Degree of carotid plaque calcification in relation to symptomatic outcome and plaque inflammation

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Objective: **We undertook this study to quantitate differences in the degree of calcification between symptomatic and asymptomatic plaques removed at carotid endarterectomy (CEA) and to determine associated extent of plaque macrophage infiltration, a histopathologic feature of plaque instability.**

Methods: **CEA plaques (n = 48) were imaged at 1.25-mm intervals with spiral computed tomography (CT; 10-15 images per plaque). Indications for CEA were transient ischemic attack (n = 16), stroke (n = 5), amaurosis (n = 4), and critical asymptomatic stenosis (n = 23). The percent area calcification for each plaque was determined in spiral CT serial sections and averaged for each plaque. In 31 of 48 plaques macrophage infiltration was quantitated in corresponding histologic sections with immunohistochemical techniques.**

Results: **The mean (± SD) age of patients with symptomatic and asymptomatic plaques was 66 ± 7 years vs 71 ± 7 years, respectively, and degree of stenosis was 76% versus 82%, respectively ($P = .05$). Atherosclerosis risk factors were similar between groups. Percent plaque area calcification was twofold greater in asymptomatic versus symptomatic plaques (48% ± 19% vs 24% ± 20%, respectively; $P < .05$). At receiver operating characteristic curve analysis, 80% of symptomatic plaques were below and 87% of asymptomatic plaques were above a cutoff point of 30% plaque area calcification. Macrophage burden was greater in the symptomatic plaques than in the asymptomatic plaques (52% vs 23%; $P < .03$). A strong inverse relationship between the degree of plaque calcification and macrophage infiltration was found in critical carotid stenoses ($r = -0.87$; $P < .001$).**

Conclusions: **Symptomatic plaques are less calcified and more inflamed than asymptomatic plaques. Regardless of clinical outcome, a strong inverse correlation was found between the extent of carotid plaque calcification and the intensity of plaque fibrous cap inflammation as determined by the degree of macrophage infiltration. Carotid plaque calcification is associated with plaque stability, and is a potential spiral CT in vivo quantitative marker for cerebrovascular ischemic event risk. (J Vasc Surg 2004;40:262-9.)**

Current management of symptomatic and asymptomatic extracranial internal carotid artery (ICA) occlusive disease is largely determined by the severity of arteriographic ICA lumen stenosis.[1-3] On the basis of North American Symptomatic Carotid Endarterectomy Trial results, use of carotid endarterectomy (CEA) to treat symptomatic ICA stenoses greater than 50% is superior to best medical therapy in preventing long-term cerebrovascular events. However, surgical or endovascular intervention to treat critical asymptomatic ICA stenoses remains controversial. In the Asymptomatic Carotid Atherosclerosis Study,[3] for example, most patients with variable degrees of ICA stenosis 60% or greater remained asymptomatic, and the benefit of CEA was not demonstrated in women.

These results suggest that other variables, such as plaque structural composition, may influence the natural history of a given ICA stenosis. The spatial distribution of specific plaque components such as the necrotic core and inflammatory foam cell distribution are conducive to carotid fibrous cap disruption and transition to a symptomatic outcome.[4] Studies of mechanisms that produce acute coronary occlusion also indicate that disruption of plaques producing 50% or less lumen stenosis is a key feature underlying such acute ischemic events.[5]

A number of in vivo carotid imaging studies have demonstrated that carotid plaque surface and structural characteristics are associated with development of thromboembolic events.[6-23] B-mode ultrasound and magnetic resonance imaging (MRI) are being increasingly used to potentially identify rupture-prone unstable carotid plaques. However, most of these studies lack satisfactory correlation with a histopathologic standard.[24,25] To date few studies have evaluated the role of spiral computed tomography (CT) in the assessment of carotid plaque stability.[26,27] Calcification is a prominent structural feature of advanced atherosclerotic plaques, and is readily detected with spiral CT. Most studies of atherosclerotic plaque calcification have focused on mechanisms in calcification of the coronary arteries or human aortas and the relation of arterial wall calcification to plaque burden[28-32]; however, the role of plaque calcification in plaque stability and predisposition to clinical events remains unclear. In this retrospective descriptive study we hypothesized that plaque calcification is a marker of plaque stability. We quantitated calcification area in symptomatic and asymp-

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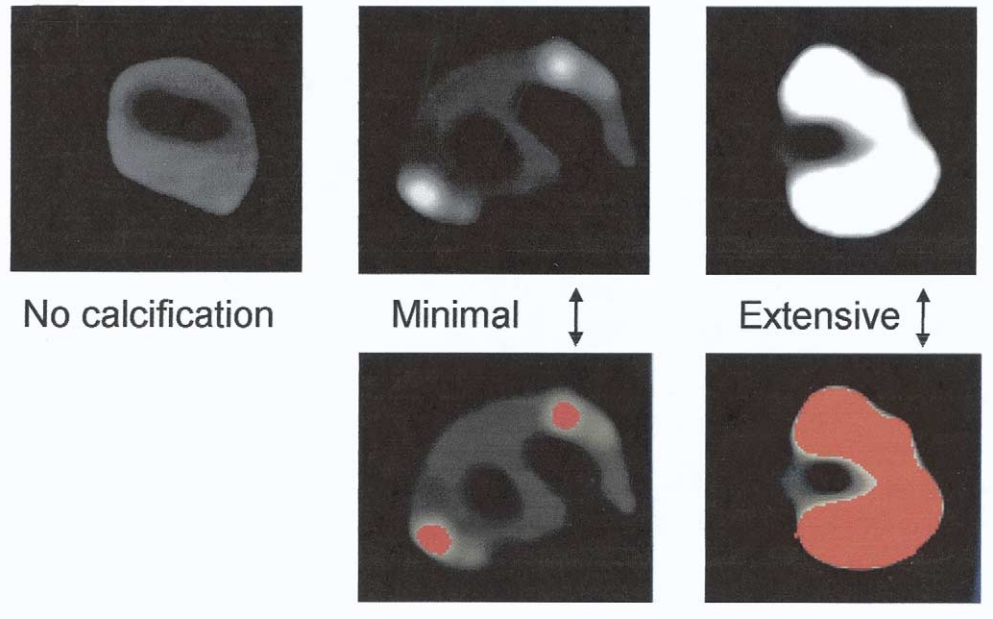

Fig 1. Spiral computed tomography cross-section images of plaques with variable degrees of calcification. Calcification area was measured with computer-assisted morphometry with color density scale analysis (calcified regions in the 0-100 range vs noncalcified regions in the 100-250 range).

tomatic carotid plaques removed at CEA using ex vivo spiral CT assessment, and investigated the associated extent of macrophage infiltration as a marker of plaque inflammation, a histopathologic feature of plaque instability.

METHODS

Patient population and CEA plaques. Forty-eight carotid bifurcation plaques were collected from 48 consecutive patients who underwent standard semi-eversion CEA to treat high-grade ICA stenosis. Patient mean (± SD) age was 66 ± 7 years and 71 ± 7 years, respectively in the asymptomatic and symptomatic groups, and mean carotid percent stenosis was 82% ± 11% and 76% ± 16%, respectively ($P > .05$). Forty percent of patients in the symptomatic group (10 of 25) and 43% of patients in the asymptomatic group (10 of 23) were women (χ^2, 0.06; $P > .05$). In all patients, preoperative color duplex ultrasound scanning of the extracranial carotid arteries was performed, and the degree of lumen stenosis was measured with established hemodynamic criteria.[33] Arch and four-vessel arteriography was performed in 46% of patients (22 of 48) for the following indications: equivocal color duplex ultrasound results due to extensive calcification or possible string sign (n = 9), severe bilateral disease (n = 7), and to exclude concomitant intracranial disease (n = 6). Clinical risk factors for atherosclerosis, including coronary artery disease, diabetes mellitus, hypertension, smoking, and hypercholesterolemia, were recorded for all patients.

Indications for CEA were transient ischemic attack (n = 16), stroke (n = 5), amaurosis fugax (n = 4), and high-grade asymptomatic carotid stenosis (n = 23). All symptoms occurred within 6 months before endarterectomy. Plaques were endarterectomized using a standard semi-eversion technique, to enable in toto removal of the specimen with preservation of plaque structural integrity and to minimize possible disruption of the plaque luminal surface.[4] The harvested specimen included the distal common carotid artery plaque in continuity with the bifurcation and its extension into the internal and external carotid arteries. Among the 48 plaques available for the study, 31 plaques (16 symptomatic, 15 asymptomatic) were formalin-fixed, and were available for both spiral CT and histopathologic studies. The remaining plaques (n = 17) were snap frozen in liquid nitrogen for future molecular analysis of plaque structural components, and were subjected only to spiral CT.

Ex vivo spiral CT assessment of carotid plaque calcification. Ex vivo spiral CT imaging at 1.25-mm intervals (Prospeed VX scanner, GE Medical Systems) was performed on all plaques. Each plaque was imaged from the proximal common carotid artery margin to the distal ICA. On average, 12 cross-sectional images (range, 8-20) were acquired for each plaque. Calcific regions of the carotid bifurcation plaque were characterized as white radiodense regions, in contrast to the remaining noncalcified regions of the plaque (gray) and lumen (radiolucent-black). Spiral CT hard copies were digitized with commercially available software (National Institutes of Health computer software), and the absolute calcification area for each spiral CT image was measured with computer-assisted morphometry with color density scale analysis (calcified regions in the 0-100 range vs noncalcified regions in the 100-250 range; Fig 1).

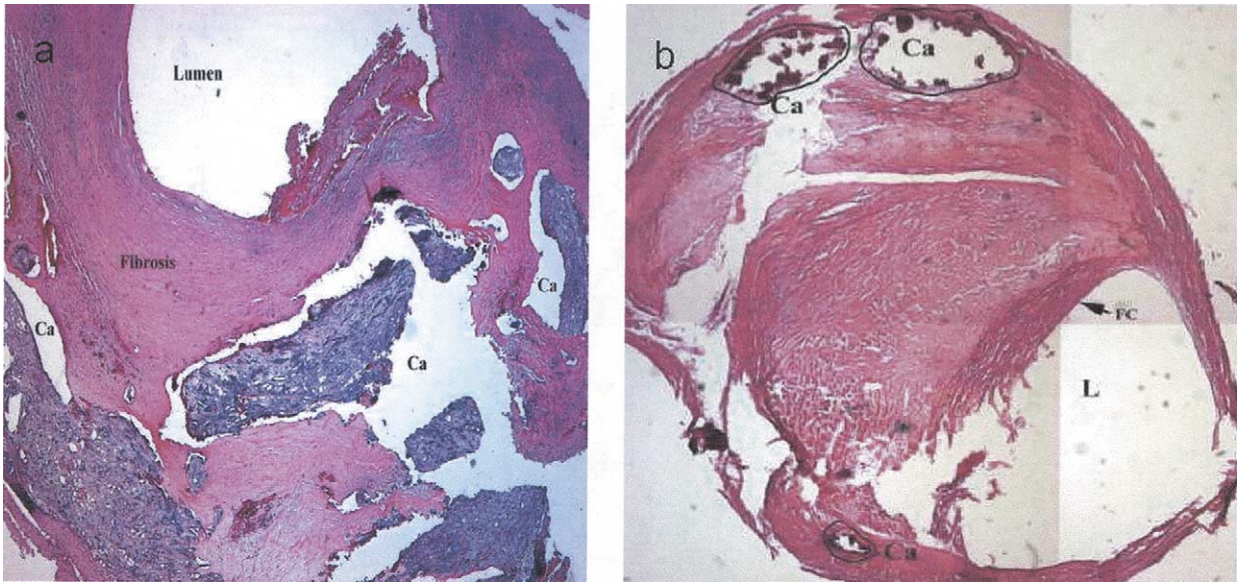

Fig 2. A, Histopathologic section of an asymptomatic plaque with extensive calcification. **B,** Region of calcification was quantitated in each section. (H&E; original magnification ×40.) *L*, Lumen; *Ca*, calcification; *FC*, fibrous cap.

Quantitative morphometry was performed in a dedicated core image analysis laboratory by dedicated personnel blinded to the clinical history pertaining to each plaque. Percent area calcification for each plaque was calculated as the ratio between the summed calcification area per section and the summed plaque total section areas at spiral CT.

Histopathologic and histomorphometric analysis. Thirty one plaques were available for histopathologic evaluation. Plaques were formalin-fixed and sectioned transversely into 3-mm blocks. On average, four blocks (range, 3-7) were available for each plaque. Each block was embedded in paraffin and sectioned at 5-μm intervals. Adjacent sections were stained with hematoxylin and eosin. In addition, immunohistochemical staining was used for detection of inflammatory cell infiltration (macrophages), as described below. The presence of atheroma, necrotic lipid core, hemorrhage, fibrosis, calcification, thrombosis, inflammatory cell infiltrate, fibrous cap integrity, and ulceration (disruption) was determined in each section to enable quantitation of percent area calcification using computer-assisted morphometric techniques (Fig 2). This investigation focused on the quantitative aspects of calcification and plaque inflammation rather than standard qualitative assessment of plaque structural features.

Immunohistochemical analysis of plaque inflammation. Immunohistochemical staining for macrophages was carried out on paraffin-embedded sections mounted on poly-L-lysin–coated slides, and air dried overnight. Sections were deparaffinized by immersing the slides in two changes of xylene for 15 minutes each and then rehydrated in 100%, 95%, and 75% ethanol for 3 minutes each. After rehydration, sections were washed in phosphate-buffered saline solution. Endogenous peroxidase activity was blocked with 3.0% hydrogen peroxide. Sections were pretreated with 10% goat serum for 20 minutes, then incubated with mouse anti-human macrophage antibody (1:30 dilution) for 1 hour at 37°C. The avidin-biotin-immunoperoxidase method was used to identify the macrophages. Incubation with 0.1% 3′,3′-diaminobezidine and hydrogen peroxide at room temperature for 5 to 10 minutes produced a brown reaction product. Sections representing each block were lightly counterstained with hematoxylin to visualize nuclei. In the fibrous cap regions including shoulder, macrophages were counted in all sections of each plaque with a micrometer (American Optical Corp) at high-power field (HPF) ×200 (Fig 3). This work was carried out by an experienced pathologist who was blinded to all clinical data. The average number of macrophages counted at HPF in each section was calculated as the macrophage count of that plaque.

Statistical analysis. For continuous demographic variables results were expressed as mean ± SD, and *t* tests were used for group comparison. For categorical demographic variables, χ^2 tests were used for group comparison. All morphometric data and cellular counts of macrophage infiltration were compared between symptomatic and asymptomatic plaques with Student *t* tests. Differences were considered significant at $P < .05$.

To classify the asymptomatic and symptomatic groups on the basis of percent area calcification, sensitivity, specificity, and positive and negative predictive values were calculated with various cutoff points of percent area calcification. A receiver operating characteristic (ROC) curve was constructed to determine the optimal threshold value.

RESULTS

Demographic characteristics and prevalence of atherosclerotic risk factors in each subgroup are summarized in

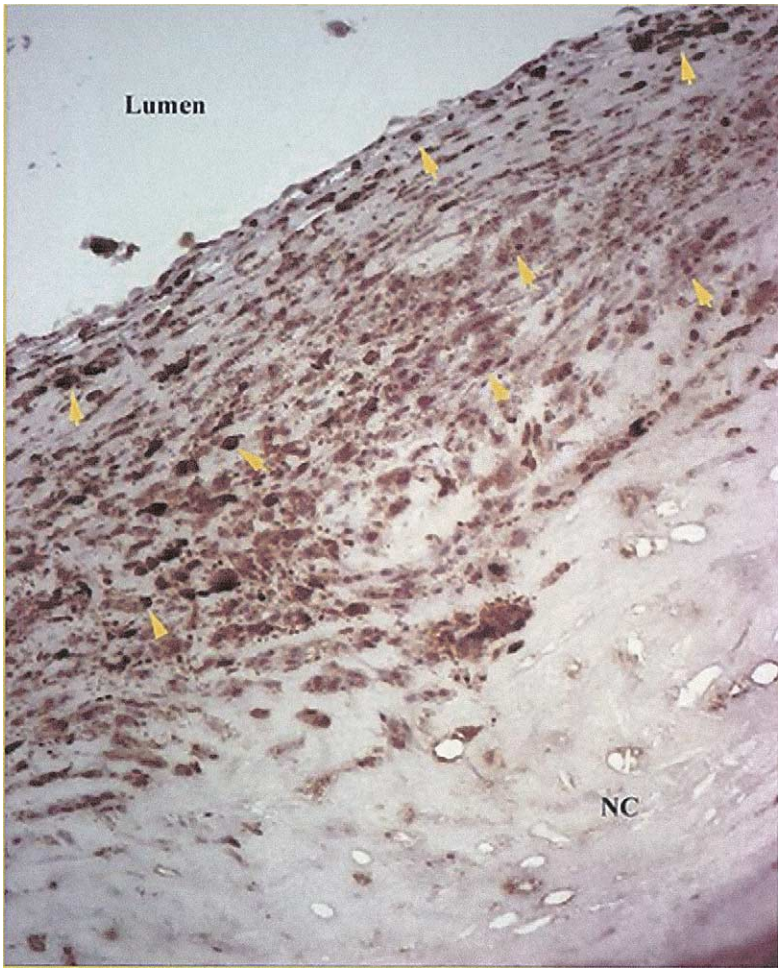

Fig 3. Macrophage infiltration *(arrowheads)* in fibrous cap region overlying the necrotic core in immunohistochemical stained section at high-power field. (Original magnification ×200.) *Arrows* point to immunostained macrophages. *NC*, Necrotic core.

the Table. The prevalence of atherosclerotic risk factors was not different between the symptomatic and asymptomatic groups, except for hypercholesterolemia, which occurred more commonly in the symptomatic group (36% vs 26%; $P < .05$). The mean degree of stenosis (at color duplex ultrasound scanning) was 76% ± 16% and 82% ± 11% in the symptomatic and asymptomatic groups, respectively ($P > .05$), as determined with pulsed Doppler flow analysis and B-mode imaging.

Spiral CT assessment of carotid plaque calcification. On average, 12 cross-sectional images (range, 8-20) were available for each plaque. Percent plaque area calcification was twofold greater in the asymptomatic group compared with the symptomatic group (48% ± 19% vs 24% ± 20%; $P < .05$). Fig 4 shows the ROC curve used to determine a calcification area threshold, which could best differentiate symptomatic from asymptomatic plaques. A cutoff of 30% plaque area calcification provided the greatest sensitivity and specificity. With 30% area calcification as the cutoff point, sensitivity was 87% and specificity was 80%. The associated positive and negative predictive values were 80% and 87%, respectively.

Histomorphometric and immunohistochemical analysis. One hundred fifty-two sections from the asymptomatic group (n = 83) and the symptomatic group (n = 64) were available for histopathologic assessment. Percent plaque area calcification was significantly greater in the asymptomatic plaques than in the symptomatic plaques (47% ± 19% vs 22% ± 21%; $P < .05$).

Macrophage infiltration was more prevalent in symptomatic plaques than in asymptomatic plaques. The mean number of macrophages per HPF per section infiltrating the region of the fibrous cap overlying the necrotic core was significantly greater in the symptomatic plaque sections compared with the asymptomatic plaque sections (297 ± 128 vs 148 ± 83; $P < .01$). More important, a strong inverse correlation ($P < .0001$) between the mean macrophage count and the percent area calcification was demonstrated in both symptomatic plaques ($r = -0.78$) and asymptomatic plaques ($r = -0.89$; Fig 5).

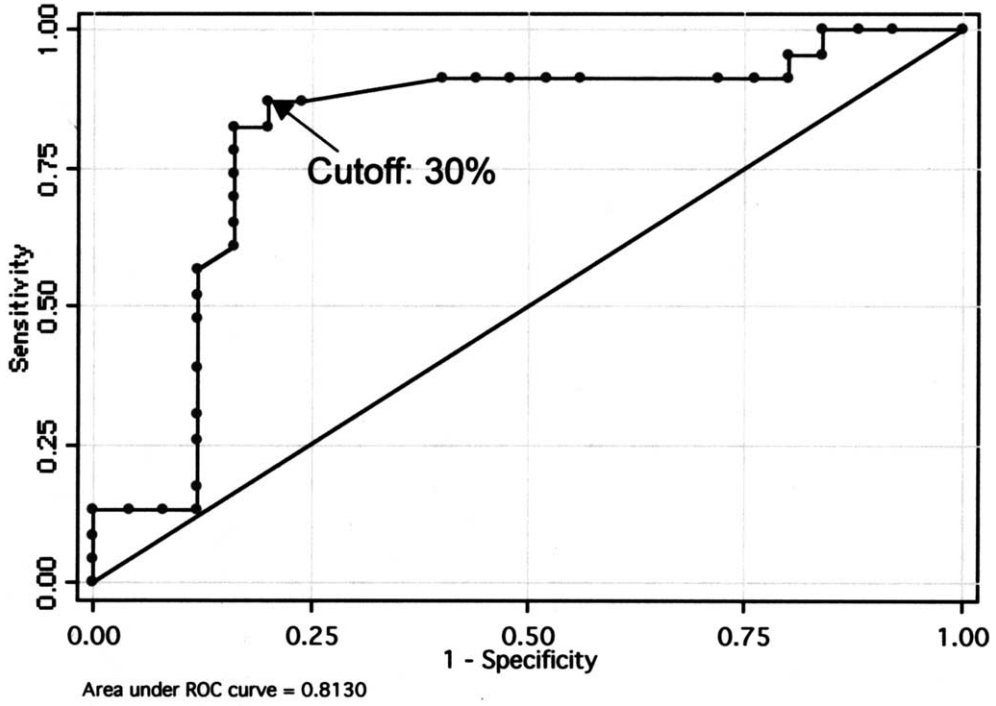

Fig 4. Receiver operating characteristic *(ROC)* curve for differentiating between asymptomatic and symptomatic groups on the basis of percent area calcification. Cutoff point of 30% *(arrows)* yielded the highest sensitivity and specificity.

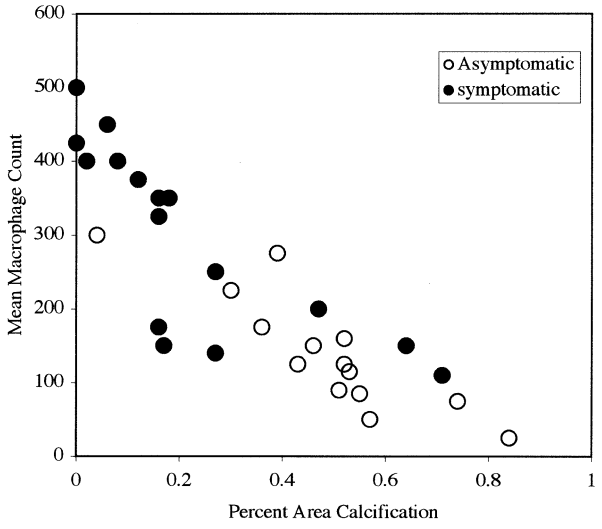

Fig 5. Correlation between mean macrophage count and percent area calcification was -0.78 ($P < .0001$) for symptomatic plaques and -0.89 ($P < .0001$) for asymptomatic plaques, indicative of the strong negative association between macrophage count and percent area calcification.

DISCUSSION

The relevance of atherosclerotic plaque calcification to plaque structural stability has been investigated most commonly in the coronary arteries. Coronary calcific deposits detected with cardiac cinefluoroscopy[31] and CT[32] appear to be associated with increased risk for coronary heart disease. Demer et al[29] proposed that regions of plaque calcification adjacent to an inflamed soft necrotic core predispose to plaque disruption and subsequent coronary artery thrombo-occlusion. This may be related to increased plaque fibrous cap peak stress at regions where hard calcific and soft necrotic plaque regions are juxtaposed.

In carotid bifurcation plaques the presence and degree of calcification has not heretofore been studied in relation to other histopathologic features that underlie plaque disruption and clinical events. In this study we investigated the association between the degree of carotid plaque calcification and the clinical findings in patients undergoing CEA to treat critical symptomatic and asymptomatic ICA stenoses. The extent of fibrous cap inflammation, a known marker of plaque instability and fibrous cap rupture, was also assessed in relation to both spiral CT and histomorphometric quantitative analysis of calcification in intact CEA plaques. Our working hypothesis is that carotid plaque calcification is pathognomonic of plaque senescence and stability.

Evidence has suggested that atherosclerotic plaque composition and anatomy are more important predictors of plaque stability and clinical outcome than merely the degree of vessel stenosis. Calcification is frequently encountered in atherosclerotic disease, and has been proposed as a stabilizing and protective factor rather than a risk factor for

Demographic data and prevalence of atherosclerotic risk factors in symptomatic and asymptomatic groups

	All patients (N = 48)		*Symptomatic group (n = 25)*		*Asymptomatic group (n = 23)*		
	n	%	*n*	%	*n*	%	P
Smoking	35	73	20	80	15	65	NS
Hypertension	34	70	18	72	16	69	NS
Diabetes	18	38	10	40	8	35	NS
Coronary artery disease	16	33	9	36	7	30	NS
Peripheral artery disease	18	38	9	36	11	50	NS
Hypercholesterolemia	15	31	9	36	6	26	<.05

NS, Not significant.

plaque rupture.[33,34] A descriptive pathologic study has been also published that details calcification in carotid atherosclerosis.[35] Over the past two decades an increasing number of echomorphologic studies have been performed to identify symptomatic carotid plaques. Many of these studies indicate that echolucent and ulcerated atherosclerotic plaques are associated with a higher risk for ischemic cerebrovascular events, whereas echo-dense plaques are more prevalent in asymptomatic plaques.[36-39] Establishing a quantitative relationship between carotid plaque calcification and plaque instability may enable better prediction of risk for ischemic events from a given plaque and the need for intervention and monitoring of the effects of medical management.

Currently available imaging, such as B-mode ultrasound scanning and MRI have been used to characterize vulnerable carotid bifurcation plaques. These techniques have promise, but are limited by lack of standard techniques and logistic difficulties when ascertaining the degree of carotid plaque calcification burden. In previous studies[26,27] spiral CT has proved to be an accurate technique for defining atherosclerotic plaque anatomy, particularly in differentiating calcified from noncalcified regions of the plaque.

In the present study we used ex vivo spiral CT to quantitate the degree of carotid plaque calcification in 48 symptomatic and asymptomatic plaques. Spiral CT and histomorphometric results demonstrate that the degree of carotid plaque calcification is twofold less in symptomatic CEA plaques compared with asymptomatic plaques, with relatively similar degrees of ICA stenosis. Calcification occupied approximately 50% of asymptomatic plaque area, and only 25% of symptomatic plaque area. At ROC curve statistic analysis a cutoff point of 30% plaque area calcification was found to discriminate between most symptomatic and asymptomatic plaques. Eighty percent of symptomatic plaques were below and 87% of asymptomatic plaques were above this cuttoff point. This is the first report to provide quantitative threshold data for differentiating symptomatic from asymptomatic carotid plaques. Nonetheless, our results indicate that approximately 15% to 20% of CEA plaques are classified incorrectly, based on the cutoff point of 30% plaque area calcification. This may be related to a number of variables, such as spatial location of the calcific region in relation to the lumen, necrotic core, and fibrous cap, and the presence of microscopic foci of fibrous cap disruption remote from heavily calcified regions in the plaque. Furthermore, some plaques that are considered asymptomatic may produce transient ischemic attacks during sleep or silent infarcts noted on CT scans. In spite of these potential drawbacks, these findings strongly suggest that with progressive carotid plaque calcification there is less likelihood for carotid plaques to disrupt and produce symptoms.

To further elucidate the potential mechanisms underlying the correlation of plaque calcification with clinical symptoms, plaque inflammatory macrophage burden was also investigated in the current study. Although the biologic events that trigger the local inflammatory response within plaque are not fully understood, the association between inflammation and atherogenesis is well known. Inflammation has been suggested to have a number of key roles, not only in initiation and progression of atherosclerosis, but also as a cause of plaque rupture.[40,41] Previously a strong relationship between carotid plaque macrophage accumulation and ischemic vascular events was shown.[42,43] Macrophages release a myriad of cytokines and proteases, such as interleukin-18, tissue factor, and matrix metalloproteinase-9, which are more prevalent in vulnerable and symptomatic plaques compared with stable and asymptomatic plaques.[44-46] Other evidence indicates co-localization of plaque oxidized low-density lipoprotein with dense macrophage infiltration, and that increased loads of oxidized low-density lipoprotein is associated with plaque instability.[47]

Our histopathologic investigations demonstrated that the macrophage burden in the cap and shoulder region of the plaque was significantly greater in symptomatic plaques than in asymptomatic plaques ($P < .01$). More important, a strong inverse correlation was found between the degree of plaque calcification and quantitative measures of macrophage burden. These findings clearly indicate that plaque calcification is a marker of regional plaque stability. Furthermore, increased macrophage density is also associated with plaque echolucency at B-mode ultrasound evaluation.[48] From a biomechanical perspective, replacement of a juxtaluminal necrotic core with calcification greatly reduces

the peak stress on the overlying fibrous cap and reduces the risk for fibrous cap disruption.[36]

Future studies directed to dissecting the molecular events that underlie plaque calcification will shed light on the biologic triggers of this pathobiologic event. More important, our findings suggest that prospective in vivo quantitative assessment of carotid bifurcation plaque calcification, and conceivably other arterial segments, with spiral CT or MRI should provide additional information for in vivo detection of asymptomatic carotid plaques that are likely to disrupt and produce cerebrovascular ischemic events.

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DISCUSSION

Dr Donald Heistad (Iowa City, Ia). Dr Bassiouny, Greg Brown, who did the original fat study, published a paper that is concordant with your findings in ATVB 2 or 3 years ago. He took patients from the fat study who had been on a statin for 10 or 15 years and their carotids were calcified, and he took a control group with the same initial lipids and they had very little calcium and lots of fat in the carotid. I think one of the implications of your findings and his, if they can be extrapolated to coronaries, is the push for detection of coronary calcification as an indication of something bad going on in the coronaries. I think your study makes sense and has important implications.

Dr Hisham S. Bassiouny. I didn't go into the differential role of calcification in different arterial segments. As you know, Demer and other investigators have proposed that plaque calcification in the coronary arterial tree is potentially deleterious because that mismatch of mechanical properties might actually lead to plaque disruption. Such hypotheses have yet to be proven and may not apply to the carotid bifurcation. I think that the behavior of coronary plaques is quite different from carotid plaques. Coronary plaques tend to disrupt at lesser degrees of stenosis, and this may be because of their composition and the biomechanics of the coronary arterial tree compared with the carotid tree. I agree with you that the question is still open for the coronary circulation, but in the carotid bifurcation, I think that if we follow these plaques and find that they are becoming progressively calcified, then the chance that they will disrupt is probably small.

Dr Munier Nazzal (Toledo, Ohio). I might have missed that in the presentation. You know, different surgeons remove different portions of the plaque. So in which part of the plaque will you calculate the percentage of calcification? This is the first question.

The other thing is the location of the calcification. Does it make a difference?

Dr Bassiouny. These are two very excellent points. Again, we have intentionally removed the plaque using the semi-eversion technique. I personally do not cut through the plaque. I dissect the plaque through the adventitia, then open the internal carotid artery distally to feather it out. As you saw in one of these gross specimens, there is a moderate plaque burden in the external carotid artery, so with spiral CT analysis we do measure the calcification in both the internal and the external carotid arteries.

With regard to your second question, the location of calcification was not assessed in this study. It is an important aspect of any study, as a focal area of calcification near the lumen behaves differently from a focal area of calcification within the adventitia. The other point is that we have outliers. We have patients who have extensive plaque calcification and have had symptoms. There is always the possibility that a focus remote from the area of calcification is inflamed and exhibits fibrous cap disruption.

Dr Kirsh Soundararajam (Omaha, Neb). I just had a comment. We at Creighton University have worked a little bit on the plaques of symptomatic and asymptomatic, and one of the works was published this year in the *Annals*, and we did find increased apoptosis and very thin fibrous cap. As part of the study, our findings were also pretty consistent with increased inflammation on symptomatic plaque. As a part of that information we also found that when we tried to culture the endothelial cells of the symptomatic plaques they wouldn't last too long. It became so clear that it was blinded, and you get a call from the lab saying that this is symptomatic. I said, I never told you that it is symptomatic, and it was so evident that these are characteristically different. I just wanted to inform you of that.

I have a couple of questions. The first one is, if you believe that the degree of calcification reflects the stability, do you think our concern in indication for endarterectomy should be focused more on the echogenicity of the plaque rather than going to the degree of stenosis of the plaque? Do you have any thoughts on that?

Dr Bassiouny. Well, that's basically the objective of these studies, to find out some in vivo characterization of plaque structure in addition to the degree of stenosis, which I think is a very simplistic way of going about things, especially in asymptomatic disease. Echolucency at ultrasound is a measure, and interestingly enough, symptomatic plaques are more echolucent and less calcified. Unfortunately, with duplex ultrasound it is challenging to consistently resolve and quantitate plaque echolucency. It is difficult to standardize echolucency measures and techniques from lab to lab. I think that imaging markers like calcification that can be readily detected with spiral CT offer a more standard measure of following asymptomatic patients, and it may be that those patients who have asymptomatic plaques devoid of calcification represent a subgroup of patients who may transition to symptoms and need intervention, while the other subgroup with heavy calcification should be left alone.

Dr Soundararajam. Were you able to categorize the degree of inflammation to the degree of symptoms? Less inflammation, fugax. Higher inflammation, stroke. Were you able to segregate your group in that order?

Dr Bassiouny. It is difficult to quantitatively achieve that goal. Symptomatic plaques had a greater number of inflamed sections than the asymptomatic plaques. The relationship between the degree of calcification and inflammation was inverse.

Dr Robert Cambria (Milwaukee, Wis). I have a quick simple question. Can you assure us that the degree of stenosis in your two groups were similar? You mentioned early on that you had different indications in the two groups, so you need to make sure that the degree of stenosis is similar.

Dr Bassiouny. The degree of stenosis in both groups was similar. The asymptomatic group had a higher degree of stenosis than the symptomatic group (82% vs 76%).