In vivo noninvasive identification of cell composition of intimal lesions: A combined approach with ultrasonography and immunocytochemistry

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Purpose: **We investigated whether differences in cellular composition of the shoulder region of carotid plaque, a cell-rich, debris-free area, can be revealed with computer-driven analysis of ultrasound scans.**

Methods: **In 26 patients referred for carotid endarterectomy, the shoulder region of plaque eligible for surgical removal was identified with ultrasound scanning. Digital images were obtained and evaluated with a specially developed computer-driven system (Medical Image Processing [MIP]). The gray level distribution of the region of interest (ROI), along with some statistical parameters exploring the spatial distribution of pixels, such as entropy and second angular moment, were analyzed. In the specimen retrieved at surgery, the area corresponding to the ROI was selected. Cryosections were tested at immunocytochemistry with monoclonal antibodies specific to smooth muscle cells (SMCs), macrophages), and lymphocytes. Computerized image analysis was performed to quantify each cellular component of the lesion.**

Results: **Mean gray levels were related positively to the content of SMCs ($r = 0.576$, $P = .002$) and negatively to the content of macrophages ($r = -0.555$, $P = .003$). Lymphocytes did not show any correlation. Prevalence of SMCs, expressed as the ratio SMC/(SMC + macrophages), was related positively with entropy ($r = 0.517$, $P = .007$) and negatively with the second angular moment ($r = -0.422$, $P = .032$). The quartiles of gray level were useful for detecting significant differences in terms of cellular composition.**

Conclusions: **Some cellular features of the shoulder region of plaque are associated with specific videodensitometric patterns evaluated with MIP. This approach enables in vivo noninvasive prediction and monitoring of cell composition of the shoulder region, and could be extended to study of the thickened intima. (J Vasc Surg 2003;38:1390-5.)**

High-resolution, B-mode ultrasound scanning coupled with color Doppler scanning is largely used as a safe, reproducible, noninvasive tool to evaluate the extent of carotid atherosclerosis.[1-3] In brief, this approach provides information about degree of lumen narrowing, the size of plaque or intima as expressed by intima-media thickness measurements, and the relative echogenic properties of the entire plaque or its parts, so that overt atherosclerotic lesions (roughly, type III through type VI, according to the American Heart Association classification) can be characterized or classified with ultrasound criteria such as echolucency and echogenicity.[1,4-6] However, initial lesions, such as diffuse intimal thickening, currently are evaluated with assessment of intima-media thickness only. In particular, it is not known whether type IIa lesions, prone to progress to advanced stages of atheroma, differ from nonprogressive type IIb lesions,[7] not only in terms of cell composition but also insofar as echographic parameters are concerned.

The search for echographic features that reflect the cellular composition of initial lesions might help in differentiating evolutive from nonevolutive lesions. In addition, this approach might allow in vivo monitoring of potential changes in cellular composition of lesions as a result of pharmacologic intervention.

Not only "visual"[1,4] but also "objective" ultrasound-based analysis of fully developed atherosclerotic lesions has been previously described.[5,8-12] However, all of these methods were tailored to investigation of the entire advanced lesion. We focused on the plaque shoulder, which is characterized by a relatively scarce extracellular component and almost no debris.[13] This region therefore mimics the structure of initial atherosclerotic lesions. Ultrasound recordings were analyzed with multipotent software specifically developed for videodensitometry studies, that is, the Medical Imaging Processing (MIP) system (Institute of Clinical Physiology, CNR, Pisa, Italy), which yields both

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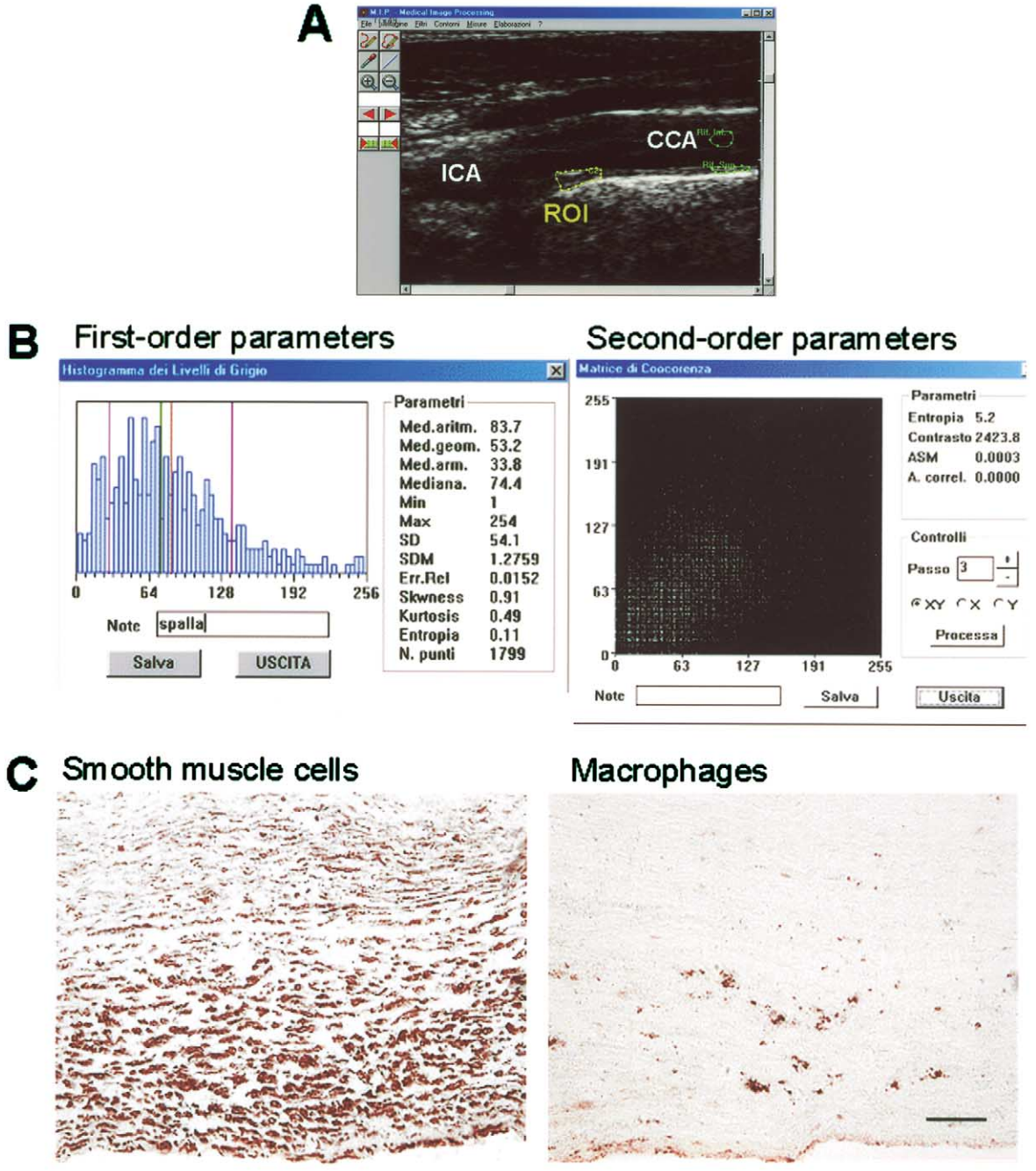

Relationship between echographic parameters and cell composition in a representative case. **A,** Echographic image of carotid artery processed for Medical Image Processing (MIP) protocol. Shoulder region of atherosclerotic lesion (thickness up to 2 mm) was chosen as the region of interest *(ROI)* and recorded in longitudinal projection. For calibration, vessel lumen (blood) was taken as blank to ROI. An adjacent portion of adventitia was considered reference tissue. *CCA,* Common carotid artery; *ICA,* internal carotid artery. **B,** MIP was used to analyze ROI by both first-order (eg, mean, SD) and second-order (co-occurrence matrix: entropy, contrast, second angular moment) parameters obtained from 256-degree gray scale. **C,** Seriate cryosections from endarterectomy specimen, which correspond to ROI (see text for details). Immunocytochemistry was performed with monoclonal antibodies SM-E7 (specific to smooth muscle cells) and HAM 56 (specific to macrophages). *Scale bar* = 50 μm.

first-order and second-order statistical parameters from the selected region of interest (ROI).[14,15]

We report that in vivo noninvasive identification of the cell composition of the plaque shoulder is feasible with routine ultrasound equipment, enabling analysis of initial atherosclerotic lesions.

METHODS

We organized a multicenter study* with third-generation ultrasound equipment from four different firms, all based on the Hewlett-Packard standard. To properly evaluate the ultrasound recordings and the corresponding endarterectomy specimens, we established specific protocols, as described below.

The study was approved by the local ethics committee. All patients gave informed consent.

Ultrasound scan acquisition and analysis protocol. Preliminary training of sonographers was carried out in the referral center at the University of Padua. Before surgery, each patient underwent ultrasound scanning of the supra-aortic trunk, recorded on half-inch super-VHS videotape with a standard acquisition protocol. Carotid arteries were evaluated with dedicated high-frequency linear probes (10 MHz; lateral resolution, 1.0-0.5 mm). In B-mode, gain level and focus were set for identification of the walls at far-wall level, and remained constant during recording. Image depth was kept constant for all recordings, and smoothing was not used. The shoulder region of the atherosclerotic plaque was chosen and recorded in longitudinal projection. The initial portion of the shoulder, no more than 5 mm, and increase in intima-media thickness to a maximum of 2 mm was considered the ROI. Intima-media thickness was defined as the distance between the lumen-intima and the media-adventitia interfaces, measured at end-diastole. Then, maintaining the ROI of the plaque in the focus of the echographic image, rotation toward the transverse projection was done. Intima-media thickness of the ROI overlapped the one previously recorded in the longitudinal projection. Gain and focus were kept stable throughout the recording procedure. Presence of shadows due to calcification was a criterion of exclusion from the study.

All recordings were evaluated by the same reader (M.P.) using a high-resolution video recorder (Panasonic AG-MD830) coupled with the computer-driven image analysis system (MIP). One optimal image from the ROI in longitudinal projection and one optimal image in transverse projection were selected. The images were digitized with resolution of 576 × 768 pixels, and 256-degree gray scale per pixel, for the study with a frame grabber and maintaining resolution of the original image stored in the super-VHS (Movie Machine II for Windows; Microsoft, Redmond, Wash). This yields the best resolution for densitometric analysis. MIP was used to analyze the ROI by both first-order (mean, SD) and second-order (co-occurrence matrix: entropy, contrast, second angular moment) statistical parameters obtained from digitized images. Insofar as algorithms to obtain second-order parameters are concerned, the gray level co-occurrence matrix can be defined as $P(d, a)$; thus Pij is the relative number of occurrences of a gray level pair (i, j) within a pixel distance d and along an angle a. Co-occurrence matrices for n distances ($d = 3$) and n angles ($a = 0$, 45, 90, and 135 degrees) were constructed. In particular, the one distance that maximizes the statistical measures computed from co-occurrence matrix was used. In general, entropy increases as tissue homogeneity decreases, and contrast increases when large gray level difference occurs among pixels. Increase in either parameter indicates plaque heterogeneity. On the other hand, the second angular moment is related negatively with entropy and hence with plaque heterogeneity.[14,15] A standard method of B-mode imaging was used: the gray scale value of the ROI was adjusted linearly so that the median value of blood was 0 and that of adventitia was 160. These two calibration steps were introduced into the videodensitometric analysis of each sample also, to adjust for variable ultrasound attenuation and different machine gain settings in different subjects.

To establish intraobserver variability of MIP measurements, analysis of a group of 16 randomly chosen scans was repeated after a few weeks. A new ROI was selected in the same arterial segment previously evaluated. Variability was measured as mean bias of repeated measurements (with 95% confidence interval [CI]), and coefficient of variation.

Preparation of endarterectomy specimens. Samples were placed in OCT (Tissue Tek OCT; Miles Inc, Elkhart, Ind), frozen in liquid nitrogen, and stored at −80°C until

Table I. Relationship between gray level (mean of longitudinal and transverse projections) and cellular composition

A. Univariate correlation

	r	P	*n*
SMC (content)	0.449	.021	26
Macrophages (content)	−0.415	.035	26
Lymphocytes (content)	−0.025	NS (.915)	20
SMC/(SMC + macrophages)	0.611	.001	26

B. Multiple regression analysis: $R^2 = 0.581$; $P = .009$; $n = 26$

	Coefficient	*Standard error*	P
Constant	41.0	8.910	—
SMC (content)	1.80	0.749	.025
Macrophages (content)	−3.84	1.764	.040

SMC, Smooth muscle cells; *NS,* not significant.

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Table II. Quartiles of gray level and cellular composition

	I (n = 6)	*II (n = 7)*	*III (n = 6)*	*IV (n = 7)*	P*
Gray level (0–255)	<29	29–40	41–59	>59	
SMC	3.79 ± 2.30	4.82 ± 3.71	10.52 ± 3.47	13.22 ± 6.68	.002
Macrophages	4.29 ± 2.85	2.15 ± 2.29	1.88 ± 1.92	2.09 ± 2.22	NS (.253)
SMC/(SMC + macrophages)	0.43 ± 0.22	0.67 ± 0.25	0.84 ± 0.18	0.87 ± 0.14	.003

SMC, Smooth muscle cells; *NS,* not significant.
*Analysis of variance.

use. The shoulder region of the plaque removed at surgery was identified macroscopically and labeled with a color marker after freezing. To ensure that the ROI identified at ultrasound examination overlapped the ROI of the specimen, we assessed the thickness of the lesion microscopically. Starting from the beginning of the shoulder, we made seriate cryosections at 500-μm intervals on a 5-mm-long portion until lesion thickness was less than 2.0 mm. When identification of the area corresponding to the ultrasound ROI was not possible, the case was excluded from the study.

Cryosections were tested at immunocytochemistry as described,[16] with monoclonal antibodies that included SM-E7, which identifies smooth muscle cells (SMCs); anti-macrophage HAM 56; and the anti-lymphocyte CD45RO. In brief, primary antibodies (except for CD45RO) were applied to freshly cut, unfixed cryosections (8 μm), and incubated at 37°C for 30 minutes. After rinsing with phosphate-buffered saline solution, the sections were treated with rabbit anti-mouse immunoglobulin G (IgG) coupled with horseradish peroxidase. Bound IgG was revealed by incubation in amino-ethyl-carbazole solution. Subsequent computerized image analysis was carried out to quantify each cellular component of the ROI, with the Leica Qwin system (Leica, Wetzlar, Germany). Cell composition was analyzed by evaluating the percentage of the areas immunoperoxidase-positive for SM-E7, HAM 56, and CD45RO, which reflects the number of cells present in the ROI.[16] The controls for indirect immunocytochemistry were mouse nonimmune IgG rather than primary antibody, and the secondary antibody alone. For each antibody, three sections per specimen (spaced 500 μm apart) and three standard microscopic fields per section were analyzed. Thus the relationship between echographic parameters and cell composition was studied (Fig).

Statistical analysis. For gray level analysis, the mean value of measurements performed in longitudinal and transverse projections was used. Other parameters evaluated with MIP were calculated only in the longitudinal projection. The univariate correlation was evaluated with the Pearson correlation coefficient with Bonferroni adjustment. Gray level also was subjected to multiple regression analysis, without forcing any item into the equation, obtaining one multiple correlation coefficient *(r)*.

To evaluate changes in cellular parameters of the primary lesions related to the gray level of the ROI, four gray level quartiles were established. Continuous variables were averaged and expressed as mean ± SD. The same procedure was used to evaluate cellular composition changes of the primary lesions, and modification of the parameter was evaluated with MIP. Four quartiles of cellular composition of the primary lesion, expressed as SMC/(SMC + macrophages), were also established. The SYSTAT package (SPSS Inc, Chicago, Ill) was used for this purpose.

Table III. Correlation between parameters of co-occurrence matrix and cellular composition

	r	P
Entropy		
SMC	0.375	NS (.059)
Macrophages	<0.286	NS (.157)
SMC/(SMC + macrophages)	0.642	.002
Contrast		
SMC	0.085	NS (.680)
Macrophages	<0.409	.038
SMC/(SMC + macrophages)	0.476	.014
Second angular moment		
SMC	<0.430	.028
Macrophages	0.050	NS (.810)
SMC/(SMC + macrophages)	<0.562	.003

SMC, Smooth muscle cells.

RESULTS

Seventy-two of 88 ultrasound recordings were considered of good quality for videodensitometric analysis. However, in 46 cases the histologic specimen was not adequate because of tissue fracturing during either surgery or the freezing procedure. Thus the final correlation analysis between cytology and videodensitometry was performed in 26 cases. Specimens used for analysis ranged from fibrous lesions to lipid-laden plaques.

First-order parameters. The mean gray level from longitudinal and transverse projections was related positively to content of SMCs ($r = 0.449$, $P = .021$) and negatively to content of macrophages ($r = -0.415$, $P = .035$; Table I). There was no relationship with content of lymphocytes. Taking into account the small number of these cells and their random distribution, the cellular composition was therefore expressed as SMC/(SMC + macrophages). This parameter was related positively to mean gray level ($r = 0.611$, $P < .001$). At multiple regression analysis, content of SMCs and macrophages in the lesion were determinants of gray level. When the longitudinal or trans-

Table IV. SMC/(SMC plus; macrophages) in endarterectomy tissue, and ultrasound parameters

	I (n = 6)	*II (n = 7)*	*III (n = 6)*	*IV (n = 7)*	P*
SMC/(SMC + macrophages)	<0.48	0.48–0.76	0.77–0.93	>0.93	
Gray level	24.0 ± 9.8	38.7 ± 15.7	50.1 ± 16.0	67.6 ± 29.8	.005
Entropy	5.57 ± 0.96	6.71 ± 0.98	6.87 ± 0.67	7.26 ± 0.96	.020
Second angular moment	125.2 ± 141.4	70.3 ± 150.8	21.2 ± 24.1	15.0 ± 16.4	NS (.243)

SMC, Smooth muscle cells; *NS,* not significant.
*Analysis of variance.

verse projections were considered separately (data not shown), a similar result was obtained.

By dividing the mean gray level yielded by the ROIs into quartiles (Table II), at increasing quartile an increase in SMC component and some trend toward decrease in macrophages was found. SMC/(SMC + macrophages) increased significantly by quartile of gray.

Second-order parameters. Presence of SMCs, and macrophages, as well, was poorly related to parameters of the co-occurrence matrix (Table III). SMC/(SMC + macrophages), however, was related positively with entropy ($r = 0.642$, $P = .002$) and contrast ($r = 0.476$, $P = .014$), and negatively with the second angular moment ($r = -0.562$, $P = .003$).

The quartiles of SMC/(SMC + macrophages) were then considered for further analysis (Table IV). With increasing quartile, a significant increase in gray level and a relationship with entropy, but not with the second angular moment, was observed.

Reproducibility. Mean bias between the two independent determinations of gray level was 1.09 ± 6.90 (95% CI, −2.58-4.77); coefficient of variation was 6.31%. Mean bias between the two independent determinations of second-order parameters was 0.069 ± 0.39 (95% CI, −0.141-0.279) for entropy, 95.8 ± 1141.8 (95% CI, −512.6-704.2) for contrast, and 23.4 ± 107.7 (95% CI, −33.9-80.8) for second angular moment; coefficient of variation was 5.74%, 11.92%, and 4.60%, respectively. Therefore, all parameters but contrast exhibited good reproducibility.

DISCUSSION

In vivo noninvasive identification of the cellular composition of selected cell-rich and debris-free parts of atherosclerotic lesions is feasible with standard ultrasound equipment. As elucidated by the Committee on Vascular Lesions of the American Heart Association, the balance of cellular types present in the initial lesion (types IIa and IIb) is of importance for propensity to evolve into overt atheroma.[7] In particular, progression-prone type IIa lesions are characterized by many layers of SMCs of the collocated adaptive thickening.[7] Use of the shoulder region of plaque as a surrogate end point of our study was a reasonable alternative, because abundant, mixed cellularity is present in this region, with relatively scarce extracellular components.[15] Nevertheless, it seems likely that the ultrasound patterns generated by the ROI stem directly from the type of extracellular tissue, which remains a main component of the intima. The intimal cells responsible for extracellular tissue synthesis (typically, SMCs for collagen and macrophages for lipid accumulation) simply mirror tissue composition. Paradoxically, previous attempts to relate texture analysis with extracellular tissue as evaluated histologically have failed,[11] probably due to loss of structural integrity as a result of the staining procedure or perhaps to specific distribution of staining. In this kind of study, fracturing of the specimen during surgery or the freezing procedure remains a major problem, which can be overcome only in part with specific training. Taking into account the purpose of our study, the availability of optimal specimens, rather than their number, is the key issue. It should also be emphasized that previous studies reported in the literature did not analyze the three main cellular components of intimal lesions.

Analysis of first-order parameters showed that mean gray level was related positively to the content of SMCs and negatively to that of macrophages (Table I). SMCs and macrophages were determinants of gray level at multiple regression analysis, whereas the few, scattered lymphocytes found in the lesions did not have a role. The cellular composition was therefore expressed by SMC/(SMC + macrophages), which reflects the prevalence of SMCs in tissue. This parameter was related positively to mean gray level, and increased significantly by quartile of gray level (Table II). When second-order parameters were analyzed, SMC/(SMC + macrophages) was related positively to entropy and contrast, and negatively to the second angular moment (Table III). Thus, with increasing ratio (ie, increasing prevalence of SMCs), homogeneity of the gray levels within the ROI decreases. When the quartiles of SMC/(SMC + macrophages) were matched with both gray level and second-order parameters, which showed good reproducibility (entropy, second angular moment), a strong relationship was found for gray level only (Table IV). We propose the quartiles of gray level as the method of choice for defining cellular content of the thickened intima, as expressed by SMC/(SMC + macrophages).

In qualitative echographic studies, standardization of B-mode imaging is a demanding issue. In our study, as well as in others,[5,6,9] mean gray level in the ROI was calibrated against blood within the vessel, which permits minimization of the effects of variable gain setting and in the adventitia layer, which normalizes for imaging depth and attenuation. As size of the ROI can to some extent influence degree of heterogeneity, we restricted ROI size to specific

limits. On the basis of reproducibility of repeated measurements, the ROI size chosen for the ultrasound study appears to be adequate. Good reproducibility was found for the gray scale, similar to that reported by Grønholdt et al[6] for histologic analysis of advanced lesions and better than that displayed by second-order parameters. Moreover, texture analysis seems to be better suited to classifying fully developed lesions than small arterial tissue areas such as thickened intima.[11]

Basically, ultrasound tissue characterization can be carried out with three different approaches: visual analysis, backscatter amplitude sampling in the frequency domain, and videodensitometric analysis of digitized, conventionally acquired images. All three approaches have been successfully applied to characterization of plaque. Whereas the efficacy of visual analysis is hampered by subjective definition of echodensity, the backscatter approach and videodensitometric analysis are more reliable and "objective." In theory, compared with two-dimensional images, the backscatter signal is not subject to nonlinear transformations in the ultrasound imaging chain, and is less dependent on changing factors from one patient to another, such as ultrasound attenuation. However, this approach is technologically demanding, and can also be affected by some methodological limitations.[17] Of interest, an acoustic densitometry system for differentiating fibrofatty from fatty components in the arterial wall, based on patented backscatter imaging technology available commercially, has been reported.[12] A practical advantage of our approach is that videodensitometric analysis is not dependent on specific, patented technology, but can be applied to any ultrasound images, regardless of the equipment used for acquisition.

In conclusion, some cellular characteristics of the shoulder region of plaque are associated with the videodensitometric pattern evaluated with MIP, the quartile of gray level in particular. This observation may enable in vivo noninvasive prediction and monitoring of cell composition of lesions at an early stage. Several potential applications can be hypothesized, including identification of initial lesions prone to progress and the effects of pharmacologic intervention.

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