Imaging of plaque perfusion using contrast-enhanced ultrasound — Clinical significance

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Summary
The identification of vulnerable and unstable carotid atherosclerotic lesions is up-to-date an important topic of research, in order to adopt the adequate strategy for preventing cerebrovascular events. Plaque inflammation, presence of adventitial vasa vasorum, intimal angiogenesis and plaque neovascularization have been identified in histological studies as indicators of the instability of the atheroma of carotid arteries in cerebrovascular patients and of coronary arteries in cardiovascular patients. Consequently, the identification "in vivo" of these pathophysiological aspects has been objective for the development of new imaging techniques. Ultrasound of carotid arteries, with ultrasound contrast agents, is not only able to provide an enhanced visualization of the arterial lumen and plaque morphology, but also allows to directly visualize adventitial vasa-vasorum and carotid plaque neovascularization. This technique and its clinical implications in the unstable plaque identification are discussed in the present paper.

Introduction
The degree of internal carotid stenosis is nowadays no more considered the only parameter to be evaluated when identifying the "plaque at risk" to be addressed to carotid endarterectomy [1–3]. Since the 1980s the characterization of the morphology of the carotid plaque has become standard for stroke risk definition and, hence, the efforts for the definition of the "unstable plaque" [1,4]. In these regards, carotid ultrasound imaging has represented the cornerstone to describe the plaque characteristics that reflect a higher risk of vulnerability [4–7]. Plaques of moderate echogenicity and with hyperechoic spots are composed of "hard" fibrous tissue and calcifications; these plaques are less harmful than heterogeneous plaques with hypechoic areas that correspond to "soft" atheromatous material consisting of cholesterol, lipid deposits, cell debris and necrotic residuals. Intraplaque hemorrhage, another cause of the sudden increase of plaque volume and rupture, is also of low echogenicity. Summarizing, the lower the degree of the echogenicity of a plaque, the higher the risk of the cap thinning and the surface endothelium rupture with subsequent ulceration, distal embolization and stroke. To reduce the biases of the subjective image interpretation, the computerized analysis of ultrasound images has also proved a reliable objective tool for identifying plaques.
A turning point in the history of atherosclerosis pathophysiological mechanisms comprehension has been the concept that “inflammation” may be linked with the disease development and progression. From histology, indeed, it was already known that while stable atheromatous lesions are characterized by a chronic inflammatory infiltrate, in vulnerable and ruptured plaques an active and acute inflammatory process regarding the surface and the plaque core takes place [10]. Consequently, adventitial vasa vasorum, intimal angiogenesis and plaque neovascularization have been considered, and confirmed by histological studies, as important predictors of unstability in atheromastic lesions of cerebro and cardiovascular patients [11—19]. Angiogenesis occurs indeed regularly within atherosclerotic plaques and atheroma vulnerability and symptomatic carotid disease have been associated with an increased number of microvessels [16] that may also be responsible of the intra-plaque hemorrhage, when the rupture of these small newly generated and vulnerable vessels within the plaque occurs.

The possibility that inflammation could represent an index of plaque vulnerability has brought the scientific interest to concentrate on imaging “in vivo” the pathophysiological “functional” status of the atheroma with the goal to identify, as early as possible, the more vulnerable ones, to adopt the adequate preventive strategy. For this reason, several conventional radiological imaging, such as Computerized Tomography Angiography, Magnetic Resonance Angiography and also 18-FDG Positron Emission Tomography have focused on the evaluation of the “plaque metabolic activity”, but — up to date — this is an evolving methodology requiring further consensus [20]. Contrast carotid ultrasound (CCU) is nowadays a well-established tool for angiogenesis detection in several fields with the principal advantage of being a simple, low cost and minimally invasive technique. Since the first data of 2006, several papers have now described the possibility to identify adventitial vasa vasorum and neovascularization also in carotid plaques [22—40], with a specific pattern of vascularization in acute atheromatous lesions [41].

Aim of this paper is to describe the methodology and the efficacy of contrast carotid ultrasound to identify plaque vascularization and to discuss the related clinical implications.

**Contrast ultrasound investigation methodology and findings**

Our experience is based on patients with carotid stenosis electively referred to our ultrasound laboratory for contrast ultrasound investigation [23,27,28,41] and from still ongoing data. The population consists of both asymptomatic patients, referred for vascular screening, as well as by symptomatic stroke patients. Plaques of different morphologies and various degree of stenosis have been investigated. According to the specific indications and guidelines for carotid endarterectomy, symptomatic and asymptomatic patients with a severe degree of stenosis were operated and histological/samples confronted with the ultrasonographic findings.

**Apparatus, plaque morphology and technique of ultrasound contrast investigation**

Ultrasound carotid duplex scanning were performed with Acuson/Siemens Sequoia 512 and Siemens S2000 systems, with standard vascular presets, and equipped with contrast multi-pulse non-harmonic imaging software “Cadence Contrast Pulse Sequencing” (CPS) technology. Linear phased array probes (6, 8 and 15 MHz for the Sequoia, 9L4 for S2000) with standard presets were used. The same machine presets were maintained constant. The technique of investigation is also reported in other published papers on this topic from our group [23,27,28,41].

Plaque echographic morphology was categorized according to criteria already well established in literature [4,7]. Plaque structure according to the echogenicity, and considered as hyperechoic with acoustic shadow, hyperechoic, isoechoic, hypoechoic, and consequently as calcific, fibrous, fibro-calcific, fibro-fatty and hemorrhagic. Plaque surface was defined as regular, irregular and ulcerated, when an excavation ≥2 mm was observed. Echogenicity was also quantified with the Gray Scale Median (GSM) computerized analysis [8], in order to better define the plaque risk. The degree of stenosis was evaluated according to European Carotid Surgery Trial (ECST) criteria [42], as percentage of the difference between the original vessel lumen diameter/area and the residual lumen diameter/area at the maximum site of stenosis, and according to blood flow velocities [4,43].

After the standard basal investigation of the plaque, contrast ultrasound investigations were performed with repeated short (0.5—1 ml) bolus injections in an antecubital vein (20 Gauge Venflon) of Sonovue (Bracco Altana Pharma, Konstanz, Germany), for a total contrast administration of up to 2.5 ml, each bolus being promptly followed by a saline flush. The 15MHz linear array probe for the Sequoia (MI 0.4—1.1) and the 9L4 MHz for the S2000 (MI 0.10) were used for the CPS continuous real-time imaging. The “Contrast Agent only” software feature, in which the image is derived only from the signals of the microbubbles, has been used. All the investigations were digitally stored and DICOM files transferred to an external PC equipped with Showcase (v 5.1, Trillium Technology) for the off-line analysis.

**Angiogenesis and neovascularization detection**

After the bolus injection, few seconds are required for the contrast to be carried through the venous system to the pulmonary filter, heart and to the carotid arterial lumen. After the contrast is detected in the carotid axis, few seconds later, mainly during the diastolic cardiac phase, probably because of the reduced local pressure on the atherosclerotic lesion, the dynamic distribution of the contrast agent inside the plaque allows the visualization of the plaque vascularization. As previously already reported elsewhere [23,27,28], vascularization was detected at the shoulder of the plaque at the adventitial layers, and in the iso-hyperechoic fibrous and fibro-fatty tissue. It is represented by little echogenic spots rapidly moving within the texture of the atheromastic lesion, easily identifiable in the real time motion, and depicting the small microvessels (Fig. 1, Clip 1). In
ulcerated plaques small vessels are constantly observed under the ulceration (Fig. 2, Clip 2). The diffusion of the contrast agent appears to be in an "outside-in" direction, namely from the external adventitial layers toward the inside of the plaque and vessel lumen [Fig. S1, online supplementary file]. Only in plaques in which the surface was fissurated or ulcerated the contrast agent appeared to have an "inside-out" direction, namely "filling" the void signal of the ulceration from the vessel lumen and better depicting the plaque surface rupture [Fig. S2, online supplementary file]. In recent atherotrombotic occlusion, vascularization, expression of the highly active remodeling process, was also observed [Fig. S3, online supplementary file]. Vascularization was not detected in the hyperechoic with acoustic shadow calcific tissue, nor in the hypoechoic necrotic and hemorrhagic areas. Moreover, plaque vascularization is present in almost every plaque, regardless the degree of stenosis.

In acute symptomatic patients a completely different pattern of vascularization was detected with ultrasound and validated by post-operative histology in a first paper published from our group [41]. In the first seconds after contrast agent administration, no vascularization seemed to be identified in the hypoechoic areas. Few seconds later, vascularization presented as a major diffuse area of contrast enhancement at the base of the plaques, due to an agglomerate of many small microvessels, difficult to differentiate from each other, while the residual hypoechoic part of the plaque, corresponding to the necrotic or hemorrhagic contents, remained avascularized. In operated patients, carotid endarterectomies were carefully performed in order to obtain the whole plaque with minimal trauma. The pathologist evaluated the removed plaques after formalin fixation: the pathologist and the sonographers discussed the regions of interest previously observed at ultrasound imaging. The intra-operative macroscopic findings confirmed the presence of the unstable plaques observed at contrast ultrasound. The microscopic findings confirmed the presence of plaque vascularization in the ultrasound contrast-enhanced areas. Symptomatic carotid plaques showed a relevant increased number of small (diameter 20—30 μm), immature microvessels in respect to asymptomatic ones, consisting with a strong neoangiogenetic activity. Angiogenesis was less represented in asymptomatic plaques that underwent surgery, with microvessels of a higher caliber (80—100 μm). Immunostaining with VEGF, MMP3, CD 31 and CD 34 depicted
a different distribution pattern between asymptomatic and symptomatic lesions: while in the former antigenic activity was of a lesser degree and localized mainly along the microvessels course, in symptomatic plaques a high antigenic fixation was observed also in the external part of the plaque, closer to the adventitial layers. In the same areas, an inflammatory infiltrate constituted by macrophagic foam cells and T lymphocytes, indicative of high plaque activity was detected, with small areas of hemorrhage expression of microvessels rupture.

From the evaluation of subsequent acute stroke patients, it has also been observed that the entity of the internal carotid stenosis may not be directly correlated with clinical symptoms: patients with smaller plaques, even without hemodynamic effect, may present plaque “harmful” characteristics and local areas of vascularization expression of intense “plaque activity”, responsible of the distal embolization. In Fig. 3 two examples are shown: in the former case, figure top, a relative small plaque with a distal ulceration is characterized by predominant vascularization in the distal part nearby the ulceration and in the second case, figure bottom, of a more complex lesion vascularization is highly expressed at the base of the plaque. All these features may then be considered expression of intense plaque remodeling — plaque “activity” that may be consequent to local acute inflammation and plaque vulnerability.

Discussion

Pathophysiological mechanisms responsible of plaque progression and developing of clinical symptoms are not yet completely understood. The role of inflammation has been hypothesized as a fundamental factor involved in the progression of the atherosclerotic plaque and the association between inflammation, atherosclerosis progression and cardiovascular events have been well established for coronary and carotid artery diseases.

The presence of newly generated blood vessels within atherosclerotic lesions has been well recognized since many decades [44], but the “in vivo” evaluation of angiogenesis has received attention, for its possible role in understanding the vulnerability of the atheroma only recently. Histological studies have indeed shown that microvessels are not usually present in the normal human intimal layers and that intima becomes vascularized only with the developing of the atherosclerotic process and when its layer grows in thickness [45]. In nearly half of the patients with a non-hemodynamic carotid stenosis addressed to medical therapy, if — and when — cerebral ischemic symptoms — be it a TIA or other — will occur, these will be without any warning [46]. Therefore, even in those patients who have a non-severe carotid stenosis, some unknown or undetected mechanisms at the level of the arterial wall produces the rupture of the plaque, with consequent embolism and

Figure 3  Top: power duplex (A) and contrast CPS imaging (B) of a small ulcerated plaque in an acute stroke patients. Contrast agents diffuse in the distal part of the plaque nearby ulceration (green arrows in B), corresponding to an area of lower echogenicity (dotted green lines in A). Bottom: color duplex (C) and contrast CPS imaging (D) of an ulcerated plaque in an acute stroke patients. Contrast agents are diffusely visualized at the base of the plaque (green arrows in D).
stroke. Nonetheless, the causes for the modifications of a "hard and stable" into a "softer and unstable" plaque are still not yet completely understood. In these regards, the role of angiogenesis and of intimal vasa vasorum may be of particular relevance. Angiogenesis has indeed also been documented in carotid atherosclerosis and in stable atherosclerotic lesions studied after carotid endarterectomy. It is believed that the absence of pericytes in some new angiogenic vessels causes these immature vessels to "leak" potentially noxious and inflammatory plasma components (hemoglobin, oxidized low-density lipoprotein cholesterol, lipoprotein, glucose, advanced glycation end products, and inflammatory cells) into the extracellular matrix of the media/intima, thus increasing the plaque volume. The ongoing deposit of plasma components appears to further reduce vessel wall oxygen diffusion, enhancing further angiogenesis, plaque inflammation. In the final phase, the plaque is enveloped in adventitial vasa vasorum and intraplaque neovascularization, a hallmark of symptomatic atherosclerosis [47]. The histological observation of a higher number of microvessels within operated symptomatic carotid artery plaques further supports this hypothesis [10,15,16]. All these data follow the observation in the cardiology field, where, angiogenesis and microvessels detected in the coronary atheromas in histological studies have proven to be strongly associated with unstable angina and myocardial infarction. Thus, the observation that, in a late phase of development, the plaque becomes richly vascularized, leading to the atheroma vulnerability increase with possibility of coronary artery occlusion and/or distal embolization, with consequent myocardial ischemic damage [9,10,13,15].

Standard ultrasound carotid duplex is one of the most diffuse and available techniques in clinical routine to assess plaque morphology and to identify the "plaque at risk". The recent application of ultrasound contrast agents to carotid plaque imaging lead to the possibility of directly visualizing adventitial vasa vasorum and plaque neovascularization "in vivo" [21—41], with the advantage of ultrasound being a simple, low cost and minimally invasive technique.

From our experience [23,27,28,41], we observed that microbubbles are visualized easily in the fibrous tissue of carotid plaques and that they correspond to the newly generated vessels, so confirming that plaques have angiogenesis that could be related to the progression and remodeling. The processes that lead to intramural hemorrhage and plaque ulcerations are other important issues that have been extensively studied. Some theories claim the hypothesis that atherosclerosis progression is due to an "outside-in" process and, effectively, intimal vessels originating from the adventitial layers have been observed much more frequently than those originating from the luminal side, resembling the microvessels than grow within tumors. This datum was also confirmed in our patients, in which the microbubbles diffusion seems to be oriented from the external adventitial layers toward the internal intimal lumen and, constantly, through a little vessel present under the plaque ulcerations. This latter observation further supports the theory that intraplaque hemorrhage and ulcerations can be related to the rupture of newly formed intraplaque microvessels, that, being immature and with a thin wall, are submitted to local triggering factors such as mechanical forces and shear stress. The histological observation that intraplaque hemorrhages are common in every atherosclerotic lesion, usually deep and not connected with the vessel lumen, is another indicator that the bleeding originates locally [48,49].

At present, the identification of the plaque vascularization with contrast ultrasound may be considered a new approach to define "in vivo" plaque vulnerability. Future development may be represented by the sonographic follow-up of the plaque vascularization, to evaluate the

![Figure 4](image.png)

**Figure 4**  Power duplex (A) of a moderate internal carotid stenosis in an asymptomatic patient. B-Mode imaging (B) with pulsed wave Doppler (small box, bottom left) shows no hemodynamic effect and GSM calculation (small box, bottom right, green dotted line) echogenicity characteristics of low stroke risk (GSM = 60). Contrast CPS imaging (C) shows a high, diffuse vascularization in the whole plaque texture.
potential benefit or specific effect of medical therapy on plaque remodeling, as regression of plaque vascularization may occur [22]. It is also our experience that vascularization is detectable not only in unstable plaques with a high grade stenosis that are addressed to carotid endarterectomy, but even in light to moderate stenosis and in asymptomatic patients [23,27,28]. The observation of apparently “stable” plaques in asymptomatic patients, determining internal carotid stenosis without indications for surgery, but with evidence of intense vascularization with contrast ultrasound, may open the discussion for further reconsidering mild, non-hemodynamic carotid stenosis, in order to better evaluate stroke risk in these cases (Fig. 4). In this view, further large-scale studies are mandatory for a complete understanding of the natural history of these vascularized lesions, to eventually adopt the adequate preventive strategy.

One limit of this approach of this technique regards the modality for the evaluation of the vascularization: at present, a method of a real numerical objective quantification of the global “plaque perfusion” is indeed not available for carotid plaques. Differently from the evaluation of the heart, in which myocardial tissue perfusion is the expression of a normal condition, and differently from small coronary plaques, in which there is a different ratio due to the size of the vessel, in carotid atherosclerosis this pattern may interest only limited regions of the plaque and therefore quantitative analysis of the mean signal enhancement derived from the whole plaque may not be expressive of the real perfusion. The finding of a “harmful” pattern of plaque vascularization may indeed be limited to a small area of the plaque, but its identification is, in our experience, highly representative of the “plaque activity”. This was confirmed in our histological and immunohistochemical specimen finding of a high angiogenesis with high density of microvessels and with a strong fixation in these areas of endothelial growth factors and inflammatory markers [41]. Moreover, the semi-quantitative evaluation of ultrasound images with time intensity curves, being arbitrary selected areas, may not be considered as really representative of plaque vascularization, also because it is evaluated in bidimensional images. The identification of these patterns then requires a very careful visual and morphological observation, by sonographers trained in this field.

**Conclusions**

Contrast carotid ultrasound is an emerging technique, easily available and quick to perform, that adds important clinical and research information of the “in vivo” pathophysiologic status, with low costs and invasiveness. In symptomatic stroke patients with carotid plaques addressed toward surgery, contrast carotid examinations could help to better analyze plaque morphology and to identify and quantify the presence and degree of neovascularization, allowing a further assessment of the cerebrovascular risk. Larger studies are needed to clarify the prognostic value of plaque vascularization detection in asymptomatic patients with non-severe carotid stenosis that are not candidates for surgery. Moreover, the identification and evaluation of plaque angiogenesis may be in the future useful to evaluate the possible effects of therapies aimed to plaque remodeling.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijperm.2012.03.017.

**References**
