ORIGINAL ARTICLE

Preliminary comparisons between in vivo ultrasonographic virtual histology and histopathological findings of endarterectomized carotid plaque

Comparações preliminares entre a histologia virtual ultrassonográfica in vivo e os achados histopatológicos da placa carotídea produto de endarterectomia

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Abstract

Background: Extracranial carotid artery atherosclerosis is a major preventable cause of strokes, the second most common cause of death in developed countries. The degree of arterial lumen stenosis is the basis for surgical indications, but does not provide information about other plaque aspects. Studies in the literature suggest that the morphological characteristics of the plaque and its composition should also be included in the assessment of this disease. **Objective:** Investigate the correlation between atherosclerotic plaque composition defined by computer-assisted analysis of ultrasound images (virtual histology - USVH) and conventional histology. **Method**: The images of twelve plaques, obtained during preoperative ultrasound scanning, were analyzed by computer, and the grey scale images were correlated with the plaque components and subsequently compared with the histological findings of the analysis of the endarterectomy specimens. **Results**: The amount of lipids and fibromuscular tissue were strongly correlated in the two tests (R=0.83 and 0.91). There were no significant correlations with amount of blood or calcium (R=0.05 and 0.19). **Conclusion**: This study confirmed the usefulness of noninvasive USVH. Further technical improvements and software developments may promote the clinical application of this method.

Keywords: atherosclerosis; carotid; ultrasound; histology.

Resumo

Contexto: A doença aterosclerótica da carótida extracraniana é uma das principais causas evitáveis de acidente vascular cerebral isquêmico (AVCi), sendo este a segunda causa mais comum de morte nos países desenvolvidos. Nos grandes estudos sobre a cirurgia carotídea, a indicação estava embasada fundamentalmente no grau de estenose arterial. Analisar somente o grau de estenose, entretanto, não revela todas as características da placa, na medida em que a morfologia e a composição da placa complementam a avaliação da doença carotídea avançada e são fundamentais para a análise e o acompanhamento da maioria das placas carotídeas tratadas clinicamente. **Objetivo**: Correlacionar a caracterização dos componentes da placa de ateroma pela histologia virtual ultrassonográfica (HVUS) com a histologia. **Métodos**: As imagens pré-operatórias obtidas por ultrassonografia transcutânea de 12 placas de ateroma de bifurcação carotídea foram submetidas a um programa de computador, o qual correlacionou os níveis de cinza com os prováveis componentes da placa da bifurcação carotídea (HVUS). Estes achados foram correlacionados com o exame anatomopatológico das placas coletadas pela cirurgia de endarterectomia. **Resultados**: O coeficiente de correlação de Pearson para os conteúdos de lipídeos e músculo/tecido fibroso foram, respectivamente, R=0,83 para gordura e R=0,91 para músculo/tecido fibroso. Quanto ao cálcio e ao sangue, foram R=0,05 e R=0,19, respectivamente. **Conclusões**: O presente trabalho corrobora a literatura demonstrando que a histologia virtual computadorizada baseada em ultrassonografia transcutânea apresenta boa correlação com os achados da histologia quanto ao conteúdo da placa. Maiores estudos para a padronização da técnica e o aperfeiçoamento do programa de análise permitirão maior uso clínico deste método.

Palavras-chave: aterosclerose carotídea; histologia; ultrassonografia.

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Financial support: The author Thiago do Carmo Silveira received an undergraduate student scientific research grant from PIBIC/CNPq.
Conflicts of interest: No conflicts of interest declared concerning the publication of this article.
Submitted on: 02.10.13. Accepted on: 04.29.13.

Study carried out at the Dr. Sandra Silveira private office and the State University of Campinas Hospital of Clinics.
This study was approved by the Research Ethics Committee of the School of Medical Sciences of Unicamp on June 22, 2010, under protocol no. 490/2010.
This study is not a clinical trial.

INTRODUCTION

Extracranial carotid artery atherosclerosis is one of the major preventable causes of strokes, the second most common cause of death in developed countries, where they are responsible for 4.5 to 5 million deaths every year[1,2]. About 50% to 80% of all strokes are ischemic[1,3], and of all ischemic strokes, 15% to 50% result from emboli and thrombi produced by the atherosclerotic plaque in the extracranial carotid artery bifurcation[1-6]. However, not all plaque with marked stenosis becomes symptomatic and leads to a stroke or transient ischemic attack (TIA), which raises questions about whether surgery should be indicated for asymptomatic patients[7].

ACAS[8], NASCET[9], ECST[10], ACST[11], and CREST[12] are large studies that investigated the use of degree of carotid artery stenosis as a tool to make surgical decisions. Currently, the characteristics of carotid stenosis are described using imaging methods, such as duplex ultrasound (US), digital subtraction angiography (DSA), CT angiography (CT-angio) and magnetic resonance angiography (MRA). The content of the atheroma may be analyzed using several of these imaging techniques, but most authors use them only to define the characteristics of anatomic plaque stenosis, while plaque morphology and composition are often not investigated[3,5]. Other complementary imaging tests, such as positron emission tomography (PET) and single photon emission CT (SPECT), may play an adjuvant role in follow-up. However, although promising, they are expensive, and access to them is limited[2,13-15].

The isolated analysis of degree of stenosis provides a limited evaluation of plaque stability. Several molecular processes, such as inflammation, lipid accumulation, proteolysis, apoptosis, angiogenesis and thrombosis, have been shown to be associated with plaque vulnerability when the risk of embolization and thrombosis increases (unstable plaque), regardless of degree of stenosis. Such vulnerability has the following characteristics: plaque ulcerations; large amount of lipids; thin fibrous cap between lipid core and arterial lumen; plaque core with necrosis; and intraplaque hemorrhage. In contrast, a high amount of fibrous contents and greater calcification may be associated with a lower risk of stroke (stable plaques). Therefore, plaque morphology and composition should be included in the evaluation of atherosclerotic diseases, which will further contribute to the knowledge about lumen narrowing[3,13,16,17].

The morphological characteristics of atheromas most often studied are: surface aspect; echogenicity; distribution of plaque content in relation to surface and degree of heterogeneity; plaque volume; and wall mobility.

In ultrasound grayscale images, plaques may be divided into echolucent (predominance of dark shades) and echogenic (predominance of light shades) according to the amount of lipids, which appear darker because they attenuate sound, or fibrous tissues, which appear lighter because they reflect sound. Plaque echogenicity may be evaluated visually using ultrasound scans, and plaques are classified as uniformly echolucent, predominantly echolucent, predominantly echogenic, uniformly echogenic, densely calcified and unclassifiable[7]. The median distribution of the brightness values of individual pixels of the gray scale image is called gray scale median (GSM), and may also be estimated. Its results indicate whether the plaque is more or less echogenic. GSM is the mid-point in the histogram generated by the pixels of the ultrasound image distributed according to brightness or echogenicity. According to Nicolaides[18], an echogenic plaque has a GSM greater than 32, although a more recent study found that the cut-off point to define plaques with high lipid contents should be 14[19]. Other authors have suggested a much higher cut-off point (74, for example) to separate plaques into echogenic and echolucent[20].

An automatic and objective classification of plaques may simplify the evaluation and identification of different types of content, such as those in the necrotic core, the fibrous cap, and the areas of hemorrhage. For that purpose, a computer program is under development to identify and measure calcium, lipids, fibromuscular tissue and blood in the carotid atherosclerotic plaque visualized on ultrasound scans, according to the brightness of image pixels and the classification defined by Lal et al.[20] (Figure 1).

This initial pilot study compared findings of the computer analyses of in vivo ultrasound examinations with those of conventional histology of the same plaque collected by endarterectomy.

METHOD

This study was approved by the Ethics and Research Committee of the institution where it was conducted, under number 490/2010, according to the norms of the Brazilian Ministry of Health (National Health Council, Resolution no. 196 of 10/10/1996, which regulates Research with Human Beings, published in the Brazilian National Gazette, 1996 Oct 16, no. 201, section 1:21082-21085).

Twelve patients received information about the study and were invited to participate. Patient characteristics are shown in Table 1. After signing an informed consent term, patients underwent B-mode ultrasound scanning of the carotid artery (in vivo ultrasound scanning) in the week before surgery. All examinations were conducted by the same operator using the same ultrasound scanner (Sonoline G40 and VF 10-5 MHz transducer, Siemens Ltd., Munich, Germany). Longitudinal views of the point of greatest luminal narrowing due to plaque were recorded in JPEG files.

After that, patients underwent endarterectomy of the carotid bifurcation. All surgeries were performed by the same surgical team and using the same technical parameters (general anesthesia, longitudinal cervicotomy and endarterectomy using the partial eversion technique, as previously

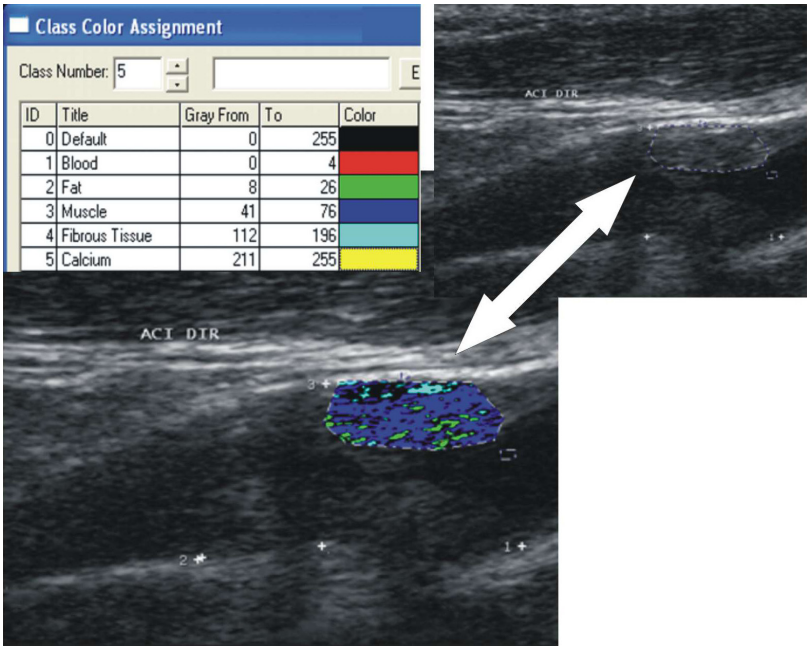

Figure 1. Images illustrating classification of a B-mode ultrasound image according to pixel brightness and following recommendations made by Lal et al.[20].

Table 1. Epidemiological data about the patients included in the study.

Patient	Sex	Age	Symptoms	Heart disease	Respiratory disease	Renal disease	Diabetes	Hypertension	Smoking
1	M	72	YES	X	X	X	X	X	X
2	M	66	YES	NO	NO	NO	NO	NO	ex-smoker
3	M	71	YES	NO	NO	NO	NO	YES	ex-smoker
4	F	75	YES	YES	NO	NO	NO	YES	ex-smoker
5	M	75	YES	NO	NO	NO	YES	YES	ex-smoker
6	M	81	YES	YES	NO	NO	YES	YES	never
7	M	79	NO	NO	NO	NO	NO	NO	ex-smoker
8	M	82	YES	YES	NO	NO	NO	YES	ex-smoker
9	M	79	NO	NO	NO	NO	YES	NO	ex-smoker
10	M	88	NO	YES	NO	NO	NO	YES	ex-smoker
11	M	65	YES	YES	NO	NO	NO	YES	ex-smoker
12	F	78	NO	NO	NO	NO	NO	YES	never

described[21,22]). Plaques were removed en bloc in the attempt to avoid fragmentation or significant damage to its original structure.

Specimens were prepared for histology and sent to the Pathological Anatomy Laboratory of our institution following usual parameters: they were fixed in formaldehyde, decalcified and embedded in paraffin blocks. Blocks were cross-sectioned to produce 3- to 4-mm-thick slices, which were stained with hematoxylin and eosin and Masson staining.

The slides were examined by two experienced pathologists, blinded to the result of ultrasound exams. Interobserver findings were not compared because each pathologist examined a different group of slides. The site for analysis was defined by visual inspection of the cross-sections, and the section with the greatest plaque volume and largest arterial lumen stenosis was chosen. Lipids, fibromuscular tissue, blood and calcium were analyzed in the tissue samples. After that, areas outlined by the pathologists were entered in the DicomWorks® 1.3.5 (2001) software, and planimetry was used to measure the percentage of each plaque component in relation to total area.

After that, two authors, who had not performed the ultrasound examination, analyzed the ultrasound images using a specific computer program; they visually inspected the images to choose the best view and the point of greatest luminal narrowing and then measured the contents of the atherosclerotic carotid plaque according to the classification described by Lal et al.[20]. The longitudinal ultrasound view was selected using criteria of quality and disease representativeness. On each longitudinal ultrasound image, an area corresponding to the site of the histological section was outlined according to a visual criterion of point of greatest arterial stenosis, made easier by the delimitation of the area using color Doppler flow. Each selected section was analyzed to define its composition of blood, calcium, fat and fibromuscular tissue. GSM was also calculated for each section.

Data were entered in an electronic spreadsheet (Excel, Microsoft 2003) and analyzed statistically using the Pearson correlation coefficient and the degree of agreement between ultrasound and histology findings of plaque composition. Figure 2 shows images of a case included in the study.

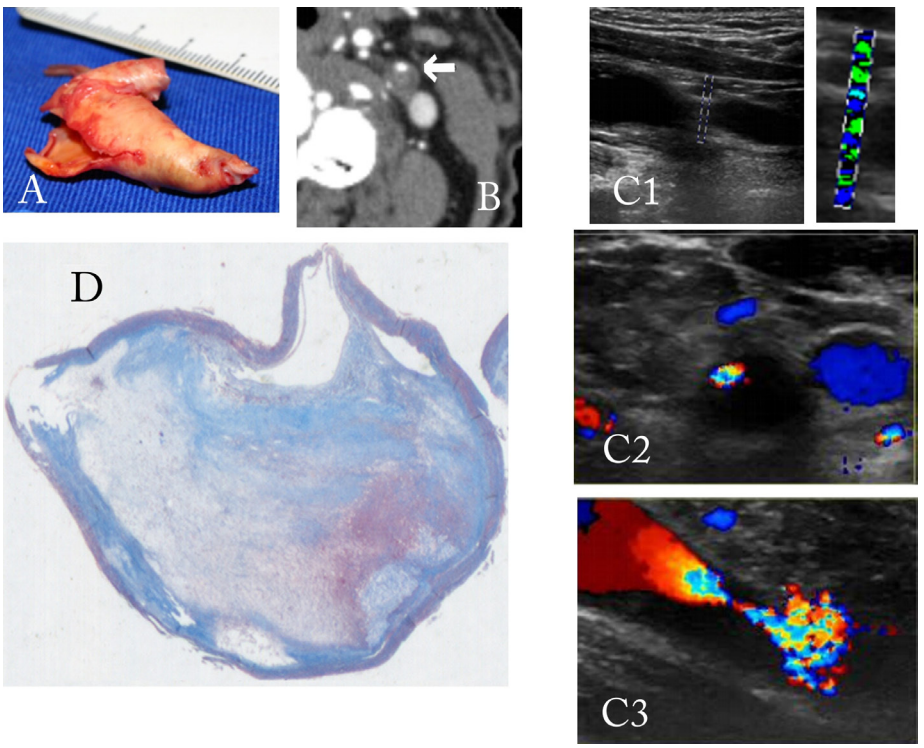

Figure 2. Image of case included in this study A: plaque obtained during endarterectomy and attempt to keep intact area to undergo histological analysis B: angio-CT scan shows degree of stenosis of internal carotid (white arrow) C1: area of maximal lumen stenosis in longitudinal section and area of image selected for analysis using virtual histology C2: same area in cross-sectional view; good correlation of plaque area and histological image C3: detection of area of maximal lumen stenosis using color Doppler.

RESULTS

Figure 3 shows an example of computer-assisted analysis of an ultrasound image of the carotid bifurcation. After standardizing gain (zero was assigned to blood and 200 for arterial adventitia), an area was outlined and the software calculated GSM and the color classification for each point within the image, according to the classification suggested in the study. Table 2 shows the percentages of each component of the atheroma according to the computer analysis and to histology. The correlations of fat and fibromuscular tissue were significant: R=0.82 and R=0.9. In contrast, calcium and blood had no significant correlations (R=0.04 and R=0.19). GSM values ranged from 20 to 69 and were in agreement with plaque characteristics in both USVH and conventional histology, as the plaques with a low GSM were classified as more lipidic. Mean GSM of asymptomatic patients was 52.3, and of symptomatic patients, 33.9 (p=0.122); these values are in agreement with those reported in other studies, in which asymptomatic patients had higher GSM values, although not statistically significant because of small sample sizes.

DISCUSSION

This pilot study found a good correlation between the analysis of echogenicity (USVH) and conventional histology, which demonstrates the potential of VHUS as a tool for the in vivo study of the composition of atheromas[20].

Almost all studies about USVH have been conducted using intravascular ultrasound[20,23], and their results showed a high correlation between ultrasound images and the components of atheromas. The advantages of studies using conventional duplex ultrasound are its low cost and the fact that it is noninvasive and may be repeated in follow-up studies with a population cohort[24], to evaluate both the drug interventions and the effects of changes in lifestyle. As the examination may be performed before a surgery, it may potentially support clinical decisions.

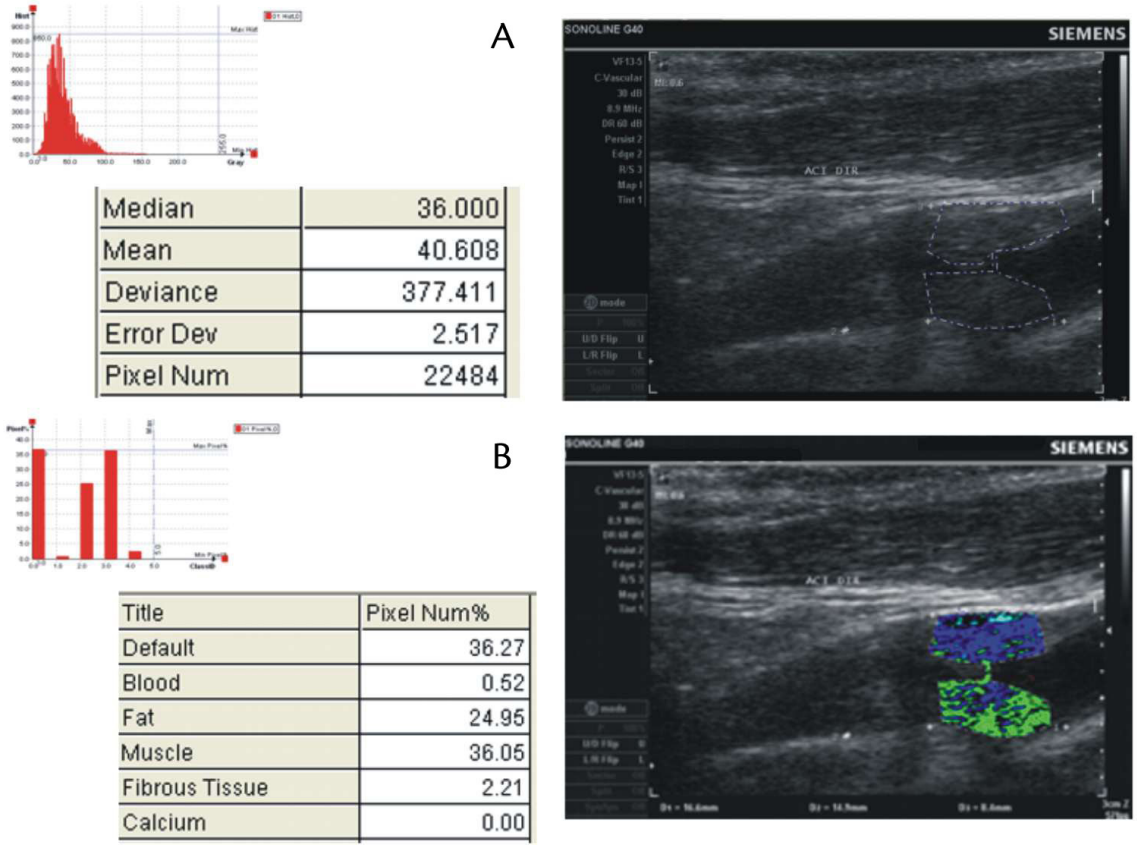

Figure 3. A: Calculation of grey scale median (GSM) in area of atheroma; B: classification of pixel brightness on same image, according to classification recommended by Lal et al.[20]; software provides percentage of each type of tissue to be found on image.

Table 2. Atheroma content (%) according to histological (H) test and the computer-assisted ultrasound (US) exam.

Plaque	Element under study								GSM
	Blood		Calcium		Fibromuscular tissue		Lipid		
	H	US	H	US	H	US	H	US	
1	0	0	12.5	1.6	75.0	82.0	12.5	16.3	56
2	0	20.3	16.7	0.2	25.0	36.0	58.3	43.5	25
3	7.7	6.10	15.4	0	15.4	15.1	61.5	78.8	20
4	22.6	8.12	6.5	0.2	29.0	44.2	41.9	47.5	34
5	20.7	10.4	10.3	0	6.9	30.2	62.1	59.4	24
6	0	0.26	0	0.8	89.9	77.6	10.1	2 21.4	44
7	0	0	0	0	56.7	50.5	43.3	49.0	34
8	0	0	2	0	61.9	51.8	36.1	48.2	37
9	0	0	0	0	78.1	99.9	21.9	0.1	65
10	0	19.7	28.9	0	59.3	51.7	11.8	28.6	41
11	0	8.2	0	0	30.1	42.9	69.9	48.9	31
12	0	0	5.19	0	90.3	95.5	4.5	4.5	69
Pearson's coefficient	**0.192737**		**0.046033**		**0.907354**		**0.828443**		

GSM: grey scale median of ultrasound image. The bottom line shows the correlation between histological findings and the computer-assisted evaluation (Pearson's coefficient).

Knowing what the lipid content of the atheroma is, as well as the location of the lipid core and the thickness of the fibrous cap, may help to make decisions about the best treatment for asymptomatic patients with stenosis of 70% to 80% of the arterial lumen[25], which is one of the topics of debate when discussing current surgical indications. It may also support decisions about which technique (open surgery or angioplasty) to use when treating patients with critical stenosis, as lesions with a higher degree of stenosis and atheromas with a higher lipid content have a greater potential to result in cerebral embolization during angioplasty, even when using cerebral protective devices, such as filters, which require that the lesion be crossed before release. In such cases, the recommendation is to use endarterectomy or cerebral protective devices with proximal blockage or flow reversion through the internal carotid artery[26]. Another potential use of in vivo plaque histology is the development of cardiovascular risk tables, which may be used in the same way as the intima-media thickness, specifically for the risk of stroke[25].

One of the limitations of this study was the fact that the classification developed by Lal et al.[20] does not evaluate all the histological structures, and according to the software, there are grey scale "default" intervals that are not assigned to any structures. Most plaques had up to 50% of unclassified material. Histology found a large amount of amorphous material (proteoglycans and fibrin), which may justify those USVH findings. It is important to keep in mind that this amorphous material should be classified and included in future studies.

Another limitation was the fact that histological analyses were conducted using plaque cross-sections of a micrometric thickness; therefore, a full evaluation of the plaque components is not possible, and only a "picture" of it at a certain point can be obtained. We tried to mitigate this limitation by choosing the point with the best correspondence with the histological section on the ultrasound image. If the entire plaque were analyzed on the longitudinal image, or even on the cross-sectional view of another point in the plaque, results would not have a similarly positive correlation. Therefore, questions are raised about whether histology should remain the criterion standard, or whether the ideal investigation should use the three-dimensional volume, as in the three-dimensional reconstruction of imaging studies (CT scans).

Currently, an important discussion seems to be which method will be more common in the future. Math values calculated using data about echogenicity of each plaque pixel, such as the grey scale median (GSM), is the technique most often used currently. Other math parameters, such as sample heterogeneity, pixel distribution along a line and the standard deviation of values distributed in a histogram, may also be objectively correlated with clinical findings and provide information about the prognosis of patients with carotid disease[19,27,28,29].

This study also found a possible correspondence between GSM and fat content (Table 2), as plaques

with lower GSMs were richer in lipids (less echogenic), which suggests that the histological analysis was actually correct and had the potential to provide more information than the GSM alone. Further studies should be conducted to evaluate the clinical values of USVH in comparison with GSM.

The colorization of the atheroma components, shown in the grey scale B-mode image, may potentially provide a better illustration of the intraplaque disease for the attending physician, whether clinician or surgeon, than the tests currently used, and might facilitate the detection of the lipidic or necrotic core, the thickness of the fibrous cap and the areas of hemorrhage. Advances in computer software have also provided opportunities for three-dimensional studies of plaques and their volume, and may potentially provide evaluations of the entire plaque and detect areas of greater interest.

One of the important method limitations was the lack of perfect correlation between ultrasound findings and plaque histology, as ultrasound is performed in vivo and the images are recorded in longitudinal sections that may or may not provide a view of the worst segment of the plaque. Moreover, the atheromatous plaques are often broken and fragmented during surgery. Additionally, in vivo images are taken with a pressurized lumen and the ex vivo histological specimen is analyzed without any luminal pressure expanding the vessel endothelium. Also, a large amount of the soft lipid content is eliminated during surgical manipulation. For the ex vivo histological analysis, the plaque should be fixed in formaldehyde, decalcified, fixed in paraffin, stained with dyes that remove lipid contents, and prepared as very thin cross-sections. Because of that, several errors are introduced both in the removal and the preparation of the plaque and in the direction of the section under analysis. Lovett et al.[13] suggested that the histological study of atheromas should be standardized. In this study, attempts were made to minimize these errors by removing the plaque with as minimal rupture as possible and using the image of the histological slide as the standard reference, choosing the area on the ultrasound image that had the same relation between the lumen and the wall, and studying a section of this region instead of the entire plaque. Therefore, the correlation of lipid contents and fibromuscular tissue in histology and USVH analysis was improved.

Both calcium contents and blood in the atheroma are difficult to evaluate because they are eliminated during slide preparation. Therefore, they should be interpreted according to the empty space that they leave in the image. Moreover, calcium produces an acoustic shadow on the US image, which prevents the accurate evaluation of the plaque contents[30].

The possibility of using artificial intelligence to study the atheroma may facilitate the routine activities of attending physicians. Computers may conduct fully automated analyses, such as the detection and analysis of the artery and the atheroma, or physicians may choose the area of interest and leave the analysis for the computer (semi-automated analysis). This method accelerates the performance and interpretation of the exam, and the histological analysis may become a routine part of exams that evaluate the degree of artery stenosis. Currently, virtual histology is conducted using the post-processing of stored images, but specific software may be included in US scanners already available in the market. Another important area for the dissemination of this method is the standardization of the exam itself and its teaching by associations involved in the qualification of vascular ultrasound specialists.

Several other methods to study the content and the activity of the atheroma have been developed, such as CT, PET-CT, MRI and scintigraphy using cell markers[3], but US will definitely have a fundamental role in patient screening because it is easy to use and widely available.

Finally, the computer program used in this study had a few problems and stopped working some times during the studies. Moreover the classification presented here does not apply to all tissues found in an atheromatous plaque. Therefore, there is room for improvement and development of a more user-friendly interface and better discrimination of plaque components. In the medical area, several authors have been working towards the improvement of this technique for the analysis of atheromas, venous thrombi, edema, renal parenchyma and thrombi in the aneurysmal sac to follow up patients after intraluminal corrections of aortic aneurysms[31-33].

CONCLUSIONS

There was a correlation between in vivo noninvasive USVH of the carotid plaque and postoperative histology to detect lipids and fibromuscular tissues; there were no significant correlations in the classification of blood or calcium. A more detailed color scale than the one used in this study, as well as a definition of standards for USVH and histological sections, should be developed for additional studies and for the practical use of

USVH in estimating stroke risk during endovascular procedures and in patient follow-up.

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Critical revision of the article: FHM, TCS, SXSC, SAFS, KM, ASCM
Final approval of the article**: FHM, TCS, SXSC, SAFS, KM, ASCM
Statistical analysis: N/A
Overall responsibility: FHM, TCS
Obtained funding: The author Thiago do Carmo Silveira eceived an undergraduate student scientific research grant from PIBIC/CNPq
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**All authors should have read and approved of the final version of the article submitted to J Vasc Bras.