CONTRAST-ENHANCED ULTRASONOGRAPHY FOR ASSESSING NEOVASCULARIZATION OF CAROTID ATHEROSCLEROTIC PLAQUE

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Neovascularization of a carotid atherosclerotic plaque (AP) is associated with an increased risk of stroke. Contrast-enhanced ultrasonography (CEUS) is a widely used method for imaging intraplaque neovascularization *in vivo*. Unfortunately, there are no standardized guidelines for CEUS interpretation. The aim of this study was to identify the most reliable method for CEUS-based assessment of AP neovascularization. Seventy-eight AP were removed during carotid endarterectomy in 73 patients, of whom 5 had AP on both sides, and examined morphologically. All patients underwent preoperative duplex scanning and CEUS; Sonovue was used as a contrast agent. AP neovascularization was assessed on a 4-grade visual scale and with 3 different quantitative methods using QLAB software. On the visual scale (method 1), poorly (37%) and moderately (51%) vascularized plaques were the most common. Quantitative analysis (data were presented as Me (Q1; Q3)) revealed that the number of blood vessels per 1 cm^2 of the plaque (method 2) was 16 (10; 26), the ratio of the total vessel area to the plaque area (method 3) was 6% (3; 9), and AP ROI (method 4) was 2.6 dB (1.8; 4.1). Significant correlations were demonstrated between the results produced by method 2 and method 3 ($p < 0.0001$), method 3 and method 2 ($p = 0.0006$), and between pathomorphological findings and the results produced by methods 1–3, especially method 2 ($p < 0.004$). AP ROI brightness did not correlate with other results. The presence of hyperechoic components (calcifications) in AP dramatically reduced the reliability of US-based intraplaque neovascularization assessment. The most accurate CEUS-based quantitative method for assessing intraplaque neovascularization is estimation of blood vessel number per 1 cm^2 of the plaque.

Keywords: atherosclerosis, carotid artery, intraplaque neovascularization, contrast-enhanced ultrasonography, Sonovue, visual scale, quantitative analysis, histopathological examination

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ОЦЕНКА НЕОВАСКУЛЯРИЗАЦИИ АТЕРОСКЛЕРОТИЧЕСКОЙ БЛЯШКИ КАРОТИДНОГО СИНУСА С ПОМОЩЬЮ КОНТРАСТ-УСИЛЕННОГО УЗИ

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Степень неоваскуляризации атеросклеротической бляшки (АСБ) каротидного синуса связывают с повышенным риском развития инсульта. Для выявления новообразованных сосудов в структуре бляшки *in vivo* широко применяют контраст-усиленное ультразвуковое исследование (КУУЗИ), однако до настоящего времени отсутствует единый подход к интерпретации результатов. Целью работы было установить наиболее надежный метод оценки неоваскуляризации АСБ каротидного синуса по данным КУУЗИ. У 73 пациентов удалено при каротидной эндартерэктомии, проанализировано, и морфологически исследовано 78 АСБ. Всем пациентам проводили стандартное дуплексное сканирование сонных артерий и КУУЗИ с введением эхоконтрастного препарата «Соновью». Неоваскуляризацию АСБ оценивали с использованием 4-балльной визуальной шкалы и трех методов количественной оценки в программе QLAB. По данным визуальной шкалы (метод 1), преобладали слабо и умеренно васкуляризированные бляшки (37% и 51% соответственно). Результаты количественной оценки (Me (Q1; Q3)): количество сосудов на 1 $см^2$ бляшки (метод 2) составило 16 (10; 26); соотношение площадей сосудов и бляшки (метод 3) — 6% (3; 9); значение ROI АСБ (метод 4) — 2,6 дБ (1,8; 4,1). Значимая корреляция отмечена: между результатами оценки по методам 2 и 3 ($p < 0,0001$); по методам 3 и 1 ($p = 0,0006$); морфологическими данными и результатами оценки по методам 1–3, особенно по методу 2 ($p < 0,004$). Значение ROI АСБ с данными других методов не коррелировало. Продемонстрировано резкое снижение надежности УЗ-оценки неоваскуляризации с увеличением объема гиперэхогенного компонента (кальцификатов) в АСБ. Наиболее точным способом количественной оценки неоваскуляризации АСБ при КУУЗИ является подсчет количества сосудов на 1 $см^2$ бляшки.

Ключевые слова: атеросклероз, сонная артерия, неоваскуляризация атеросклеротической бляшки, контраст-усиленное ультразвуковое исследование, препарат «Соновью», визуальная шкала, количественный анализ, морфологическое исследование

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Carotid sinus (CS) atherosclerosis accounts for up to one-third of all ischemic strokes. The most common causes of stroke in these cases are atherothrombosis, thromboembolism or atheroembolism of cerebral arteries, associated with unstable atherosclerotic plaque (AP) [1, 2]. Pathologic studies have demonstrated that unstable AP are usually characterized by a large atheromatous core, thin or ulcerated fibrous cap, hemorrhage and pronounced inflammation [2, 3]. Recently, neovascularization has been increasingly recognized as the key factor in promoting AP instability and atherosclerosis progression [1, 3–6].

Contrast-enhanced ultrasonography (CEUS) is one of the most widely used techniques for assessing the degree of neovascularization *in vivo*. Since CEUS was first applied to visualize carotid plaque neovascularization in 2003 [7], its accuracy and reliability have been confirmed by multiple animal and human studies that demonstrated a high correlation between ultrasonography findings and histopathologic data [8–9].

Although CEUS has been exploited as an AP neovascularization imaging technique for over 15 years, there is still no consensus as to what approaches should be used to interpret the acquired data. The majority of CEUS-based studies employ qualitative or semi-quantitative scoring systems; however, there is broad agreement that such scoring approach is somewhat biased and unsuitable for the dynamic assessment of atherosclerosis progression. This indicates the need for a uniform, precise and validated scoring system [6, 10]. Methods used for quantitative assessment of CEUS findings are still a matter of ongoing debate [9–11]. Besides, studies comparing CEUS findings and histopathologic data are rare and their results often require further validation.

There is a pressing need for a uniform, accurate and reliable approach to the assessment of CEUS findings in light of emerging ultrasound contrast agents for *in vivo* molecular imaging of vascular phenotypes and targeted drug delivery that are currently in preclinical trials [12, 13]. Novel ultrasound contrast agents and drug delivery systems open new horizons for effective personalized strategies of prevention, diagnosis and treatment of carotid artery disease. Their adoption into clinical practice may be complicated by the absence of a uniform approach to CEUS data analysis.

This study aimed to identify a reliable, informative and clinically friendly method for CEUS-based assessment of carotid AP neovascularization.

METHODS

Studied population

The study was conducted at the Research Center of Neurology in 2015–2018. Eligible patients had atherosclerotic lesions of the carotid sinus and indications for carotid endarterectomy described in the Russian National guidelines for the management of patients with brachiocephalic artery disease [14]. The following exclusion criterion was applied: heavily calcified plaques detected on ultrasonography (> 50% of the total plaque area) casting an acoustic shadow that prevented accurate estimates of AP neovascularization. The study recruited a total of 73 patients (50 men and 23 women aged 40 to 79 years; the mean age was 63 ± 8 years) with ≥ 50% atherosclerotic carotid stenosis (50–90%, the mean value was 70 ± 16%) according to NASCET criteria [15]. All patients underwent carotid endarterectomy at the Research Center of Neurology between January 1, 2015 and December 31, 2017; the intervention was bilateral in 5 patients. The removed plaques were examined histopathologically. A total of 78 plaques were analyzed. Stenosis was symptomatic in 25 (32%) and asymptomatic in 53 (68%) patients.

Conventional and contracts-enhanced ultrasonography examinations

Preoperatively, the patients underwent duplex ultrasound scanning of the carotid arteries and CEUS of the identified carotid atherosclerotic plaques in the longitudinal projection. Examinations were performed using an iU22 scanner (Philips Healthcare NV; Netherlands) equipped with an L9-3 linear array probe.

Duplex ultrasound scanning was performed in order to determine plaque echogenicity, the degree of carotid stenosis and the best visible plaque aspect for the subsequent CEUS examination. Plaques were stratified into 4 groups based on their echogenicity as proposed by A. Gray-Weale [16]: group 1, uniformly hypoechoic; group 2, heterogeneous, predominantly hypoechoic; group 3, heterogeneous, predominantly hyperechoic, and group 4, uniformly hyperechoic.

For CEUS, 2.4 ml of SonoVue contrast agent (Bracco; Italy) dissolved in 5 ml of 0.9% normal saline were administered into a patient's peripheral vein via a bolus injection; 5 ml of normal saline were subsequently injected intravenously through the same catheter. The scan was performed in the Contrast General mode, at a low mechanic index (0.06) and 85% signal enhancement. The probe was held in a fixed position until the arterial lumen was well contrasted; then the angle of the probe was slowly changed to facilitate visualization of the entire plaque. Video clips were recorded for 2 minutes from the moment the patient received the SonoVue injection.

The clips were analyzed off-line using QLAB software (Philips Healthcare NV; Netherlands). Plaque neovascularization was inferred from time-variant dynamic signal enhancement (dynamic hyperechoic signals, DHS) in the plaque caused by nonlinear responses from the microbubbles; hyperechoic signals that did not change over time were interpreted as calcifications. The degree of plaque neovascularization was assessed in QLAB using the following methods.

1. Method 1: semiquantitative assessment on the 4-grade scale: 0 — no DHS; 1 — single DHS; 2 — a moderate number of DHS; 3 — a substantial number of DHS.

2. Three quantitative methods (see the Figure): a still frame showing the maximal number of blood vessels was selected from the CEUS cine loop. The frame was analyzed as described below.

a) method 2: the number of DHS per 1 cm^2 of the plaque was counted. The contours of the plaque were delineated manually and DHS were counted within the circled area. The obtained number was divided by the automatically computed area of the plaque;

b) method 3: the ratio of the total DHS area to the plaque area was calculated and expressed as %. The contours of the plaque and all DHS were manually delineated on the selected still frame. The total DHS area was divided by the plaque area and multiplied by 100%;

c) method 4: plaque ROI (signal intensity) was determined. The area of interest, i.e. the entire plaque, was circled manually on the selected still frame; hyperechoic signals that did not change over time (calcifications) were excluded where possible, and the software automatically computed ROI brightness expressed in dB units.

Histopathological examination

A total of 13 atherosclerotic plaques fragmented during surgery were excluded from the histopathological analysis. The

rest 65 removed plaques were fixed in 10% neutral buffered formalin (pH 7.4), cut into 4 to 9 (depending on the plaque size) transverse 0.3 cm-thick blocks and embedded in paraffin. Five-µm sections of each block were stained with hematoxylin-eosin and van Gieson's stain and then scanned using Aperio AT2 (Leica Biosystems; Germany) at ×400 magnification.

Plaque neovascularization was analyzed in Aperio ImageScope ver. 11.2.0.780 (Leica Biosystems; Germany). Blood vessels were defined as structures that had an endothelial lining and a lumen. To calculate the total vessel density per 1 cm^2 of the plaque, we divided the total number of blood vessels contained in all studied slides by the total area of those slides. Additionally blood vessel density was analyzed for the vessels of certain diameter (< 20, ≥ 20, ≥ 30, ≥ 40, ≥ 50 µm) due to the limitations of CEUS spatial resolution. In noncircular sections, the diameter of the blood vessel was inferred from its transverse size at its widest site.

Statistical analysis

Statistical analysis was done in Statistica 10.0 (StatSoft; USA). Statistical differences and correlations were calculated using nonparametric Mann–Whitney U test and Spearman's correlation coefficient. Differences were considered significant at $p = 0.05$. In this work the data are presented as a median (Me) (quartile Q1; quartile Q3).

RESULTS

On the duplex ultrasound scans, the majority of plaques were heterogenous (81%) and predominantly hypoechoic (51%) (Table 1). Small or medium-sized calcifications were observed in 67% of the plaques. Blood vessels were detected in all studied plaques; none of the applied methods revealed any significant differences between different groups of AP (classification by Gray-Weale [16]) in terms of plaque neovascularization (see Table 1).

Semiquantitative analysis (method 1) demonstrated that the general group of plaques was dominated by AP with a moderate or low DHS number (2 points and 1 point on the visual scale, respectively) that amounted to 51% and 37% of all studied AP, respectively. Plaques with a substantial number of DHS (3 points on the applied scale) were seen > 3 times as rare, making 12% of all AP. When comparing ultrasonography findings and morphological data, we noticed that the more points the plaque scored on the applied scale, the more blood vessels it contained per 1 cm^2 of its area. However, the difference in the degree of AP neovascularization was significant only for the plaques characterized by a low number of hyperechoic signals (Table 2).

All of the applied quantitative methods revealed considerable variability in the degree of AP vascularization: the number of DHS per 1 cm^2 of the plaque (method 2) was 16 signals/cm^2 (10; 26); the ratio of the total DHS area to the plaque area (method 3) was 6% (3; 9); AP ROI (method 4) was 2.6 dB (1.8; 4.1). A direct correlation was established between the results produced by methods 2 and 3 ($R = 0.45$; $p = 0.000034$), and between the results of methods 3 and 1 ($R = 0.38$; $p = 0.0006$). ROI values were not correlated with the results produced by other assessment methods.

We have discovered a significant correlation between the histopathologic data and the results of CEUS-based AP neovascularization assessment aided by the applied methods 1, 2 and 3; the correlation was especially high for method 2 (DHS number per 1 cm^2 of the plaque) (Table 3). Method 2 allowed us to directly compare ultrasonography and histopathologic findings and determine the mean diameter of blood vessels that were visible on CEUS — 30 µm (22; 37).

In order to assess the impact of hyperechoic AP components on CEUS results, we attempted to correlate CEUS and histopathologic data in 3 groups of plaques with different echogenicity (Table 4). We found that the greater was the degree of the hyperechoic component, the weaker was

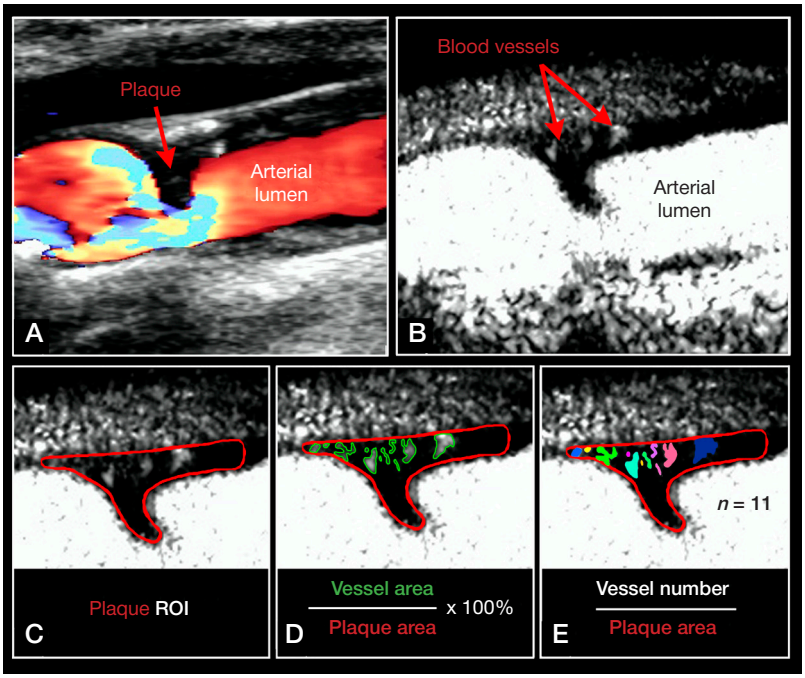

Fig. Quantitative methods for the assessment of carotid atherosclerotic plaque neovascularization from contrast-enhanced ultrasonography data. **A**. A heterogeneous, predominantly hypoechoic atherosclerotic plaque on a conventional Color Doppler Image. **B**. Contrast-enhanced ultrasonography: a predominantly hypoechoic atherosclerotic plaque with single hyperechoic echogenic components (blood vessels, shown by arrows), hyperechoic arterial lumen and surrounding tissue. **C**–**E**. Quantitative analysis of intraplaque neovascularization on a still frame showing the max number of blood vessels (the contour of the plaque is shown in red): ultrasound signal intensity (ROI) (**C**); the ratio of the total vessel area to the plaque area (blood vessels are shown in green) (**D**); blood vessel number per 1 cm^2 of the plaque (blood vessels are shown in different colors) (**E**)

the correlation between CEUS findings and vessel density (histopathologic examination) and the lower was the reliability of US-based assessment of AP neovascularization. For example, the DHS number per 1 cm^2 of the plaque (CEUS, method 2) that had the highest correlation with the results of the histopathologic examination in the general group of plaques demonstrated an even higher correlation in the group of predominantly hypoechoic plaques, whereas for other plaque groups the correlation analysis produced dubious results (see Table 4). The ratio of the total DHS area to the plaque area (method 3) was correlated with the histopathologic findings only for predominantly hypoechoic plaques (Table 4). The correlation analysis between semiquantative scores and histopathologic data in different groups of plaques produced controversial results (Table 4).

DISCUSSION

Visual scales for CEUS data interpretation have received a lot of attention in the literature because they are simple, time-saving, do not require any software for quantitative analysis, and, therefore, can be used in the clinical setting. So far, over 10 different approaches to semiquantitative assessment of AP neovascularization have been described based on visual 2- to 5-grade scales. The majority of such scales take into account both the number and location of dynamic hyperechoic signals [8, 17–22]; scales that rely solely on the number of DHS are rare [17, 23, 24]. The problem with type 1 scales is that an increase in the number of DHS is expected to be directly dependent on signal propagation from the adventitial side of the plaque to its surface. This complicated the choice of an adequate scale for our study, because the identified patterns of AP neovascularization did not fit into any of the considered scales. Therefore, we decided to use a simple one-parameter 4-grade scale for DHS count that was similar to the one described in the literature [24]. Its author proposed that plaques with large artery-like vessels should be classified as having pronounced vascularization (grade 3) with no elaboration on the acoustic characteristics of those artery-like vessels. In our study, the results produced by CEUS and histopathologic examinations revealed the presence of poorly vascularized AP with large artery-like vessels and abundantly vascularized AP that did not contain large artery-like vessels; therefore, we decided to ignore blood vessel size when conducting semiquantitative assessment.

Vessel density in AP was measured during the histopathologic examination and then compared between 3 groups of plaques with different degree of neovascularization assessed on a 4-grade visual scale. The difference was significant only between the group of poorly vascularized plaques with single DHS and the groups of plaques with a moderate or high number of DHS. At the same time, CEUS data assessed on the applied 4-grade visual scale were correlated significantly with histopathologic data, as was the case with other visual scales described in the literature [8, 20, 23, 24]. However, the correlation analysis of plaque groups characterized by different echogenicity produced controversial results, which rendered the applied method of semiquantitative assessment unreliable. This could be explained by a small sample size, a subjective approach to establishing the degree of neovascularization in the absence of clear grading criteria, or frequently occurring calcifications in AP leading to under- or overestimation of the neovascularization degree [25]. Duplex scanning detected the presence of small and medium-sized calcifications in 67% of AP that may have been mistaken for blood vessels on CEUS. The difficulty in discriminating between blood vessels and small calcifications was associated with similarity between their visualization patterns first discovered in this study. The majority

Table 1. Neovascularization of carotid plaques of different types (classification by Gray-Weale)

	Plaque structure			
	Group 1	**Group 2**	**Group 3**	**Group 4**
Number of plaques	3	40	23	12
Of them, morphologically studied	2	33	20	10
Neovascularization, Me (Q1; Q3)				
Contrast-enhanced ultrasonography				
Method 1 (scored points)	1	1 (1; 2)	1 (1; 2)	2 (1; 2)
Method 2 (signal/cm^2)	9 (5; 13)	13 (10.5; 25)	20 (11; 29)	20.5 (9.5; 33.5)
Method 3 (%)	3 (0.4; 5)	6 (3; 7)	7 (3; 11)	8.5 (5; 15)
Method 4 (dB)	2.8 (2.2; 3.1)	2.7 (1.6; 4.2)	2.4 (1.9; 5.5)	2.7 (2.1; 3.4)
Histopathological examination, number of vessels per 1 cm^2 of the plaque	62. 111	161 (96; 253)	90 (61; 305)	230 (125; 300)

Table 2. Results of the semiquantitative analysis of contrast-enhanced ultrasonography data compared to the vessel density determined during the histopathologic examination (* — $p \leq 0.03$)

	AP neovascularization score on the semiquantitative scale (contrast-enhanced ultrasonography)		
Number of blood vessels of a specific diameter per 1 cm^2 of the plaque, Me (Q1; Q3)	1 point	2 points	3 points
	(n = 40)	(n = 29)	(n = 9)
All blood vessels	**108.6 (55.3; 182.4)***	168.6 (125; 356.8)	370 (229; 485)
Blood vessels < 20 μm in diameter	**66.5 (40.8; 111.4)***	117.4 (70.8; 216.8)	277.3 (174.5; 332)
Blood vessels ≥ 20 μm in diameter	**30.5 (9.6; 54.7)***	55.8 (38; 90.2)	90.2 (38.4; 131.8)
Blood vessels ≥ 30 μm in diameter	**13.2 (2.4; 26.1)***	25.5 (12.8; 46.7)	41.4 (13.4; 50.3)
Blood vessels ≥ 40 μm in diameter	**5.5 (1.2; 13.9)***	11.9 (6.2; 23.1)	17.5 (5.8; 25.4)
Blood vessels ≥ 50 μm in diameter	**2.2 (0; 7.6)***	5.9 (3.4; 12.6)	8.8 (2.9; 15.2)

of small and medium-sized calcifications became visible on CEUS only when the contrast agent reached the plaque vasculature, which might be associated with a change in tissue reflectance in those areas [26]. Besides, our histopathologic examination revealed that blood vessels were often located in close proximity to calcifications, which also complicated their identification on CEUS due to a limited resolution capacity of the scanner.

The literature describes 3 principally different approaches to quantitative analysis of CEUS data, all of which were applied in this study. The most common approach relies on the assessment of signal intensity in the region of interest (a contrasted plaque); other include the ratio of the total DHS area to the plaque area and DHS number per 1 cm^2 of the plaque. We did not find any correlation between plaque ROI brightness and vessel density. ROI was not correlated with the results of other CEUS-based neovascularization assessment methods. Some authors have reported a correlation between ROI-based plaque neovascularization assessment and vessel density verified by a histopathologic examination [20, 27, 28]. However, those studies had limitations, such as a small sample size, or employed a less accurate semiquantitative approach to the assessment of plaque neovascularization during a histopathologic examination. Other researchers have established a correlation between the intensity of the signal during CEUS and the results of a histopathologic examination for stable plaques only [8]. The intensity of the US signal is affected by a variety of factors, including tissue reflectance, the degree of plaque calcification (specifically, the presence of small or powdery calcifications that cannot be excluded from the analyzed site), predominant location of the plaque on the anterior or posterior artery wall; brightness and contrast properties of the image that cannot be standardized, etc. [2, 25, 26]. All those factors may have contributed to the outcome we got. Besides, the authors of all articles cited above used a corrected (but not absolute) value of US signal intensity: the ratio of plaque ROI to the arterial lumen [8, 17] or to the intact adjacent vascular wall [27]; the difference between plaque ROI values before and after the injection of a contrast agent [18, 20]; complex algorithms that took into account a number of factors

Table 3. The correlation analysis of data on plaque neovascularization obtained from contrast-enhanced ultrasonography and the histopathologic examination (n = 65)

Histopathologic examination — density of blood vessels of a specified diameter	Contrast-enhanced ultrasonography — the degree of AP neovascularization assessed with different methods							
	Method 1		Method 2		Method 3		Method 4	
	R	*p*	*R*	*p*	*R*	*p*	*R*	*p*
All blood vessels	**0.45**	**0.00019**	**0.41**	**0.00069**	0.23	0.06545	–0.04	0.75
< 20 μm	**0.43**	**0.00033**	**0.36**	**0.0034**	0.18	0.15532	–0.07	0.6
≥ 20 μm	**0.45**	**0.00017**	**0.52**	**0.00001**	**0.37**	**0.00257**	0	0.99
≥ 30 μm	**0.41**	**0.00068**	**0.57**	**0**	**0.36**	**0.00338**	0.03	0.82
≥ 40 μm	**0.41**	**0.00074**	**0.6**	**0**	**0.35**	**0.00438**	0.02	0.89
≥ 50 μm	**0.4**	**0.00102**	**0.6**	**0**	**0.32**	**0.01103**	0.03	0.81

Table 4. The correlation analysis of data on neovascularization in different types of plaques obtained from contrast-enhanced ultrasonography and the histopathologic examination (classification by Gray-Weale)

Histopathological examination — density of blood vessels of a specified diameter	Contrast-enhanced ultrasonography — the degree of AP neovascularization assessed with different methods					
	Method 1		Method 2		Method 3	
	R	*p*	*R*	*p*	*R*	*p*
Heterogeneous, predominantly hypoechoic plaques, group 2 (n = 33)						
All blood vessels	0.34	0.05493	**0.43**	**0.01164**	0.06	0.73935
< 20 μm	0.3	0.08642	**0.35**	**0.04485**	-0.01	0.96716
≥ 20 μm	**0.41**	**0.01825**	**0.67**	**0.00002**	0.33	0.06705
≥ 30 μm	0.34	0.05633	**0.72**	**0**	0.3	0.0897
≥ 40 μm	**0.4**	**0.02162**	**0.74**	**0**	**0.43**	**0.01507**
≥ 50 μm	**0.45**	**0.00857**	**0.79**	**0**	**0.47**	**0.00718**
Heterogeneous, predominantly hyperechoic plaques, group 3 (n = 20)						
All blood vessels	**0.5**	**0.02512**	0.41	0.07403	0.14	0.5446
< 20 μm	**0.47**	**0.03701**	**0.45**	**0.04716**	0.21	0.38029
≥ 20 μm	**0.52**	**0.01815**	0.41	0.07345	0.22	0.35255
≥ 30 μm	**0.51**	**0.02294**	0.43	0.06146	0.15	0.52769
≥ 40 μm	0.38	0.10226	0.41	0.07068	0.12	0.60956
≥ 50 μm	0.34	0.1424	0.4	0.0782	0.15	0.51773
Uniformly hyperechoic plaques, group 4 (n = 10)						
All blood vessels	0.62	0.05444	0.21	0.5667	0.41	0.23349
< 20 μm	**0.71**	**0.02047**	0.06	0.86751	0.27	0.44295
≥ 20 μm	0.43	0.21702	0.36	0.3088	0.43	0.21862
≥ 30 μm	0.13	0.7209	0.46	0.17886	0.3	0.4017
≥ 40 μm	0.25	0.49232	**0.67**	**0.03451**	0.21	0.55384
≥ 50 μm	0.22	0.53903	0.61	0.06125	0.18	0.61791

[12, 22], etc. [28]. We intentionally used the absolute ROI value that can be determined during scanning without additional calculations because it was deemed comparable to the visual scale in terms of time and convenience and at the same time allowed performing dynamic assessment of atherosclerosis progression. However, the obtained results suggest that in order to use ROI as a quantitative method for assessing AP neovascularization, one need to take into account a variety of factors and apply correction coefficients.

The ratio of the total DHS area to the plaque area (method 3) did not provide information on the total vessel density in the plaque or the density of small 20 μm vessels that amounted to 96% of all intraplaque vessels [29]. However, CEUS data were correlated with the density of larger vessels (≥ 20 μm and ≥ 40 μm, respectively) determined during the histopathologic analysis in the general group of plaques and the subgroup of predominantly hypoechoic plaques. The analysis of plaques characterized by different echogenicity demonstrated that this assessment method should not be recommended for hyperechoic plaques because there was no correlation between CEUS and histopathologic data for groups 3 and 4 (classification by Gray-Weale). This can be explained by over- or underestimation of neovascularization degree from CEUS data in the plaques that contained calcifications, as described above. The authors of the method reported a high correlation of CEUS data with the total plaque vessel density assessed during a morphological examination [10]. We did not observe such correlation in our study, which is probably because we used a commercial QLAB package and delineated the area of DHS manually whereas A. Hoogi et al. used a specially developed automated algorithm based on Matlab software (Mathworks). Besides, the accuracy of manual DHS delineation can decrease significantly as the signal area (the vessel size) becomes lower. However, considering the reports of a high correlation been the density of AP blood vessels of different diameters [29] and our data supporting the possibility of reliable CEUS-based identification of vessels over 30 μm in diameter, the applied method can be used for quantitative assessment of AP neovascularization in the absence of a pronounced hyperechoic component. We recommend using an automated algorithm in order to improve the accuracy of measurements.

DHS number per 1 cm^2 of the plaque was well correlated with histopathologic findings both in the general group of plaques and the subgroup with predominantly hypoechoic component. Similar to other quantitative approaches, the results of this method for the group of hyperechoic plaques were not convincing, suggesting a need for developing a complex automated algorithm for accurate assessment of neovascularization in type 3 and type 4 plaques. There are no reports on the comparison of histopathologic data and CEUS findings assessed using this approach. A similar method was used to assess neovascularization from CEUS data [12], but the authors of that work did not verify CEUS results by histology and used an automated MevisLab-based algorithm. They compared the results of semiquantitative and quantitative analyses of plaque neovascularization based on ROI, the area and number of DHS in the plaque and showed a correlation between the ratio of total DHS area to plaque area, DHS number per 1 cm^2 of the plaque and the results of visual assessment; the correlation turned out to be even higher when hyperechoic AP were excluded from the analysis.

CONCLUSIONS

CEUS is an effective, rapid and reliable technique for assessing the degree of neovascularization of carotid AP that do not contain hyperechoic components. The analysis can be performed in standard QLAB software. The most reliable and convenient method for quantitative assessment of AP neovascularization is DHS number per 1 cm^2 of the plaque on a still frame that contains the maximal number of visible signals. Its results were well correlated with the histopathologic findings. The ratio of the total DHS area to the plaque area can also be an option, but this method is more time-consuming and less reliable. We do not recommend using absolute ROI brightness of the plaque for assessing intraplaque neovascularization. Semiquantitative assessment on a 2–3 grade visual scale should be used as a qualitative express method for detecting the presence of blood vessels in the plaque. Hyperechoic AP components have a significant impact on CEUS results. This indicates a need for an algorithm that can automatically detect and exclude from the analysis not only large but also small and medium-sized calcifications.

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