A mechanistic analysis of the role of microcalcifications in atherosclerotic plaque stability: potential implications for plaque rupture

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1Department of Biomedical Engineering, The City College of New York, The City University of New York, New York, New York; 2Graduate Center, The City University of New York, New York, New York; 3Department of Cardiology, College of Medicine, New York University, New York, New York; 4Department of Pathology, New York Medical College, Valhalla, New York; and 5CVPath Institute, Inc., Gaithersburg, Maryland

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Maldonado N, Kelly-Arnold A, Vengrenyuk Y, Laudier D, Fallon JT, Virmani R, Cardoso L, Weinbaum S. A mechanistic analysis of the role of microcalcifications in atherosclerotic plaque stability: potential implications for plaque rupture. Am J Physiol Heart Circ Physiol 303: H619–H628, 2012. First published July 9, 2012; doi:10.1152/ajpheart.00036.2012.—The role of microcalcifications (μCalcs) in the biomechanics of vulnerable plaque rupture is examined. Our laboratory previously proposed (Ref. 44), using a very limited tissue sample, that μCalcs embedded in the fibrous cap proper could significantly increase cap instability. This study has been greatly expanded. Ninety-two human coronary arteries containing 62 fibroatheroma were examined using high-resolution microcomputed tomography at 6.7-μm resolution and undecalcified histology with special emphasis on calcified particles <50 μm in diameter. Our results reveal the presence of thousands of μCalcs, the vast majority in lipid pools where they are not dangerous. However, 81 μCalcs were also observed in the fibrous caps of nine of the fibroatheroma. All 81 of these μCalcs were analyzed using three-dimensional finite-element analysis, and the results were used to develop important new clinical criteria for cap stability. These criteria include variation of the Young’s modulus of the μCalc and surrounding tissue, μCalc size, and clustering. We found that local tissue stress could be increased fivefold when μCalcs were closely spaced, and the peak circumferential stress in the thinnest nonruptured cap (66 μm) if no μCalcs were present was only 107 kPa, far less than the proposed minimum rupture threshold of 300 kPa. These results and histology suggest that there are numerous μCalcs <15 μm in the caps, not visible at 6.7-μm resolution, and that our failure to find any nonruptured caps between 30 and 66 μm is a strong indication that many of these caps contained μCalcs.

coronary artery calcification; coronary computed tomography; vulnerable plaque; finite-element analysis

THE RUPTURE OF VULNERABLE FIBROATHEROMA IN CORONARY ARTERIES CAUSES MORE THAN ONE-HALF OF CARDIOVASCULAR DEATHS, yet the mechanisms as to why some caps rupture and some others do not are unresolved. The most common criterion used to define a fibroatheroma as “vulnerable” is the thickness of its fibrous cap and lesion size. Burke and coworkers have defined a vulnerable plaque as one with a relatively large necrotic core, infiltrated with macrophages, but sparse in smooth muscle cells, with an overlining thin fibrous cap of <65-μm thickness (8). Other researchers have defined vulnerable plaques as having thicknesses of up to 100 μm (27) and in the case of exertion up to 160-μm thickness (39). Although coronary calcification is clinically related to poor prognosis and is used as a marker of the advancement of the disease (28), it has not been successfully correlated with plaque rupture, and its role as a clinical marker of disease is controversial (6, 18, 20, 42).

Extensive studies have concentrated on identifying the triggers for cap rupture. Pathologists have emphasized the role of inflammation, matrix metalloproteinases, macrophages, and smooth muscle cell apoptosis (46) and the presence of neovascularization and intraplaque hemorrhage in advanced coronary lesions (19). It is generally accepted that plaque rupture occurs in the fibrous cap at the location at which the tissue’s stress exceeds a certain critical peak circumferential stress (PCS) (10, 26), and that local tissue properties play a significant role (3). Numerical studies using initially two-dimensional (2D) (10, 36) and, more recently, three-dimensional (3D) finite-element analysis (FEA) (32, 33) and fluid-structure interaction models (4, 24, 40, 41, 47) have attempted to relate PCS to tissue properties and plaque geometry, more specifically cap thickness and necrotic core size. The early 2D studies indicated that PCS occurred at the cap shoulders in regions of high necrotic core curvature (10, 36), but experiments indicated that as many as 40% of ruptures occurred in the center of the cap, where the PCS was significantly reduced (27, 36). Cheng et al. (10) have proposed that the average PCS for rupture is 545 kPa, and the minimum threshold is 300 kPa.

Vengrenyuk et al. (44) have proposed an alternative hypothesis for cap rupture, namely that the fibrous cap was destabilized by microcalcifications (μCalcs), as small as a single calcified macrophage in the cap proper. Their theoretical model for a single small spherical inclusion predicted that, for a cap of uniform thickness, the PCS would be nearly double the local background stress. Rupture was hypothesized to occur due to interfacial debonding. This hypothesis was used to explain why ruptures could occur in the center of the cap, and why caps of >65-μm thickness could have a PCS >545 kPa in a region in which the background stress was <300 kPa. In a subsequent 3D FEA study, Vengrenyuk et al. (43) showed that the thickness criterion could be significantly increased if the μCalcs within the cap were elongated, supporting the observations in Loree et al. (26) and Tanaka et al. (39) that caps >100-μm thickness could rupture.

Vengrenyuk et al. (44) also attempted to experimentally demonstrate the feasibility of their hypothesis using high-resolution microcomputed tomography (HR-μCT) to see if they could identify μCalcs in the cap proper. Spotty or speckled areas of calcification had been observed previously using
intravascular ultrasound (12) and reported in histological studies (14, 46), but quantification and characterization of the \( \mu \text{Calcs} \) were difficult due to the decalcification that was necessary for tissue sectioning. Experiments demonstrated the existence of \( \mu \text{Calcs} \) in the cap proper of human coronary arteries (44); however, this finding was based on a very limited sample set, which included only five fibroatheroma, only one of which had \( \mu \text{Calcs} \) in its cap. The stress distribution surrounding the \( \mu \text{Calcs} \) found in this specimen are examined in detail using 3D FEA and 3D FSI analysis in Refs. 43 and 34, respectively.

The primary objective of the present study is to evaluate the clinical relevance of the \( \mu \text{Calc} \) hypothesis for a much larger sample of all three major coronary vessels: the left anterior descending artery (LAD), the left circumflex artery (LCX), and the right coronary artery (RCA). Detailed results are presented for 92 arteries, covering 460 cm of vessel length, with 62 atherosclerotic lesions, primarily from individuals who had died of heart disease. Nine of these lesions (15%) contained \( \mu \text{Calcs} \) in the cap proper when viewed at 6.7-\( \mu \)m resolution. In total, 81 \( \mu \text{Calcs} \) were observed, and all 81 were analyzed using a refined FEA, wherein it was possible to examine the effect of both clustering and position within the cap, shoulders vs. central region, local cap thickness, and heterogeneity of tissue properties. This much larger sample has made it possible to define new criteria for cap stability, which suggests that rupture is triggered, not by interfacial debonding of calcified macrophages, as proposed in Ref. 44, but by the explosive growth of small voids in the tissue in the vicinity of closely clustered \( \mu \text{Calcs} \) whose size in many cases is smaller than can be detected at 6.7-\( \mu \)m resolution HR-\( \mu \text{CT} \) used in the present study and Ref. 43.

MATERIALS AND METHODS

Specimens. Atherosclerotic whole human hearts were obtained from the National Disease Research Interchange. Donors were 67 \( \pm \) 8 yr old, 88% were men, and 72% had a cardiovascular-related death. The reported cause of death was cardiac arrest 37%, myocardial infarction 22%, cerebrovascular accident 3%, other cardiac 14%, and noncardiac-related reasons 28%. Ninety-six human coronary arteries were harvested from 32 formaldehyde-fixed whole hearts; four coronary arteries were excluded from the study due to the presence of a stent. Both left and right coronary arteries were dissected preserving their ostium and segments from the RCA, the LAD, and the LCX. Of the 92 dissected specimens, 77 were 50 mm in length and 15 extended to 100 mm. During dissection, HR-\( \mu \text{CT} \) scanning, and preparation for histological processing, specimens were kept moist with phosphate-buffered saline.

HR-\( \mu \text{CT} \) scanning. Coronary specimens were scanned using a HR-\( \mu \text{CT} \) system (SkyScan 1172). The right and left coronaries were imaged in separate scans: one containing the RCA only, and the second scan containing the main stem of the left coronary artery branching into the anterior interventricular and circumflex arteries. HR-\( \mu \text{CT} \) system energy settings were chosen to increase the contrast between soft tissue, atheroma’s lipid content, and the mineralized tissue. Each scan produced \( \approx 8,000 \) 2D slices of \( 2000 \times 2000 \) in-plane matrix, with 6.7-\( \mu \)m isotropic voxel resolution and 8-bit gray levels.

A standard Hounsfield unit (HU) calibration procedure was performed in the SkyScan analysis software (CTAn version 1.10.1) using a water and air phantom, assigning 0 HU and 1,000 HU to water and air, respectively. Hydroxyapatite phantoms (1-mm diameter rods containing 250 and 750 mg/cm\(^3\) hydroxyapatite) were scanned using the same protocol. The 250 and 750 mg/cm\(^3\) hydroxyapatite calibration standards resulted in 1,324 and 3,031 HU and were used to density calibrate the gray color levels of images, allowing measurements of tissue mineral density (TMD) on each calcified object within the artery. The calcified volume fraction (CVF) was calculated as the ratio between the calcified tissue volume \( V_c \) (after thresholding) and total tissue volume \( V_t \). CVF(%) = 100 \( (V_c/V_t) \), and is reported as a percentage.

The cumulative length of all artery segments included in this study is approximately equivalent to 460 cm of tissue, and, after reconstruction, 512,000 cross-sectional images were analyzed. The \( \mu \text{CT} \) cross-sectional images from each sample were sequentially analyzed by two observers using DataViewer to identify atheromas based on its morphology. Before quantitative assessment of calcified objects, objects appearing on HR-\( \mu \text{CT} \) images were confirmed to be calcifications by comparing with histological staining for phosphate and calcium.

Histology. Arteries of interest, previously identified by HR-\( \mu \text{CT} \) images, were processed for histology. A tissue-processing protocol previously developed for high-fidelity histological analysis of tendon (23) and undecalcified bone (25) was adapted for the calcified coronary arteries. Coronary specimens were fixed with 10% neutral buffered formalin for 48 h and dehydrated for a total of 24 h. Samples were cleared, infiltrated, and embedded in a hydrophobic methacrylate resin over a period of 3 days at a temperature of 35°C. To coregister histology sections with HR-\( \mu \text{CT} \), embedded samples were scanned once again, and the images were used to locate the region of interest relative to the edge of the plastic block. Large calcifications within the sample were used as landmarks to locate the desired area and orientation of the cut. A gross cut was performed in the plastic block using a low-speed diamond saw. Thin (4-6 \( \mu \)m) sections were cut using a motorized rotary microtome (Leica RM2165, Nussloch, Germany). Sections were mounted on chrome alum-coated slides, covered with a thin, nonstick plastic, clamped with a slide press, and dried in an incubator at 37°C overnight. Before staining, tissue sections were deplasticized and stained to highlight overall morphology and detailed regions of calcification using Alizarin Red S or von Kossa staining. The \( \mu \text{CT} \) image that best corresponded to the histological section was found and compared with the morphology of the histology section. This process was facilitated by the shape of calcifications within the \( \mu \text{CT} \) image and the histological section.

Statistical analysis. All data are reported as means \( \pm \) SD (error bars in Figs. 2-4). ANOVA was used to compare the CVF among epicardial coronary arteries (LAD, LCX, RCA), as well as for the comparison of \( \mu \text{Calc} \) sizes. Differences among groups were considered statistically significant at \( P < 0.01 \). All of the statistical analyses were performed using Prism5 statistical software (GraphPad Software).

Reconstruction and 3D FEA. To analyze the stress concentration created by the \( \mu \text{Calcs} \) in the fibrous cap, FEA was performed using ABAQUS (version 6.10, Simulia, Providence, RI). The HR-\( \mu \text{CT} \) data sets obtained from nine coronary segments containing \( \mu \text{Calcs} \) in their cap were imported into Mimics software (version 13.0, Materialize). The regions corresponding to the lipid core and calcifications were segmented based on mass density calibrated images and used to generate 3D surface meshes. These meshes were then exported to ABAQUS, where a volumetric tetrahedral mesh was created.

Material properties were then assigned using an incompressible neo-Hookean isotropic model, previously used to model coronary arteries and human fibrous plaques (32). In this model, the strain-energy function is \( W = C(I_1 - 3) \), where \( C \) is a material constant, such that the initial Young’s modulus \( E = 6 \times C \), and \( I_1 \) is the first invariant of the strain tensor. Simulations were carried out in atheromas that contained \( \mu \text{Calcs} \) in the cap proper. First, the \( \mu \text{Calcs} \) were replaced by soft tissue, \( E \) (Young’s modulus of the tissue) = 500 kPa, stress concentration was calculated, and then \( \mu \text{Calcs} \) were considered, assuming the calcified tissue Young’s modulus \( (E_{\text{calc}}) = 10 \) GPa to evaluate the effect on the stress distribution in the surrounding material. The value of Young’s modulus of the lipid core \( (E_{\text{lipid}}) \)
was obtained from the literature and was assumed in this study to be $E_{\text{wall}} = 5$ kPa.

To obtain a greatly detailed stress analysis within the area of interest at the $\mu$Calcs level, a submodeling technique was used (43). The stress distribution in a segment of the coronary was first calculated with a relatively coarse mesh (~30-μm element edge) and used as background stress in a submodel, where the stresses were recalculated just in the fibrous cap with a finer mesh (~3-μm element edge length). This technique dramatically reduced the computational effort and allowed sufficient refining of the area around the $\mu$Calcs. A study was performed to determine the optimum element type and size for FEA, with special emphasis on the edge size of the mesh at the interfacial boundaries of the $\mu$Calcs.

At the lumen of the artery, a pressure of 110 mmHg (14.6 kPa) was applied, deforming the artery and creating a tensile stress in the fibrous cap. The circumferential stress distribution was calculated in the fibrous cap, considering presence and absence of $\mu$Calcs. The results were then compared and are reported as the stress concentration around the $\mu$Calcs.

RESULTS

Experimental results. Atheromas are distinguished in HR-μCT by the thickening of the vessel wall, the appearance of a typical semilunar core region, and the different gray color intensity between lipid, soft, and calcified tissues. Figure 1A shows a HR-μCT image (6.7-μm resolution) of a typical atheroma in which calcified tissue is shown in white color, light gray color represents soft tissue, and dark gray color corresponds to lipid within the atheroma.

Presence of calcified tissue in HR-μCT images was validated by comparing CT images with corresponding histological sections stained with von Kossa (Fig. 1B) and Alizarin Red S (Fig. 1C) at ×1 magnification. Numerous calcifications of multiple sizes are revealed in undecalcified, plastic embedded, histological thin sections analyzed for the presence of calcium with Alizarin Red S and for phosphate with von Kossa staining. Note the punctate bright inclusions close to the border of the lipid pool in Fig. 1A. Similar details can be seen on histological sections stained with Alizarin Red S in Fig. 1C, where the distribution of the punctuate micro-inclusions is similar to the HR-μCT image. Additional details are shown in Fig. 1D. This von Kossa stain at higher magnification reveals the presence of many $\mu$Calcs 1–10 μm in diameter, invisible at the current 6.7-μm HR-μCT resolution.

Soft tissue is shown in gray color by HR-μCT, providing excellent depiction of the blood vessel morphology, including the lumen, periphery of the vessel, the core and the cap of the atheroma. The atheroma’s core displays a distinctive gray color that is darker than for the rest of the soft tissue in the core (necrotic) or the vessel wall (Fig. 1A). This identification of lipid is based on the lower X-ray absorption characteristic of fat with respect to soft tissues. Moreover, an intrinsic tissue density/composition control is provided within the sample by the presence of lipid in the adventitia, which displays a similar dark gray color as the lipid in the core, which is captured in detail with HR-μCT. The core is thus identifiable by the presence of lipid (dark gray color) and also by the presence of calcifications at the border of the core. Identification of the core permitted distinguishing of the atheroma’s cap.

The automated 3D analysis of the coronary arteries using CTAn revealed numerous calcified particles of different sizes, with diameters ranging from 15 μm to 1,000 μm. On average, each sample contained 4,199 ± 3,807 calcified particles. The thousands of calcifications with effective diameter of <200 μm, invisible using current in vivo imaging techniques, represent ~97% of the total number of calcifications. The remaining 3% consisted of calcifications between 200- and 1,000-μm effective diameter. Figure 2A shows the mean number of calcifications found in the coronaries, classified by size (equivalent spherical diameter) of each object. Data obtained from the 3D analysis of the samples led us to divide the observed calcifications into three groups based on size, as shown in Fig. 2B. The $\mu$Calcs < 50 μm comprised the first group and were found to be the most numerous, 84.5% of the total number of calcifications.

TMD was assessed for calcified objects of different sizes in five different atheromas randomly selected (Fig. 3). The results indicate the degree of mineralization differs, with large calcifications being significantly more calcified with TMD = 1.419 ± 65 mg/cm³ vs. $\mu$Calcs TMD = 787 ± 64 mg/cm³. The higher degree of calcification in macrocalcifications is indicative of a longer time of calcification, as opposed to $\mu$Calcs, which exhibit a lower TMD, since they have been calcifying for a shorter period of time. A partial volume effect contributes to differences in TMD; the existence of calcifications even smaller than the 6.7-μm pixel size affects TMD measurement of $\mu$Calcs. HR-μCT images suggest that large calcifications are possibly formed as aggregates of smaller particles. Measurements of the CVF demonstrate that the LAD was found to be the most calcified artery segment among the three coronary arteries, as shown in Fig. 3D ($P < 0.01$). This observation is in agreement with a previous report on the correlation of calcification and plaque burden (38); the increased plaque burden at the branching point of the left coronary is often
attributed to the fluid flow pattern and high cell turnover in regions of low fluid shear stress and high wall curvature (9).

The spatial distribution of \( \mu \text{Calcs} \) in the LAD, LCX, and RCA coronary segments, quantified from the right and left coronary ostium, is summarized in Fig. 4. Proximal segments, 50 mm long from the ostium, were analyzed in 92 arteries, while the dissection of 100-mm-long segments was possible only in 15 arteries. The axial distribution of calcifications in the LAD and RCA was found abundant in the proximal 60 mm and are much less frequent in the distal 40 mm. The distribution of \( \mu \text{Calcs} \) in the LCX was abundant along the whole 100-mm length analyzed. Large standard deviations indicating inter-specimen variability are due to the presence of heavily calcified plaques, where, in some cases, thousands of \( \mu \text{Calcs} \) can be seen in calcifying lipid pools.

Sixty-two atheromas at different stages of disease were identified from the HR-\( \mu \text{CT} \) images of the 92 proximal segments. Of the thousands of \( \mu \text{Calcs} \) found in the coronary arteries, the majority of them reside at the borders of necrotic cores or inside lipid pools surrounding calcifications of larger size, but in 9 (15\%) of the plaques analyzed in this study, 81 \( \mu \text{Calcs} \) (9 \( \pm \) 4.5) >15 \( \mu \text{m} \) were present in the fibrous cap proper, where they could be biomechanically dangerous. The location of these \( \mu \text{Calcs} \) in the cap is presented in Table 1 for each of the nine caps, assuming the cap is divided in center and shoulders, where the center occupies \( \sim \) 50\% of the cap length. A total of 47 (58\%) of the \( \mu \text{Calcs} \) were detected in the cap shoulders, and 34 (42\%) in the central region. The reported cause of death of the donors of these nine plaques where \( \mu \text{Calcs} \) were visible at 6.7-\( \mu \text{m} \) resolution was myocardial infarction in four cases, cardiac arrest in three cases, and two noncardiovascular deaths. However, as the study is limited to nonruptured samples, the analyzed atheromas are not to be considered the culprit lesion.

Figure 5A shows a 3D reconstruction of an artery segment, including a fibroatheroma with a fibrous cap. Calcifications of
segments, where μCalcs could be observed at 6.7-μm resolution. In agreement with results presented previously by Ven-grenyuk et al. (43, 44), a 2.5 ± 0.7-fold increase in the cap local circumferential stress was observed at the tensile poles of these roughly spherical calcifications with a size variation of 28 ± 13.6 μm. Figure 5B shows a typical stress distribution at the interface of a μCalc in the cap. The high stress induced at the poles is due to the mismatch in mechanical properties between the calcification and the fibrous tissue.

Table 1 summarizes the results from FEA for the nine segments with caps containing μCalcs visible at 6.7-μm resolution. Data are reported as follows: per cap, mean value and standard deviation for all nine caps, maximal PCS concentration created by μCalcs, the PCS generated in the cap, the location of μCalcs within the cap, i.e., at the center or shoulder region, and the minimum cap thickness. Importantly, the calculated stress concentration produced by μCalcs increased up to a factor 5 when they were in close proximity along the tensile axis. Figure 6 shows this effect for a cap with four μCalcs visible at the same level, the PCS is about two times that of the background at the interface of a single μCal as in Fig. 5, but, when μCalcs are in close proximity, this effect is greatly increased and the calculated PCS at the interface is approximately five times that of the background (sample 6, Table 1). This result demonstrates that two μCalcs in close proximity could render a nonvulnerable plaque highly vulnerable.

We have also analyzed the 53 atheromas, where no μCalcs were observed at the present 6.7-μm resolution. The thinnest nonruptured cap in this group was 66 μm. FEA revealed that the PCS in this cap was only 107 kPa, far below the widely used 300-kPa threshold proposed in Refs. 10 and 13. FEA shows this cap would have to thin to ~30 μm to achieve this threshold tissue stress required for rupture. The distribution of the cap thicknesses in this group of 53 nonruptured atheromas was as follows: 21% between 66 and 80 μm, 7% between 80 and 100 μm, 9% between 100 and 120 μm, 7% between 120 and 150 μm, 11% between 150 and 200 μm, and 45% >200 μm. Note that there was not a single nonruptured cap between 30 and 66 μm in our experimental dataset, where FEA predicts there should be, if cap thickness were the only criterion for cap rupture. As will be discussed later, a likely explanation is that caps between 30 and 66 μm have all ruptured, due not just to cap thickness, but to the presence of μCalcs, which have more than doubled the PCS. It is also likely that there are far more than nine nonruptured caps with μCalcs, but these μCalcs are <15 μm and thus not visible at 6.7-μm resolution. The μCalcs and soft tissue composition act synergistically to increase the PCS and render the cap more vulnerable to rupture. Figure 7 shows the effect on PCS of a 25-μm μCalc embedded in fibrous caps of 120-μm thickness over a twofold increase or decrease in intimal stiffness (E_{intima}) from a mean value of 500 kPa. Increases in E_{intima} from 500 to 1,000 kPa modified PCS only 20%, whereas the presence of a μCalc increased PCS >80% over the same range of E_{intima}. Similar results are observed for a decrease in E_{intima} to 250 kPa.

**DISCUSSION**

This study is the first HR-μCT analysis to attempt to quantify the size and spatial distribution of a large sample of...
calcifications in the three main human coronary arteries: the LAD, LCX and RCA. Data are presented from the proximal region (first 50 mm, starting from coronary ostium) for 92 vessels and the distal regions of 15 vessels. A special emphasis is placed on μCalcs of <50 μm and, in particular, those that lie in the fibrous cap proper. The study is largely limited to nonruptured fibroatheroma because of the difficulty in distinguishing the tissue at a rupture site from its adjacent thrombus in HR-μCT. While previous studies using intravascular ultrasound, optical coherence tomography, and MRI (15, 20) have attempted to quantify coronary calcifications, these studies do not clearly identify discrete particles below 50-μm diameter; therefore, these μCalcs appeared as cloudy areas, which have been often referred to as spotty and speckled calcifications (note optical coherence tomography has a resolution of 10–50 μm, but X-ray CT is much more sensitive to differences in tissue mineralization) (21, 22). Although histological studies (7, 35, 37, 46) are able to demonstrate the existence of μCalcs, common histological sectioning procedures involve decalcification and are not easily amenable to 3D reconstruction. In addition, available coronary calcium scores, which are based on existing in vivo imaging techniques, would also significantly underestimate the presence of μCalcs, because, even though they represent 84.5% of the total number of calcifications in an artery (see Fig. 2), their contribution to the total Vc is <0.1%.

Of the thousands of μCalcs found in a coronary artery, only the ones in the cap proper have an adverse effect on biomechanical stability. The existence of μCalcs in lipid pools or necrotic cores have little influence on mechanical stability, since they can be thought of as floating debris, which does not contribute to local tissue stress. In the past, it has proved very difficult to separate the μCalcs that resided in the necrotic core from those that were present in the fibrous cap of a vulnerable

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μCalcs, microcalcifications; PCS, peak circumferential stress.

| Fig. 5. A: three-dimensional reconstruction of an artery segment with a fibroatheroma, showing calcifications of various sizes, and the corresponding two-dimensional inverse gray-scale HR-μCT image showing μCalcs in the fibrous cap proper (dark spots). Scale bar is 500 μm. B: an approximately spherical μCalc in the cap corresponding to the highlighted area in A. Regions of high stress concentration appear in the cap at the poles of the μCalc, creating a 2.1-fold increase in the local stress in the above sample. |

| Fig. 6. Stress distribution calculated in a cap, where four μCalcs are visible at the same level, showing how calcifications in close proximity introduce a high-stress concentration of about five times the circumferential stress of the background, while relatively isolated μCalcs increase the PCS a factor of two. |
Microcalcifications in Vulnerable Plaque Rupture

Fig. 7. Effect on PCS of a 25-μm μCalc embedded in a fibrous cap of 120 μm thickness for twofold changes in elastic moduli (intimal stiffness). Changes in the intimal stiffness increase peak circumferential stress ~30%, whereas the presence of a μCalc increases peak circumferential stress >80%.

Perhaps the most important contribution of the present study is the insight gained from the detailed analysis of the 81 μCalc that resided in the caps of the 9 lesions, where μCalc could be seen at 6.7-μm resolution. This sample of 81 μCalc is large enough to provide potential new clinical guidelines as to cap stability. The single fibrous cap analyzed in Refs. 43 and 44 contained three nearly spherical μCalc several diameters distant from one another. FEA revealed that these μCalc all exhibited a roughly twofold increase in tissue stress along the poles of their tensile axis. In marked contrast, the present analysis shows that tissue stress concentrations five times the background stress can result from the close clustering of μCalc. Second, none of the 81 μCalc examined produced a local peak tissue stress exceeding 275 kPa (specimen 8, Table 1). This confirms the frequently cited minimum threshold of 300 kPa proposed in Ref. 10 for cap rupture. Third, the distribution of the total 81 μCalc in the 9 caps between shoulders and the central region was nearly the same as the observed location of rupture in Ref. 27, where 40% of the ruptures were observed in the central region of the cap. This is a necessary, but not a sufficient, condition for the μCalc to be the primary cause of cap rupture: if the percentage of μCalc in the center of the cap were to differ significantly from 40%, one would have to seriously question this hypothesis. Fourth, of the 53 fibrous caps observed, where no μCalc could be seen at 6.7-μm resolution, the thinnest was 66 μm. The PCS calculated for this lesion was only 107 kPa, far below the 300-kPa threshold for rupture. While previous 2D FEA studies (10, 13) have predicted a 300-kPa PCS in 50- to 70-μm caps, it is important to note that 2D structural analysis has been shown to overestimate the amplitude of the PCS by approximately a factor of two compared with 3D analyses, and that 3D models predict quite well the site of PCS concentration, which is not the case when considering 2D computations (31). Thus the often-cited association of 50- to 70-μm caps and a 300-kPa minimum threshold for rupture needs to be revised using 3D FEA. The present results indicate a 107-kPa PCS for a 66-μm cap. This is based on a greatly refined multiscale 3D analysis of an actual atheroma geometry generated at 6.7-μm HR-μCT resolution.

The widely cited observation in Ref. 8 that 95% of caps that rupture are <65 μm is not due only to cap thickness, but, as we shall show next, a combination of both cap thickness and μCalc clustering. It is also likely that the majority of caps contain μCalc, and that, at 6.7-μm resolution, only the larger of these μCalc (>15 μm) are visible. The average size of the μCalc observed in the present study was 28 ± 13 μm. As stated in RESULTS, there should have been numerous nonruptured caps with thickness between 30 and 66 μm, if the caps were truly devoid of μCalc. The fact that no such nonruptured caps were found suggests that all caps in this thickness range have ruptured. This could only occur if the rupture threshold of 300 kPa was reached. Since large variations in tissue properties produce only modest changes in PCS (see Fig. 7), the most likely explanation is that these thinner caps did contain μCalc, which, as shown in Table 1, can amplify local tissue stress by a factor of two to five, depending largely on their closeness of spacing. Since PCS in a cap of 65 μm is ~100 kPa, it is evident that even a small cluster of μCalc has the capability of increasing the PCS to exceed the 300 kPa threshold.

Furthermore, the nine atheromas observed in the present study where μCalc were seen is very likely a gross underestimate of caps with μCalc, since Fig. 1D clearly shows that there are numerous μCalc between 1 and 10 μm that are just not visible at 6.7-μm resolution. The present calculations indicate that a cap would have to thin to 30 μm, if it were to rupture without μCalc. Yet, in the 62 atheromas observed, not a single nonruptured cap of <66 μm was observed. A thin cap can reach the rupture threshold due to a single μCalc, but a thick cap can also rupture if the μCalc are in close proximity, provided the 300-kPa threshold value is exceeded. Comparison of specimens 2 and 5 in Table 1 indicates that minimum cap thickness can be a very misleading indicator of PCS or cap stability, since both of these caps have the same minimum 110-μm thickness. In fact, the PCS amplification of specimen 2 is 2.5, and specimen 5 is 3.4, yet the PCS of specimen 2, 103 kPa, is more than twice that of specimen 5, 48 kPa. The crucial difference is that the μCalc in specimen 5 are clustered in a region where the cap thickness is >200 μm, whereas those is specimen 2 are close to the location of minimum cap thickness. The high PCS in specimen 8 is due to a large necrotic core underlying the fibrous cap; this finding supports results in Ref. 33, where core thickness and cap thickness have a combined effect on PCS.

The authors proposed in Ref. 44 that the mechanism for rupture is an explosive expansion of a small void (air bubble) near the tensile poles of the μCalc, which triggers interfacial debonding or cavitation in the vicinity of the...
inclusion. In the latter case, a small void less than one-tenth the size of the inclusion initiates an internal rupture in the tissue near the particle surface, which spreads inward and then brings about debonding. A detailed theoretical analysis of these two mechanisms is beyond the scope of the present paper, but important simple quantitative predictions can be made based on the theory in Gent (16) for the growth of a small void in the tissue in the vicinity of a \( \mu \text{Calc} \). For the cavitation-induced mode of failure, the applied tensile stress must exceed a critical value of \( \sigma = 5 E_t/6 \), where \( E_t \) is typically estimated to be \( \sim 500 \text{ kPa} \). The cavitation threshold would be \( 416 \text{ kPa} \), a value close to the average between 300 and 545 kPa, the widely used threshold and average values for cap rupture proposed in Ref. 10. A full analysis of this void growth is more complicated, since the mechanism that prevails depends on the size of the microinclusion and the energy released when the void expands. The presence of such voids has been observed (N. Maldonado, A. Kelly-Arnold, L. Cardoso, and S. Weinbaum, unpublished observations). Larger inclusions would allow for the growth of a void at the interface, as observed for millimeter size beads in polymeric materials (16), whereas a void of \(<500 \text{ nm} \) diameter in the tissue near the inclusion would involve an additional surface energy \( 2\gamma/a \), where \( \gamma \) is the surface energy of the tissue, and \( a \) is the radius of the void.

The rupture mechanism just described casts a new light on the interpretation of the frequently quoted thickness criterion in Burke et al. (8) for cap rupture, where the mean \( \pm \) SD is given as \( 23 \pm 19 \text{ \mu m} \), with 95% of the ruptured caps being \(<65 \text{ \mu m} \). If one examines the tears, they are nearly all at an acute angle, where the tissue is severely tapered toward its thin edge [see Fig. 1, Burke et al. (8)]. This type of tear could easily be triggered by the explosive growth of a void along the local tensile axis of the tissue, with the actual thickness of the cap being significantly larger than its tapered edge at the tear.

Vengreyuk et al. (43, 44) assumed that cap rupture was triggered by interfacial debonding associated with a single calcified or group of calcified macrophages. This was based on the observation that the smallest \( \mu \text{Calc}s \) they could have observed in the extremely limited sample of three \( \mu \text{Calc}s \) was \( \sim 20 \text{ \mu m} \). It is now clear from the present study, where numerous smaller \( \mu \text{Calc}s \) are visible in nondecalcified histology in Fig. 1, that \( \mu \text{Calc}s \) can be far smaller and most likely grow as agglomerations of calcified matrix vesicles, which, as discussed next, are both much smaller and far more numerous.

In a related study of cap stability, Wenk et al. (47) consider local regions at the shoulder and center of the fibrous cap containing large numbers of calcified 300-nm matrix vesicles using effective medium theory. These local regions are assumed to be associated with inflammation. In this approach, small circular regions of 0.45-mm diameter in the cap proper, containing of the order of \( 10^6 \) calcified matrix vesicles (roughly 1% of the tissue volume), are analyzed using “homogenization” theory to determine the increase in the effective \( E_t \) due to the inclusion of numerous rigid particles. This theory predicts increases in local tissue stress in the cap, which are of the same order (35%) as the results shown in Fig. 7 for a twofold increase in \( E_t \). The density of the tiny calcified vesicles in these local regions is based on the data in Ref. 5. It is highly unlikely that the individual tiny inclusions themselves could lead to cap rupture since, as noted previously, miniscule voids one-tenth the size of the calcified vesicles would introduce a large surface energy, preventing these very small voids from growing.

This study, combined with previous 3D FEA and fluid-structure interaction models (4, 34, 43, 47) and ex vivo animal models (17), indicates the necessity for including \( \mu \text{Calc}s \) in any realistic calculation of the PCS. The cap thickness and lipid/necrotic core size are clearly not the only determinants of plaque vulnerability. While there is a synergistic effect among \( \mu \text{Calc}s \), cap thickness, and soft tissue composition, and each of these factors contributes to the rupture threshold in human coronary atheroma, the results in Fig. 7 indicate that the \( E_t \) of the soft tissue in the intima and the \( E_{\text{calc}} \) itself have a far smaller effect on the PCS than the presence of \( \mu \text{Calc}s \). The results in Fig. 7 are based on a single \( \mu \text{Calc} \) of 25-\( \mu \text{m} \) diameter in fibrous cap of 120-\( \mu \text{m} \) thickness, where the presence of the \( \mu \text{Calc} \) produces a roughly 80% increase in PCS. Based on Table 1, this increase in PCS clearly would have been much greater if there had been a cluster of \( \mu \text{Calc}s \) in a region of high background stress. While increasing \( E_t \) increases the PCS, it also increases the critical value of the tissue stress required for the explosive growth of a small void in the tissue, thus delaying cap rupture. These two effects nearly counterbalance one another. In contrast to \( E_t \), the \( E_{\text{calc}} \) has a small effect on the local stress amplification, provided \( E_{\text{calc}} > 10 \) times that of the surrounding tissue (not shown).

Advanced atherosclerotic lesions are considered a great challenge for histology. Traditional preparation involves the use of acid solution or calcium chelating agents to soften heavily calcified areas for microtome cutting. Although there is a good correlation between the remaining calcified matrix and radiographic large calcification morphology (7), the effect of decalcification on \( \mu \text{Calc}s \) is much greater. The polymethyl methacrylate embedded undecalcified histology presented herein and shown in Fig. 1 has been developed for the histological preservation of bone tissues (23). It provides a major advance in our ability to preserve \( \mu \text{Calc}s \), as well as large...
calcified areas, and thus comparison of HR-μCT and histological images.

The present results indicate the distribution of the μCalcs along the longitudinal axis of the vessel measured from the ostium is similar to the axial distribution of plaques in the LAD and RCA (11, 38), although the reason the axial distribution in the distal 40 mm of the LCX differs significantly from the results in Ref. 11 is currently unknown. This difference requires further investigation and an increased number of distal LCX samples than the n = 5 used in this study.

All of the 92 samples analyzed, both heavily calcified plaques and atheromas in early stages, exhibit μCalcs. These findings are supported by recent pathology reports associating the presence of μCalcs and atheromas in type I and II intimal lesions (37). This latter report emphasized that calcified matrix vesicles 100–300 nm in diameter seemed to precede changes in the intimal content of calcification-regulating proteins and are associated with inflammation in the early stages of lesion formation (2).

One of the present limitations of HR-μCT imaging is that it is hard to capture cap rupture, since it is often embedded within a thrombus. Thus, unless the μCalc that has caused the rupture is retained at the rupture site and the thrombus has washed downstream, it is difficult to clearly separate a ruptured cap from its thrombus in HR-μCT images. Occasionally, a μCalc is clearly visible at the precise location of the rupture site. Such an event is captured in a LAD artery in Fig. 8, where the thrombus washed downstream, clotting the artery 1.5 mm distal from the rupture site, allowing preservation of the ruptured geometry by perfusion fixation. The presence of the μCalc in Fig. 8 and its localization strongly suggest rupture as either a cavitation-induced, or interfacial debonding of the, microinclusion. In the rest of the culprit lesions identified in this study, the thrombus formation coincided with the rupture site, and since the X-ray absorption (color image intensity) of both the fibrous cap and thrombus are similar, the culprit μCalc could not be assessed. An alternative approach for capturing the cap rupture process would be to stretch vessels ex vivo and then examine the ruptured lesions in HR-μCT. In this way, one avoids the thrombus formation and the possible wash out of the μCalc that we hypothesize is the cause of the stress-induced cavitation or interfacial debonding that leads to rupture.

In summary, we show that μCalcs in coronary atherosclerotic plaques are abundant, and when located in the fibrous cap proper, they introduce a stress concentration effect that increases the risk of rupture due to cavitation and/or interfacial debonding. The HR-μCT images allowed us to measure particles <50 μm, giving us insight into the origin of larger calcifications as an agglomeration of smaller calcifications. Increasing the resolution even further could provide greater detail on the delimitation of necrotic cores and the identification of many smaller μCalcs in the fibrous caps that were missed in the present study. Such refinement may make it possible to relate cellular level μCalcs described herein to the calcified matrix vesicles observed using molecular imaging techniques (1, 2, 29, 30) and confirm the evidence herein that nearly all caps with <65-μm thickness contain small μCalcs that could lead to rupture. Finally, from a clinical viewpoint, it is not the μCalcs per se that are dangerous, but their locations in the cap relative to the location of the minimum cap thickness and their spacing. This suggests that, if an adequate in vivo imaging technique could be developed, one might be able to use FEA to assess cap stability.

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REFERENCES

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