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Assessment of neovascularization in atherosclerotic carotid sinus plaques using quantitative contrast-enhanced ultrasound perfusion imaging

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Aim. To assess the prospects of using quantitative contrast-enhanced ultrasound perfusion imaging of atherosclerotic carotid sinus plaques.

Material and methods. The study included 5 men and 1 woman (59-76 years old, median 72) with symptomatic coronary sinus atherosclerosis. The inclusion criterion was history of ischemic stroke due to internal carotid artery lesion (NASCET ≥60%). We performed contrast-enhanced ultrasound perfusion imaging of the carotid arteries, endarterectomy, studying pathomorphology of the removed plaque with the calculation of the neovascular density and the total number of neovessels with a diameter <40 μm. Neovascularization was assessed by quantitative contrast-enhanced ultrasound 20 seconds after the 1 ml infusion of Sonovia (Bracco, Italy) and subsequent application of the flash. The analysis of dynamics of ultrasonic signal intensity in the atherosclerotic plaque was carried out by creating the curves of the ultrasonic signal intensity (dB)/time (s) over 3 segments of the cross section of the internal carotid artery long axis. The automatic calculation of the intensity dynamics took into account the parameter values in the studied areas within 20 s after the flash. The calculated coefficients (A, B, β) of the exponential equation for 3 atherosclerotic segments were recorded.

Results. Perfusion and neovascularization were assessed in 27 segments of atherosclerotic plaques. The correlation relationships between the ultrasonic parameters of plaque perfusion and the severity of neovascularization were assessed according to the histological data. Significant correlations of the β coefficient exponential curve and histological parameters characterizing the prevalence of "young" vessels (<40 microns) in the atherosclerotic plaque were revealed. Spearman's R for the density of neovessels was 0,54; for the number of neovessels with a diameter <40 μm — -0,66 (p<0,01).

Conclusion. Diagnosis of atherosclerotic plaque neovascularization becomes possible to quantify, assessing not only the presence of neovascular vessels, but also the perfusion intensity. The novel approach replaces the qualitative and semi-quantitative method for calculating the number of carotid plaques neovessels *in vivo*.

Key words: carotid stenosis, perfusion, neovascularization, quantitative analysis.

Relationships and Activities: none.

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The idea that *vasa vasorum* is involved in the pathophysiology of atherosclerosis was firstly described in studies by W. Köester (1876) and M. Winternitz (1938), which showed that the atherosclerotic segments of the coronary arteries had a rich vascular network from adventitia to intima [1, 2]. As atherosclerosis progresses, a decrease in oxygen diffusion reduces the nutrition of arterial wall. Physiological compensation causes an intima-media thickening that exceeds the oxygen diffusion threshold, causing ischemia and subsequent activation of the continuous release of angiogenic growth factors [3]. The absence of pericytes in the new vessels is accompanied by the diffusion of potentially harmful plasma components (oxidized low-density lipoprotein cholesterol, glucose, advanced glycation end-products, inflammatory cells) into the extracellular intima matrix, which increases the volume of atherosclerotic plaque [4].

Deposition of plasma components further reduces the oxygen diffusion to the vascular wall, causing a continued increase in angiogenesis. Ultimately, the plaque is enveloped by external membrane. Neovascularization inside plaques becomes a sign of symptomatic atherosclerosis [5]. New capillaries of the vascular wall (neo-vessels) are already detected in type II plaques. They originate mainly from adventitia, less often from the lumen of the major vessel; the spread of blood vessels to the intima is a sign of vascular malformation and is associated with risk of plaque ulceration [6]. Formation of microvessels in plaque, on the one hand, is a sign of reparation; on the other hand, vessels in the surface plaque layer destabilizes it, increasing the risk of ulceration and rupture [7].

A direct and indirect assessment of vascular wall perfusion, as well as direct visualization of neovessels, can provide assessing the response to antiatherosclerotic therapy and improve risk stratification. There are three levels of blood flow assessment inside an atherosclerotic affected vascular wall: vascular, interstitial and cellular. The vascular level can be assessed using optical coherence tomography and contrast-enhanced ultrasound. The interstitial level can be studied using contrast-enhanced magnetic resonance imaging. Cellular perfusion is characterized by active metabolic processes between the capillary and the structural components of the plaque. There are still no methods for assessing cellular perfusion of atherosclerotic plaques. Contrast-enhanced ultrasound seems to be the most accessible and non-invasive method for studying the perfusion of atherosclerotic plaques in the aorta and major arteries.

The aim was to assess the prospects of using quantitative contrast-enhanced ultrasound perfusion imaging of atherosclerotic carotid sinus plaques

Material and methods

The pilot open-label uncontrolled study with 1 woman and 5 men (59-76 years of age, median — 74 years) with symptomatic hemodynamically significant atherosclerotic lesion of bifurcation of the common and internal carotid arteries (CA) was performed. Local ethics committee approved this study. All patients signed informed consent. There were following inclusion criteria: previous carotid ischemic stroke >6 months prior to examination, corresponding to a significant atherosclerotic lesion (NASCET ⩾60%) of internal CA. All subjects underwent contrast-enhanced ultrasound of CA using Logiq E9 Ultrasound System (GE, USA) with the assessment of internal CA stenosis using the NASCET approach [8]. Characteristics of perfusion in atherosclerotic plaque were studied.

A quantitative assessment of perfusion in atherosclerotic plaques was performed by dynamically assessing the intensity of ultrasound waves as they travel through tissue after an intravenous bolus injection of 1 ml Sonovia (Bracco, Italy) followed by 0,9% sodium chloride bolus injection (5 ml). Sonography was performed by generating cross-sectional long-axis image of internal CA in the plaque area, as well as in the proximal and distal vessel segments without atherosclerotic lesions of at least 5 mm wide each. Video clips were recorded after adequate filling of the vessel with contrast agent. Perfusion was evaluated after applying a high-energy ultrasonic pulses (flash) destroying the bubbles of contrast agent followed by visualization of the vessel. Quantitative calculation of the ultrasonic intensity of 5 areas was carried out using specialized software for tissue intensive curve analysis.

The software of the ultrasound system automatically analyzed the changes in intensity of the ultrasonic signal and built up scatter diagrams of the acoustic intensity values before and after applying a flash for each analyzed segment. In addition, the exponential equation for the dependence of the estimated parameter with time was calculated. The dynamics curve of the signal intensity value was built up within 20 seconds after applying flash. The exponential equation was as follows: $y=A(1-e-\beta t)+B$, where y is acoustic intensity of the signal; t — time; A, B, β — coefficients of the exponential equation. Exponential coefficients (A, B, β) of the acoustic intensity for 20 seconds were recorded for three atherosclerotic segments, proximal and distal areas CA lumen. For each measurement series, the data were presented graphically as 5 dynamical curves of acoustic intensity. The coefficients obtained for each of the analyzed segments were given in the table (Figure 1).

In three patients, ultrasound revealed concentric atherosclerotic plaques of internal CA. Two patients

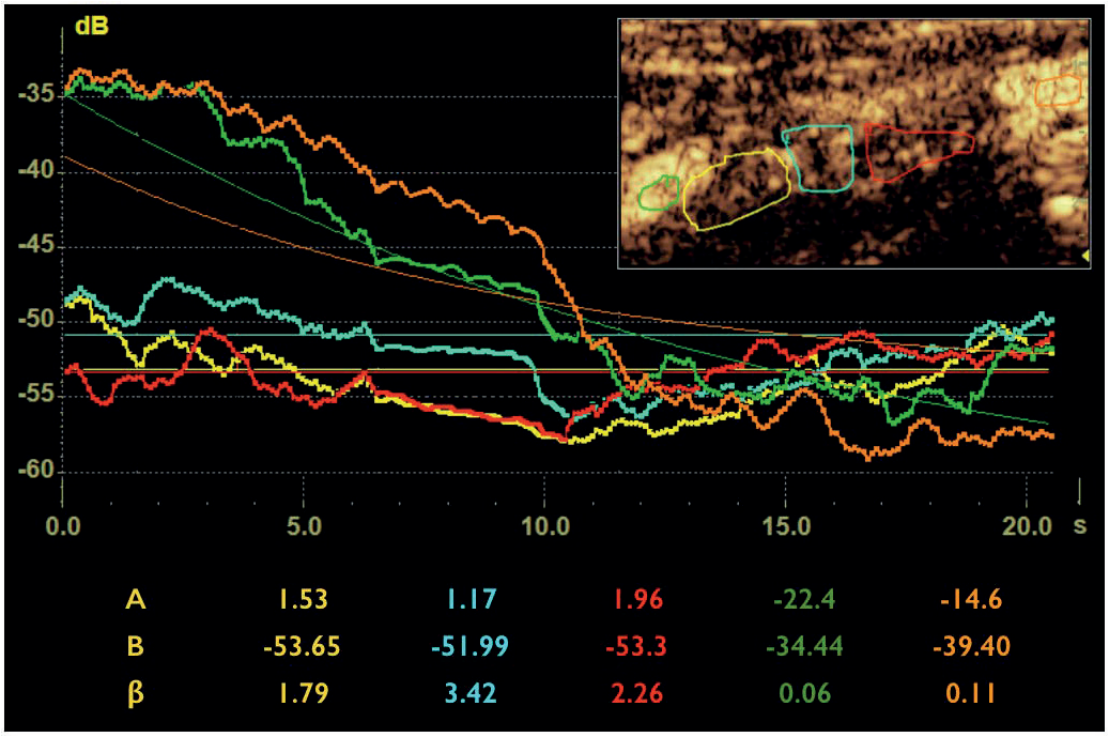

Figure 1. An example of a TIC analysis results in patient M. with symptomatic hemodynamically significant stenosis of the right internal CA.
Note: at the top right is a scan of a longitudinal section of the internal carotid artery during contrast-enhanced ultrasound. Five areas for evaluating the acoustic intensity were allocated. The orange and green areas correspond to the proximal and distal parts of the internal CA. The red, turquoise and yellow areas correspond to the proximal, middle and distal parts of the atherosclerotic plaque. The center presents graphs of the dynamics of the acoustic intensity in the studied areas for 20 seconds after flash. The curve colors correspond to the colors of the areas shown on the scan. Thin curves is modeling the exponential dependence of the acoustic intensity on the estimated areas of the same color. Below are the coefficients of the equation for each of the 5 areas.

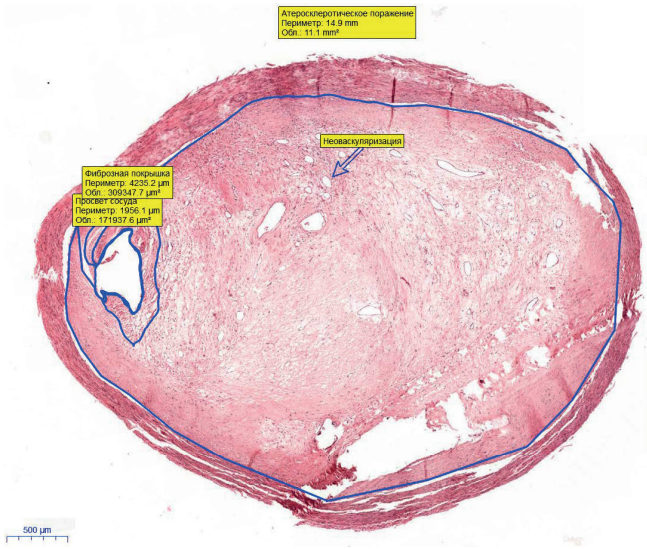

Figure 2. Morphology of the atherosclerotic plaque of patient M. with symptomatic hemodynamically significant stenosis of the right internal CA.
Note: All-vessel density was 84,782 $\mu m^2/cm^2$. Vessels with a diameter of <40 µm predominate; their cross-section number was 97 versus 24 for vessels with a diameter of ⩾40 µm. At the same time, the vessel density with a diameter of <40 µm was only 27492 $\mu m^2/cm^2$ (32%).

had plaque of the posterior wall, one — the anterior wall of the internal CA. In the case of concentric lesion, front and back of the plaque in the central, proximal and distal segments were analyzed.

Within 2 weeks after examination, each patient underwent endarterectomy. Morphological characteristics of removed atherosclerotic plaques was studied with an assessment of neovascularization in cross sections at 3 levels (distal, central and proximal) calculating 2 parameters: neovascularization density in a plaque, total number of neovessels with a cross-section diameter of more and less than 40 µm (Figure 2). In total, an analysis of 27 segments of atherosclerotic plaques was performed.

Statistical analysis was performed with Spearman's rank correlation coefficient using the Statistica 8.0 software package (StatSoft, USA). The data obtained are presented as median, maximum, minimum value, Spearman's rank correlation coefficients. Differences were considered significant at $p<0,05$.

Results

The data of 6 patients with symptomatic hemodynamically significant lesions of the carotid sinus were

Table 1

The values of the correlation coefficients (R, Spearman) between the estimated plaque perfusion and ultrasound and histological parameters of the stenotic area of the internal CA

	A		B		β
The degree of stenosis of internal CA (NASCET)	-	p>0,05	-	p>0,05	-
Peak systolic blood flow	-	p>0,05	-	p>0,05	0,44
Neovessel density	-	p>0,05	-	p>0,05	0,54
The number of neovesels with a diameter of ⩾40 μm	-	p>0,05	-	p>0,05	-
The number of neovessels with a diameter of <40 μm	-	p>0,05	-	p>0,05	-0,66
Density of neovessels with a diameter of ⩾40 μm	-	p>0,05	-	p>0,05	0,57
Density of neovessels with a diameter of <40 μm	-	p>0,05	-	p>0,05	-

Note: Significant correlation coefficients are presented.

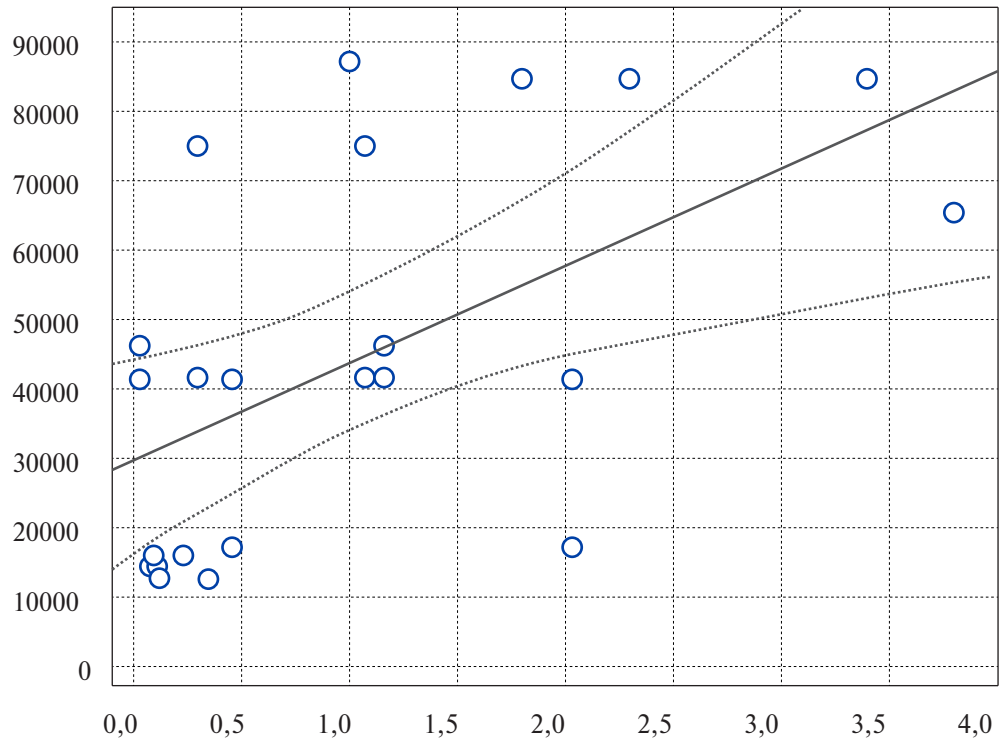

Figure 3. Correlation analysis. Scatterplot matrix of acoustic intensity in segments of atherosclerotic plaques and β coefficient of the exponential equation. Spearman R=0,54; p=0,008.

analyzed. Perfusion and morphology parameters of 27 segments of atherosclerotic plaques were evaluated. The median degree of stenosis was 75% (70-80%). The median peak systolic velocity in the internal CA was 250 cm/s (230-507 cm/s). The median density of plaque neovascularization was 41676 $\mu m^2/cm^2$ (12711-87334 $\mu m^2/cm^2$). The median number of vessels with a diameter of <40 μm amounted to 107 (55-189). The median number of vessels with a diameter of ⩾40 μm amounted to 25 (11-62). The median neovascularization density of atherosclerotic plaque by vessels with a diameter of <40 μm was 10165 $\mu m^2/cm^2$ (7647-27491 $\mu m^2/cm^2$), and by vessels ⩾40 μm — 32695 $\mu m^2/cm^2$ (643-74558 $\mu m^2/cm^2$).

The correlation between the ultrasonic parameters of plaque perfusion and the level of neovascularization was studied according to histology of the material taken during endarterectomy, the estimated severity of stenosis and the peak systolic flow velocity in internal CA (Table 1). Significant correlations of

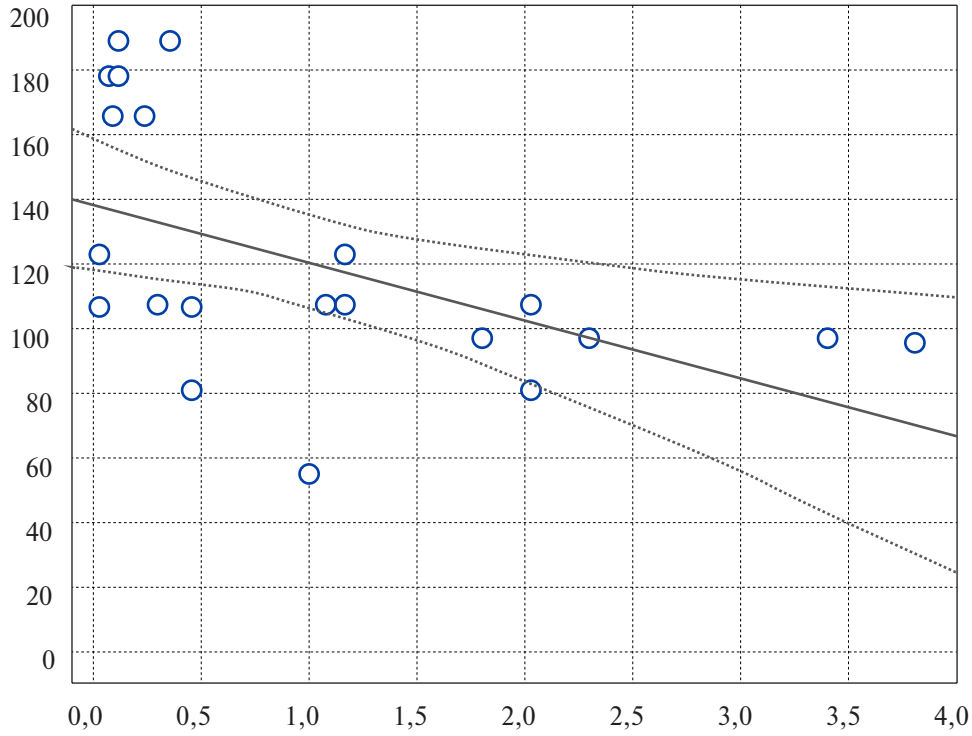

Figure 4. Correlation analysis. The scatterplot matrix of the number of neovessels with a diameter of <40 μm and β coefficient of the exponential equation. Spearman R=-0,66; p<0,001.

the β coefficient exponential curve of dependence of the acoustic intensity on time and the peak systolic flow velocity in the internal CA, neovascularization density and the number of neovessels with a diameter of <40 μm were revealed (Figs. 3, 4). The relationship between the β coefficient and the number of neovessels with a diameter of ⩾40 μm was not significant.

Discussion

The data obtained confirm the usefulness of quantitative perfusion contrast-enhanced ultrasound to assess the severity of plaque neovascularization in carotid sinus. Among the parameters of the exponential equation, significant correlations were obtained only for the β coefficient. β coefficient had a direct moderate correlation with all-vessel density of plaque tissue and neovessels with a diameter of ⩾40 μm, inverse moderate correlation with the number of neovessels with a diameter of <40 μm. Additionally, a moderate direct relationship between the β coefficient and peak systolic velocity in the internal CA was revealed as a parameter characterizing the severity of stenosis.

The first data showing a significant relationship between neovessels of an atherosclerotic plaque and cardiovascular events was presented in 2010 by Hellings W, et al. [9]. The authors examined 818 patients with symptomatic atherosclerosis of CA and previous endarterectomy. A morphology of removed carotid plaques was assessed for neovascularization, calcification, connective tissue and lipid degeneration. Follow-up was carried out for 3 years after endarterectomy. Authors assessed cardiovascular mortality, surgery, number of non-fatal myocardial infarction and strokes. Kaplan-Meier survival analysis was performed for two groups of patients with mildly and highly expressed morphological manifestations of atherosclerosis in removed plaques. It turned out that high plaque vessel density was associated with increased risk of developing endpoints (odds ratio (OR) 1,4 (1,1-1,9)). At the same time, the severity of calcification, connective tissue and lipid degeneration was not associated with a significant increase in the risk of cardiovascular events.

A number of further studies evaluated the severity of neovascularization by the semi-quantitative method by counting the number of microvessels visualized during a contrast-enhanced investigation [10]. Most researchers believe that the severe neovascularization, the higher the cardiovascular risk [9, 11-13]. The division of vessels into "small" (<20 μm) and "large" (>40 μm), however, can change the prospective assessment of atherosclerosis course. It is hypothesized that the predominance of "small" neovessels indicates a high activity of atherosclerotic (or even

inflammatory) process. "Large" vessels indicate a high reparative potential and stabilization of the plaque [12].

The first publications on the quantitative assessment of plaque perfusion and morphology were presented only in 2014 [11, 14]. The technique involved cross-section contrast-enhanced scanning and assessing the absolute values of acoustic intensity in the artery lumen, as well as the central and peripheral parts of the plaque. The study showed that the maximum values of acoustic intensity were recorded in the artery lumen, the minimum — in the central part of the plaque. In symptomatic patients, the ultrasound intensity in the peripheral plaque area is significantly higher (10,8±3,7 dB) than in asymptomatic (7,7±2,4 dB, $p<0,01$).

The method for quantifying neovascularization presented in this study is a novel approach. Firstly, it is proposed to use the short-term increase in the mechanical index (flash) to destroy all the bubbles of contrast agent. This provides equalizing the acoustic intensity in all segments and an assessment over the next 20 seconds of changes in filling the contrast agent. Secondly, the acoustic intensity is estimated not by absolute values, but by the exponential coefficients of the equations created as a result of dynamical observation of filling with a contrast agent. The method allows to simplify the assessment of results and to eliminate the influence of random errors during investigation.

Morphological assessment of atherosclerotic lesions of CA, carried out by a number of authors, show that almost every plaque has signs of neovascularization [12]. The number, dimension and density of neovessels are determined by many factors, among which age and the severity of systemic microinflammation are most important. However, there is no generally accepted point on the negative impact of neovascularization on the course of atherosclerosis. Probably, the predominance of "small" vessels is a reflection of intensive angiogenesis due to microinflammation and the active formation of atherosclerotic plaque. Large-diameter vessels are more often found closer to the adventitia. Detection of such vessels can be associated with a stable course of atherosclerosis. It is vessels of large diameter ($\geqslant$100 μm) that can be well visualized during contrast-enhanced ultrasound. The severity of neovascularization by small-diameter neovessels is better assessed by the novel quantitative approach presented in this study [15]. Interrelation of the processes of neovessel formation in atherosclerotic plaque, as well as their potential negative impact on vascular complications, remains to be further studied.

Unfortunately, our study is limited by the relatively small sample size, which do not allow us to evaluate all forms of atherosclerotic lesions in the common and internal CA, taking into account its severity, the plaque stability, the course of dyslipidemia, other cardiovascular diseases and related complications, the age, constitutional and gender differences of patients. Ultrasonic sections do not fully consist with morphology data. However, taking into account the potential benefits and simplicity of obtaining data on the plaque perfusion in CA, further testing a novel diagnostic approach and accumulating statistical data is an important problem.

Conclusion

Diagnosis of atherosclerotic plaque neovascularization becomes possible to quantify, assessing not only the presence of neovascular vessels, but also the perfusion intensity. The novel approach replaces the qualitative and semi-quantitative method for calculating the number of carotid plaques neovessels *in vivo*. A direct moderate correlation was found between the severity of plaque neovascularization in the carotid sinus according to morphological data and the intensity of blood supply according to contrast-enhanced ultrasound. Dynamic perfusion contrast-enhanced ultrasound of the carotid sinus provides quantifying the severity of plaque neovascularization and identifying areas of plaques with a maximum neovessel density.

Relationships and Activities: none.

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