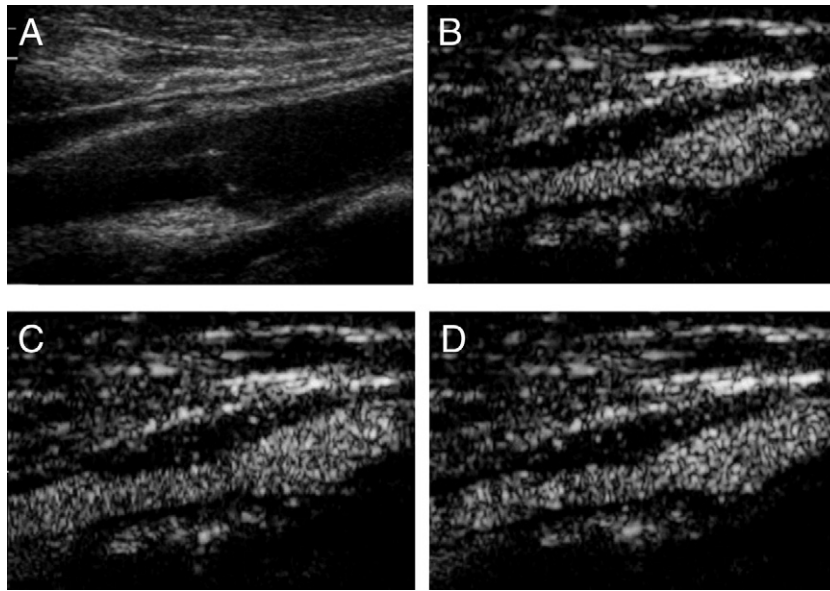
Another important observation of our study is that echolucent plaques show a greater contrast-agent enhancement compared with echogenic ones. More echolucent plaques are known to have more vulnerable pathological features and to bear a higher risk of cerebrovascular events (20,30,31). Plaque neovascularization was found to be more extensive in symptomatic and pathologically vulnerable carotid plaques (8,9). In a series of carotid surgical specimens, McCarthy et al. (10) found a close correlation between the number of plaque neovessels and clinical manifestations; moreover, plaque hemorrhage and rupture were also associated with increased neovascularization. Our observation of a positive relationship between plaque echolucency and contrast-agent enhancement is in agreement with these reported studies, as higher risk lesions are likely to have a greater degree of neovascularization. Neovascularization is extensive in diabetic atherosclerosis (32), but fibrocalcific plaques in diabetic patients are no longer vascularized, suggesting that microvessel involution may be a marker for plaque stabilization (33). This is in accordance with our results showing less extensive neovascularization in more echogenic (i.e., more fibrocalcific) lesions.

The degree of stenosis was not a significant predictor of contrast-agent enhancement in our study. Hyperplasia of vasa vasorum and neovascularization in atherosclerosis may be driven by hypoxia (34) caused by arterial wall thickening, which could be greater in more stenotic lesions, but this does not appear to be the only mechanism; inflammation (35) and activation of toll-like receptors probably represent another important pathway to promote angiogenesis in



**Figure 4** Grade of Contrast-Agent Enhancement According to Plaque Echogenicity

The proportion of plaques with grade 2 contrast-agent enhancement (red) is greater in more echolucent plaques.



**Figure 5** Hypochoic Carotid Plaque With Extensive (Grade 2) Contrast-Agent Enhancement

**Panel A** shows a hypochoic plaque at the origin of internal carotid artery. After injection of Optison (**panels B to D**) an extensive contrast-agent enhancement can be observed, represented by several bright spots within the plaque itself (also see Online Video 1).

atherosclerotic lesions (36). Nevertheless, a larger study is required to assess the factors associated to the visualization of plaque neovessels.

Given the low number of symptomatic patients included in our study, we cannot draw any conclusion on the association between clinical manifestations and contrast-agent enhancement, but this should be an area of further research, as the noninvasive detection of neovascularization may represent an important tool in plaque characterization. Highly vascularized lesions identified by contrast-enhanced ultrasound imaging, even if determining only an intermediate degree of stenosis, could theoretically bear a more severe risk of neurological events. Interestingly, plaque echolucency does not correlate with histological density of vasa vasorum, suggesting that contrast-enhanced ultrasound imaging may identify among hypochoic carotid lesions a subgroup of highly vascularized, potentially vulnerable plaques. Plaque neovascularization may also become an important marker for assessing the result of antiatherosclerotic therapies beyond IMT. Experimental studies have shown that plaque neovascularization can regress and that this can happen despite a persistently increased arterial wall thickness (37); therefore, changes in neovascularization could occur earlier than IMT reductions and contrast-enhanced ultrasound imaging could be able to monitor this phenomenon in the clinical setting. Indeed, Feinstein (14) has described a very interesting case of regression of vasa vasorum after aggressive statin therapy in a diabetic patient.

**Study limitations.** Our study has several limitations. We have used a semiquantitative approach for the evaluation of contrast-agent enhancement; dedicated approaches for microvascular imaging may greatly facilitate image analysis. At the time of this study, we cannot define all the factors, such as dynamic regulation of local microcirculation, that influence the ability to visualize plaque neovessels beyond their density. Similarly we cannot determine the dimensions of the vessels we specifically observe. Further studies are required to confirm our findings in different groups of patients and possibly in other vascular districts. Prospective clinical studies are also needed to evaluate the potential impact of contrast-enhanced ultrasound imaging of plaque neovascularization in determining the risk of cerebrovascular events and in monitoring the effect of antiatherosclerotic therapies.

## Conclusions

Carotid plaque contrast-agent enhancement with sonographic agents correlates with histological density of neovessels and, therefore, is a promising tool to study plaque vasa vasorum in the clinical setting. Neovascularization assessed by contrast-enhanced ultrasound imaging is associated with plaque echolucency, a well-accepted marker of high-risk lesions, and does not depend on the degree of stenosis. Echolucency by itself does not correlate with histological density of vasa vasorum, suggesting that contrast-enhanced ultrasound imaging may identify among hypochoic carotid lesions a subgroup of highly vascularized, potentially vulnerable plaques. Contrast-enhanced carotid ultrasound im-

aging may therefore be a new tool for plaque risk stratification, beyond the simple evaluation of stenosis and echogenicity, and for the assessment of progression and regression of atherosclerosis. Further studies are necessary to evaluate the potential clinical usefulness of carotid contrast-enhanced ultrasound imaging to better stratify the risk of cerebrovascular events and to monitor the response to antiatherosclerotic therapy. (18)

#### Acknowledgments

The authors wish to thank Dr. Chiara Foglieni for her assistance in carotid surgical specimen collection and Dr. Giliola Calori for her assistance in statistical analysis.

**Reprint request and correspondence:** Dr. Stefano Coli, Department of Cardiothoracic and Vascular Diseases, University Vita-Salute, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy. E-mail: coli.stefano@hsr.it.

#### REFERENCES

- Köester W. Endarteriitis and arteriitis. *Berl Klin Wochenschr* 1876; 12:454-5.
- Winternitz MC, Thomas RM, LeCompte PM. *The Biology of Arteriosclerosis*. Springfield, IL: Charles C Thomas, 1938.
- Barger AC, Beeuwkes R 3rd, Lainey LL, Silverman KJ. Hypothesis: vasa vasorum and neovascularization of human coronary arteries. A possible role in the pathophysiology of atherosclerosis. *N Engl J Med* 1984;310:175-7.
- Herrmann J, Lerman LO, Rodriguez-Porcel M, et al. Coronary vasa vasorum neovascularization precedes epicardial endothelial dysfunction in experimental hypercholesterolemia. *Cardiovasc Res* 2001;51: 762-6.
- Jeziorska M, Woolley DE. Neovascularization in early atherosclerotic lesions of human carotid arteries: its potential contribution to plaque development. *Hum Pathol* 1999;30:919-25.
- Moulton KS, Heller E, Konerding MA, Flynn E, Palinski W, Folkman J. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. *Circulation* 1999;99:1726-32.
- Kumamoto M, Nakashima Y, Sueishi K. Intimal neovascularization in human coronary atherosclerosis: its origin and pathophysiological significance. *Hum Pathol* 1995;26:450-6.
- Moreno PR, Purushothaman KR, Fuster V, et al. Plaque neovascularization is increased in ruptured atherosclerotic lesions of human aorta: implications for plaque vulnerability. *Circulation* 2004;110:2032-8.
- Fleiner M, Kummer M, Mirlacher M, et al. Arterial neovascularization and inflammation in vulnerable patients. Early and late signs of symptomatic atherosclerosis. *Circulation* 2004;110:2843-50.
- McCarthy MJ, Loftus IM, Thompson MM, et al. Angiogenesis and the atherosclerotic carotid plaque: an association between symptomatology and plaque morphology. *J Vasc Surg* 1999;30:261-8.
- O'Brien KD, McDonald TO, Chait A, Allen MD, Alpers CE. Neovascular expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content. *Circulation* 1996;93:672-82.
- Kolodgie FD, Gold HK, Burke AP, et al. Intraplaque hemorrhage and progression of coronary atheroma. *N Engl J Med* 2003;349:2316-25.
- Kerwin W, Hooker A, Spilker M, et al. Quantitative magnetic resonance imaging analysis of neovascularization volume in carotid atherosclerotic plaque. *Circulation* 2003;107:851-6.
- Feinstein SB. Contrast ultrasound imaging of the carotid artery vasa vasorum and atherosclerotic plaque neovascularization. *J Am Coll Cardiol* 2006;48:236-43.
- Feinstein SB. The powerful microbubble: from bench to bedside, from intravascular indicator to therapeutic delivery system, and beyond. *Am J Physiol Heart Circ Physiol* 2004;287:H450-7.
- De Bray JM, Baud JM, Dauzat M. Consensus concerning the morphology and the risk of carotid plaques. *Cerebrovasc Dis* 1997;7: 289-96.
- Grant EG, Benson CB, Moneta GL, et al. Carotid artery stenosis: gray-scale and Doppler US diagnosis—Society of Radiologists in Ultrasound Consensus Conference. *Radiology* 2003;229:340-6.
- Kwon HM, Sangiorgi GU, Ritman EL, et al. Enhanced coronary vasa vasorum neovascularization in experimental hypercholesterolemia. *J Clin Invest* 1998;101:1551-6.
- Aherne WA, Dunnill MS. *Morphometry*. London, UK: Edward Arnold Publishers, Ltd, 1982:24-26.
- Nordestgaard BG, Gronholdt ML, Sillesen H. Echolucent rupture-prone plaques. *Curr Opin Lipidol* 2003;14:505-12.
- Macioch JE, Katsamakis CD, Robin J, et al. Effect of contrast enhancement on measurement of carotid artery intimal medial thickness. *Vasc Med* 2004;9:7-12.
- Kono Y, Pinnell SP, Sirlin CB, et al. Carotid arteries: contrast-enhanced US angiography—preliminary clinical experience. *Radiology* 2004;230:561-8.
- Rajaram V, Pandhya S, Patel S, et al. Role of surrogate markers in assessing patients with diabetes mellitus and the metabolic syndrome and in evaluating lipid-lowering therapy. *Am J Cardiol* 2004;93:32C-48C.
- Hancock J, Dittrich H, Jewitt DE, Monaghan MJ. Evaluation of myocardial, hepatic, and renal perfusion in a variety of clinical conditions using an intravenous ultrasound contrast agent (Optison) and second harmonic imaging. *Heart* 1999;81:636-41.
- Cosgrove D. Angiogenesis imaging—ultrasound. *Br J Radiol* 2003; 76:S43-9.
- Vicenzini E, Giannoni MF, Puccinelli F, et al. Detection of carotid adventitial vasa vasorum and plaque vascularization with ultrasound cadence contrast pulse sequencing technique and echo-contrast agent. *Stroke* 2007;38:2841-3.
- Shah F, Balan P, Weinber M, et al. Contrast-enhanced ultrasound imaging of atherosclerotic plaque neovascularization: a new surrogate marker of atherosclerosis? *Vasc Med* 2007;12:291-7.
- Iordanescu I, Becker C, Zetter B, et al. Tumor vascularity: evaluation in a murine model with contrast-enhanced color Doppler US effect of angiogenesis inhibitors. *Radiology* 2002;222:460-7.
- Lassau N, Koscielny S, Opolon P, et al. Evaluation of contrast-enhanced color Doppler ultrasound for the quantification of angiogenesis in vivo. *Invest Radiol* 2001;36:50-5.
- Gronholdt ML, Nordestgaard BG, Bentzon J, et al. Macrophages are associated with lipid-rich carotid artery plaques, echolucency on B-mode imaging, and elevated plasma lipid levels. *J Vasc Surg* 2002;35:137-45.
- Mathiesen EB, Bønaa KH, Joakimsen O, on behalf of the Tromsø study. Echolucent plaques are associated with high risk of cerebrovascular events in carotid stenosis. *Circulation* 2001;103:2171-5.
- Moreno PR, Fuster V. New aspects in the pathogenesis of diabetic atherothrombosis. *J Am Coll Cardiol* 2004;44:2293-300.
- Moreno PR, Purushothaman KR, Sirol M, Levy AP, Fuster V. Neovascularization in human atherosclerosis. *Circulation* 2006;113: 2245-52.
- Carmeliet P. Angiogenesis in health and disease. *Nat Med* 2003;9: 653-60.
- Moulton K, Vakili K, Zurawski D, et al. Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. *Proc Natl Acad Sci U S A*. 2003;100:4736-41.
- Frantz S, Vincent KA, Feron O, Kelly RA. Innate immunity and angiogenesis. *Circ Res* 2005;96:15-26.
- Williams JK, Armstrong ML, Heistad DD. Vasa vasorum in atherosclerotic coronary arteries: responses to vasoactive stimuli and regression of atherosclerosis. *Circ Res* 1988;62:515-23.

**Key Words:** contrast-enhanced ultrasound imaging ■ vasa vasorum ■ atherosclerosis ■ carotid artery ■ imaging.

#### ▶ APPENDIX

For an accompanying video, please see the online version of this article.