Relative Value of Normalized Sonographic In Vitro Analysis of Arteriosclerotic Plaques of Internal Carotid Artery

Christian Denzel, MD; Klaus Balzer, MD; Klaus-Michael Müller, MD; Franz Fellner, MD; Claudia Fellner, PhD; Werner Lang, MD

Background and Purpose—A close correlation between B-mode sonography and the histopathology and surface structure of plaque is rarely seen in vivo with high-grade stenoses of the extracranial carotid artery. The goal of this study was to determine whether normalized gray scale analysis and surface analysis of the plaque are capable of characterizing the attributes correctly.

Methods—Optimized B-mode images of 107 carotid endarterectomy specimens were captured, and the gray scale median (GSM) was calculated. The specimens were classified histologically into 3 groups: (1) calcium-rich hard plaques, (2) lipid-rich soft plaques, and (3) combined plaques. The surfaces of the plaques were classified as smooth or rough on the basis of standardized reference samples. The endoluminal surface was digitally documented in vitro by videoendoscopy and again classified into the same categories. All stages of the investigation were performed by 2 observers at 2 different times.

Results—Evaluation of the GSM showed close interobserver and intraobserver correlation ($P<0.01$, $R>0.8$). However, there was only 46% agreement between the GSM and the histopathological findings. In both in vitro angioscopy ($k=0.936$, $P<0.001$) and sonographic evaluation ($k=0.842$, $P<0.001$), there was a high correlation between the observers with regard to the evaluable specimens. In 73%, agreement was observed between the sonographic image and angioscopy.

Conclusions—Normalized gray scale analysis and evaluation of the plaque surface in an in vitro study make possible observer-independent evaluation. The composition of the plaque cannot be visualized with sufficient accuracy by sonographic GSM analysis. This also applies to the correlation between sonography and actual surface composition of the plaque. (Stroke. 2003;34:1901-1906.)

Key Words: carotid stenosis $\square$ pathology $\square$ ultrasonography

Randomized controlled clinical studies have illustrated the benefits of carotid endarterectomy (CEA) in the prevention of stroke. In these studies the degree of stenosis was the sole selection criterion.$^4$-$^8$ In addition to degree of stenosis, surface attributes as well as plaque morphology are seen as potential criteria for stratifying patients into different risk level categories.$^7$ The association between lipid-rich plaques with ulcerated surfaces and the increased danger of embolization has been described previously.$^8$ One noninvasive technique for diagnosing plaque composition and surface characteristics is sonography. Together with computer-aided calculation of the gray scale median (GSM), a method that showed a high degree of observer-independent comparability has been found.$^7$ Nevertheless, a correlation between sonographic findings and the histopathological report has rarely been possible in vivo.$^9$-$^{10}$

The objective of this study was to investigate the reproducibility of B-mode sonography in an artifact-free, in vitro model. A second goal was to determine whether this standardized technique shows a correlation between computer-aided GSM analysis of the CEA specimen and a comparable histological classification. Finally, comparisons of a normalized B-mode image with findings of in vitro angioscopy sought to show whether the surface attributes of plaque in the internal carotid artery can be displayed on sonography.

Subjects and Methods
We included in the investigation 107 consecutive plaques from the internal carotid artery (74 men, 33 women; mean age, 69 years) with a degree of stenosis $>70%$ according to North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria that were harvested by eversion endarterectomy, taking care not to cause any artificial lesions.$^1$ Specimens that could not be excised en bloc were
not evaluated. The specimens were preserved in a 10% formalin solution for further examination. The patients’ clinical stages were recorded but not evaluated because the sole purpose of this study was to investigate correct visualization of the plaque on sonography.

The apparatus settings, calibration of the B-mode, and course of the investigation were standardized as follows.

**Sonographic Scan Settings**
To evaluate morphology and surface attributes, the plaques were scanned with a 10-MHz linear probe in the best possible longitudinal projection (Logic 500, General Electric Company). The scanner was set at a medium frame rate, with time gain compensation vertical to blood vessel, linear postprocessing curve, minimal persistence, and maximum dynamic range (in this material 60 dB). The plaques, fixed in formalin, were transferred to a special chamber that was filled with a 0.9% saline solution and scanned at a distance of 1.5 cm. The B-scan images were stored on a magnetic optical disk and analyzed with a graphics program (Adobe Photoshop D1-6.0, Adobe Systems Incorporated).

**Standardization of B-Scan Images and Calibration**
Because, in contrast to in vivo investigations on CEA specimens, no adventitia is present for calibrating the gray scale analysis, a pilot phase was conducted to determine the linear gray scale adjustment for calcium with the use of pieces of chalk (Figure 1; GSM for NaCl solution, 0 to 5; GSM for calcium, 250). In these normalized images, the circumferance of the plaque was then captured, and the GSM was calculated. This describes the percentage of the pixels and serves as a measure of the echogenicity of the entire plaque (Figure 2).

**Course of Investigation**

**Sonographic Imaging of Plaque Morphology**
To show that echogenicity is not altered by fixation in formalin, a pilot phase was conducted in which 16 cadaverous internal carotid artery specimens were taken and immersed in physiological saline, and the GSM was calculated for the adventitia in the B-mode image. The specimens were then fixed in a formalin solution, and, after 3 days of incubation, the GSM was again calculated for the adventitia.

**Normalized GSM**
The GSM was calculated with the use of a graphics program (Adobe Photoshop 6.0, Adobe Systems Incorporated).

To compare the GSM with the histological findings, the GSM was divided into 3 categories on the basis of the first (GSM=74) and third (GSM=93) quartiles of all the specimens investigated.

**Plaque Surface in Sonography**
To determine the plaque surface, a B-mode image of the CEA specimen was taken in longitudinal projection. These frames were then assessed to judge whether or not the plaque surface was evaluable. The surfaces were then classified as smooth or rough with the use of a series of standardized reference specimens. Plaques whose surfaces showed no signs of ulceration or fissures were classified as smooth. All the other plaques were classified as rough.

**Imaging Plaque Morphology by In Vitro Angioscopy**
The endoluminal surfaces of the specimens fixed in formalin were viewed in vitro with a 2.7-mm videendoscope (HOPKINS straight forward telescope, 30°, KARL STORZ GmbH & Co KG) and digitized. Surfaces totally free of any ulceration were classified as smooth. All other specimens with irregular structures were classified as ulcerous. The specimens were classified with the use of a series of standardized reference specimens. In vitro angioscopy was considered the reference method for evaluating the surface.11

With the exception of histology, all stages of the investigation were assessed by 2 observers (C.D., W.L.). The assessments were performed in random order and repeated after an interval of 4 weeks. In case of discrepancies between the observers, a majority decision was made by a third observer. All the observers were blinded in terms of the patients’ clinical data and the results of the histological findings.

**Plaque Morphology and Histological Examination**
The specimens fixed in formalin were examined by light microscopy (hematoxylin-eosin and elastic van Gieson staining). All the sections were examined for the presence of atheromas, necrotic core, hemorrhage, fibrosis, and calcification. For the evaluation, the classification was reduced to 3 groups: (1) calcium-rich fibrous hard plaques, (2) combined plaques, and (3) lipid-rich soft plaques.12

**Statistical Analysis**
Statistical analysis was performed with the use of a Microsoft Excel database and SPSS for Windows (version 10.0.5, SPSS Inc).
nonparametric Spearman rank order test was applied to calculate the distribution of the GSMs between the various observers. Cohen’s $\kappa$ test was applied to compare the different investigation techniques with the same categories between lines and columns. The lowest level of significance was set at $P=0.05$.

**Results**

**Comparison of Adventitia Before and After Fixation in Formalin**

The adventitia of 16 freshly harvested internal carotid artery specimens in physiological saline had a mean GSM of 70 (range, 40 to 106; SD ±21). After fixation in formalin, the mean GSM was 77 (range, 51 to 113; SD ±19). Comparisons of the individual specimens before and after fixation in formalin showed no significant difference for the GSM ($P<0.01$, $R=0.794$).

**Interobserver and Intraobserver Correlation**

The results for the GSMs of the different observers and different times showed highly significant agreement ($P<0.01$). The correlation coefficients ($\kappa$) were $>0.87$ in all cases (observer C.D./observer W.L. at time 1, $\kappa=0.906$; C.D./W.L. at time 2, $\kappa=0.878$; C.D. between times 1 and 2, $\kappa=0.927$; W.L. between times 1 and 2, $\kappa=0.957$ (Figure 3).

**Histological Plaque Analysis**

The mean GSM for all plaques was 75 (range, 18 to 131; SD ±19). Nineteen of the 28 plaques with a GSM $<65$ were shown by histopathology to be soft plaques. The 53 GSM figures between 65 and 87 were confirmed as combined plaque in 26 cases. Of the 26 GSM figures $>87$, histology confirmed that 5 were cases of hard plaque.

Overall, the results agreed with those of histopathology in 50 of 107 cases (46.7%) ($\kappa=0.168$, $P=0.013$; Figure 4).

**Surface Structure**

**Angioscopy**

One hundred four of the 107 specimens examined by in vitro angioscopy were evaluated as the same by the 2 observers (97.2%). Observer 1 classified 71 plaques (66.4%) as smooth and 36 (33.6%) as rough. Observer 2 saw a smooth surface in...
74 (69.1%) of the plaques and a rough surface in 33 (Table 1). Both observers made a statistically equivalent assessment of the plaque surface (\(\kappa=0.936, P<0.001\)).

**Sonography**

The plaque surfaces of 67 (62.6%) of the specimens were evaluable from the B-mode images. Forty specimens (37.3%) were classified as not evaluable by one or both observers, mainly because of a high degree of stenosis or segmental acoustic shadows due to calcification.

Observer C.D. considered 42 (62.6%) of the evaluable plaque surfaces as smooth and 25 (37.3%) as rough. Observer W.L. saw a smooth surface in 41 specimens (61.2%) and a rough surface in 26 (38.8%).

The observers agreed on 62 specimens (92.5%). Five surfaces (7.5%) were classified differently on the basis of their B-mode images (Table 2).

Overall there was a highly significant correlation between the observers (\(\kappa=0.842, P<0.001\)).

**Comparison Between Surface Sonography and In Vitro Angioscopy**

A comparison between the sonographic findings and in vitro angioscopy was possible for 67 (62.6%) of the 107 specimens (Figure 5). In this instance, 49 (73.1%) were in agreement, and in 18 cases (26.8%) there were differences between sonographic findings and in vitro angioscopy (\(\kappa=0.421, P<0.001\); Table 3).

**Discussion**

**GSM Analysis**

In addition to estimation of the degree of stenosis, reliable in vivo analysis of the internal structure and the surface attributes of plaques in the internal carotid artery is becoming increasingly important. Hypoechoic carotid plaques present a higher risk of ipsilateral strokes than hyperechoic plaques. Duplex sonography is essentially suitable for assessing the degree of stenosis and for visualizing the echogenicity and the surface attributes of the plaque. However, endeavors to evaluate the structure of the plaque by means of different categories of echogenicity have failed because of the inadequate intraobserver and interobserver correlation.

Normalized computer-aided gray scale analysis considerably improved sonographic plaque analysis. Before gray scale analysis can be performed, the digitized data must be transferred to a graphics program; processed printouts of scanned images are inadequate. Although digitization compensates for the observer-dependent errors in B-mode analysis, it does not provide sufficient correlation with the histopathological findings. Thus, a number of studies have been conducted to investigate the relative value of normalized gray scale analysis. Therefore, plaque analysis was performed in vitro in this study to discover whether the variability described is attributable to the special investigation conditions in vivo and hence does not occur under standardized conditions in vitro.

In addition, a number of parameters were further optimized compared with previous investigations, as follows. (1) The surgical eversion endarterectomy technique guarantees artifact-free specimens. (2) In vitro patient-independent sonography has been further standardized (eg, to rule out movement artifacts caused by movement of the vessel wall or the patient or scans near the probe in an optimal coupling medium). (3) Normalization and calibration of the gray scale analysis are ensured by a uniform apparatus setup and assessment for all plaques (eg, standardized fluids and standardized calcium block for determining the correction factor). In contrast to this, each in vivo investigation is calibrated

**Table 1. Classification of the Plaque Surface as Smooth or Rough at In Vitro Angioscopy by 2 Independent Observers**

<table>
<thead>
<tr>
<th>CD</th>
<th>Smooth</th>
<th>Rough</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>WL 2 smooth</td>
<td>71</td>
<td></td>
<td>71</td>
</tr>
<tr>
<td>WL 2 rough</td>
<td>3</td>
<td>33</td>
<td>36</td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>33</td>
<td>107</td>
</tr>
</tbody>
</table>

\(\kappa=0.936; P<0.001\).

**Table 2. Classification of the Plaque Surface as Smooth or Rough in B-Mode Images by 2 Independent Observers**

<table>
<thead>
<tr>
<th>WL</th>
<th>Smooth</th>
<th>Rough</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD smooth</td>
<td>39</td>
<td>3</td>
<td>42</td>
</tr>
<tr>
<td>CD rough</td>
<td>2</td>
<td>23</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>26</td>
<td>67</td>
</tr>
</tbody>
</table>

\(\kappa=0.842; P<0.001\).

**Figure 5. Images of endoluminal plaque surfaces on sonography, in vitro angioscopy, and histopathology. Series A originates from a patient with a smooth plaque surface; series B, from a patient with a rough plaque surface.**

**Table 3. Comparison of the Examination Methods in In Vitro Angioscopy and Sonographic Visualization of Plaque in B-Mode Images in Respect of the Endoluminal Surface Attributes**

<table>
<thead>
<tr>
<th>Sonography</th>
<th>Smooth</th>
<th>Rough</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angioscopy smooth</td>
<td>34</td>
<td>12</td>
<td>46</td>
</tr>
<tr>
<td>Angioscopy rough</td>
<td>6</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>27</td>
<td>67</td>
</tr>
</tbody>
</table>

\(\kappa=0.421; P<0.001\).
individually. (4) Macroscopic evaluation with an angioscope as the reference standard for the surface analysis is included.

The only way in which these conventional conventional B-mode images could be improved in vitro would be to use a multiangle compound imaging method, with reduced angle dependence, reduced random variation (speckle), and improved delineation of the plaque outline.22

Because the present investigation was conducted to illustrate the connections between sonographic diagnostics and histopathology together with macroscopic surface analysis, a conscious decision was made not to analyze the patient data. The histological evaluation was restricted to the 3 main categories: soft plaque, combined plaque, and hard plaque. Although more parameters were recorded (eg, presence of atheroma, necrotic core, hemorrhage, fibrosis), these were excluded from the evaluation to simplify the categories. Normalized GSM analysis also simplifies plaque analysis by reducing all the sonographic data to a mean GSM figure. Individual regions of interest (eg, a histologically confirmed necrotic core) are not visualized but are indicated by a change in the mean GSM figures.19,23

Similar to the results of other studies,19,24,25 the present investigation shows that GSM analyses are outstandingly reproducible. However, there was no correlation between the GSM data and classified histopathologies of the plaques. The reason for this is that the GSM analysis figures represent the average for the whole arteriosclerotic area. No percentage figure can be given for the lipid or calcium fractions. Results published by Tegos et al26 show that GSM analysis is not capable of differentiating between calcified and uncalcified plaques. Echomorphology can determine neither the lipid nor the calcium fraction; only hemorrhages are identifiable. Symptomatic hypoechoic plaques in the internal carotid artery exhibit hemorrhage more often,13,21 which, in our opinion, explains the relatively close correlation between the low GSM figures for echolucent plaques and clinical symptoms. In previous symptomatic patients, hypoechoic carotid plaque predicted a 5.1-fold higher risk of ipsilateral ischemic strokes, while a hypoechoic plaque with 80% to 99% stenosis presented an 8-fold risk compared with a hyperechoic plaque with 50% to 79% stenosis. This is equivalent to an absolute risk increase of 28%.15 Since it has been shown that, apart from hemorrhage, the lipid fraction has a major influence on the echogenicity of plaque1 but that GSM cannot determine either the calcium fraction or the echomorphology,21 the relative value of GSM analysis is limited. The present study also shows that the correlation is unsatisfactory, even under ideal sonography conditions and even if histopathology is restricted to 3 groups. To date, all the investigations have used only representative sections through the plaques and have not considered the overall distribution in a volume model. Studies with 3-dimensional sonography are promising in this respect.27 The value of GSM analysis may also be increased by analyzing the pixel distribution over certain regions of interest within the plaque.23 Thus, further studies must be conducted to investigate whether, under less ideal in vivo sonography conditions as well, these detailed analyses are possible and whether this might also be successful with

plaques with a high percentage of calcium with acoustic shadowing.

Plaque Surface
Ulcereated plaques in high-grade stenosis present a significantly higher risk of ipsilateral stroke.17,27,28 The significance of ulcers in isolation remains the subject of debate because other investigators were only able to establish connections between clinical symptoms and plaque ulceration for stenoses of <50%,29 and >20% of the sonographically diagnosed lesions showed changes over time, predominantly as regression to a smooth surface.27 However, evaluation of the plaque surface is included in the risk evaluation.4,30 With the help of sonography, plaque surfaces can be characterized and ulceration recognized.14,27 However, it has been shown that the reliability of evaluations of ulceration declines as the degree of stenosis rises.31 In the cohort of stenoses >70% discussed here, there was very close agreement between the observers on the endoluminal surface analysis of the evaluable specimens. Sonography was capable of reliably visualizing the attributes of the plaque surface among the evaluable findings. By virtue of direct visualization of the endoluminal surface, in vitro angiography is an ideal reference technique for sonography. In addition to assessing the internal structure of the plaque, B-mode sonography of its surface can contribute toward risk assessment of evaluable findings. However, it must be assumed that in vivo examinations will provide a lower degree of evalability. Improved sonographic surface diagnostics would be possible with intravascular ultrasonography or 3-dimensional sonography. Experience has already been gained with in vivo intravascular ultrasonography of the coronary arteries.32 On account of the possible iatrogenic embolization of plaque material during the investigation, intravascular ultrasonography of the internal carotid artery will only be indicated for in vivo investigations during or after percutaneous angioplasty and/or stenting. The results of 3-dimensional sonography are promising despite specific limitations such as swallowing artifacts and vessel kinking. However, definite evidence of treatment optimization by 3-dimensional sonography in asymptomatic patients will be unlikely in view of the 1200 to 1400 patients needed in each study arm.27

In conclusion, normalized image analysis of carotid plaques shows close interobserver and intraobserver correlation. However, in vitro no correlation was seen between GSM and histology or between the sonographic surface analysis and macroscopic evaluation of the surface structure. The discrepancy between the results of GSM and the histology findings shows that the GSM value does not reflect all aspects of plaque histology. If we want to continue using sonography for visualizing plaque morphology, then specific areas of the plaque will have to examined more closely.

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References
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