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Imaging Atherosclerotic Plaque Calcification: Translating Biology

Grant Bailey^{1,2}, Judith Meadows^{1,2}, and Alan R. Morrison^{3,4}

¹ Department of Internal Medicine (Section of Cardiovascular Medicine), Yale Cardiovascular Research Center, Yale University School of Medicine, New Haven, CT 06511, USA

² VA Connecticut Healthcare System, 950 Campbell Avenue, West Haven, CT 06516, USA

³ Department of Internal Medicine (Section of Cardiovascular Medicine), Alpert Medical School at Brown University, Providence, RI 02903, USA

⁴ Providence VA Medical Center, 830 Chalkstone Avenue, Providence, RI 02908, USA

Abstract

Calcification of atherosclerotic lesions was long thought to be an age - related, passive process, but increasingly data has revealed that atherosclerotic calcification is a more active process, involving complex signaling pathways and bone-like genetic programs. Initially, imaging of atherosclerotic calcification was limited to gross assessment of calcium burden, which is associated with total atherosclerotic burden and risk of cardiovascular mortality and of all cause mortality. More recently, sophisticated molecular imaging studies of the various processes involved in calcification have begun to elucidate information about plaque calcium composition and consequent vulnerability to rupture, leading to hard cardiovascular events like myocardial infarction. As such, there has been renewed interest in imaging calcification to advance risk assessment accuracy in an evolving era of precision medicine. Here we summarize recent advances in our understanding of the biologic process of atherosclerotic calcification as well as some of the molecular imaging tools used to assess it.

Keywords

Calcification; Atherosclerosis; Molecular imaging; Vulnerable plaque; Sodium fluoride PET; Coronary artery disease

Alan R. Morrison alan_morrison@brown.edu.

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Conflict of Interest Grant Bailey, Judith Meadows, and Alan R. Morrison declare that they have no conflict of interest.

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Introduction

According to the Molecular Imaging Center of Excellence and the Society of Nuclear Medicine (SNM), molecular imaging is the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in humans and other living organisms [1]. Though calcification is a hallmark of atherosclerosis, it was long viewed as a passive process [2, 3]. Calcification was considered simply a marker of burden, and early efforts in molecular imaging of atherosclerosis focused more on characterizing inflammation, apoptosis, hemorrhage, and neovascularization, all “active processes” that might yield insight into plaque vulnerability [4]. Increasingly, calcification is recognized to be the convergence of a number of highly regulated biologic processes that reflect a common inflammatory phenotypic expression and that may contain valuable information regarding targets for therapies designed to modify the natural history of coronary artery disease (CAD) [5, 6].

Even today, the direct role of calcification in plaque vulnerability is poorly defined, and thus the development of novel molecular imaging probes and measures targeting vascular calcification provides the opportunity both to study this interesting biologic process and to identify ideal imaging methods that can quantify the vulnerability of atherosclerotic plaque to rupture. Computed tomography (CT) has been the gold standard for patient risk assessment through the identification of macrocalcification in the coronary arterial tree, and is a readily available clinical imaging technique. It has superb spatial resolution; however, as we will discuss below, sensitivity for microcalcification (calcium nodules $<50\text{ }\mu\text{m}$ in diameter) associated with higher risk plaques is limited, and research aimed at identifying early stages of calcification where plaque vulnerability may be higher may require the merger of CT with other modalities like positron emission tomography (PET). PET has much higher sensitivity for microcalcification, but lacks spatial resolution (limited to approximately 5 mm), requiring combined use with CT for adequate signal localization [4]. Recent advances in optical coherence tomography (OCT), ligand imaging using in vivo near infrared fluorescence (NIRF), and intravascular ultrasound (IVUS) have allowed for primarily invasive imaging analyses of the biologic process of calcification. Though we will touch upon it, the role of magnetic resonance imaging (MRI) with regard to the process of calcification is still being defined. Irrespective of the modality, the major goals in imaging the calcification process are to gain a better understanding of the pathogenesis of atherosclerosis in order to develop earlier detection methods, to identify new treatment targets, to accurately assess plaque vulnerability, and to determine early CAD preventive therapy options.

Calcification: a Complex, Active Biological Process

Osteogenesis is the conversion of mesenchymal tissue into organized calcified bone. Mesenchymal-derived osteoblasts secrete a collagen-proteoglycan matrix capable of binding calcium and phosphate salts, forming hydroxyapatite minerals. A major breakthrough in understanding vascular calcification was the identification that it consisted of biologic and genetic processes quite similar to bone development and metabolism [3, 7]. Bone-forming genes and bone-forming signaling pathways are highly upregulated in calcifying vascular

plaques supporting the notion that calcification is a highly active and regulated process that requires a complex interaction between multiple cell types, including endothelial, mesenchymal, and hematopoietic cells, all of which are responding to mechanical, inflammatory, and metabolic cues [5, 8–15].

Initial studies linking inflammation and vascular calcification have demonstrated that calcifying vascular cells or primary smooth muscle cells can express osteoblast-like markers such as alkaline phosphatase (ALP) in response to inflammatory stimuli, including lipopolysaccharide (LPS), tumor necrosis factor- α (TNF- α), or reactive oxygen species (ROS) [8, 13]. There is a close association between inflammatory macrophages and vascular cells that appears to require macrophage-secreted factors like TNF- α , or in some situations, cell-cell contact to promote vascular calcification [8–10].

Several studies have linked mesenchymal cell signaling pathways to the upregulation of the osteogenic gene program, including a number of transcription factors that can induce differentiation of vascular cells into an osteoblast-like phenotype [13]. The osteogenic protein, BMP2, has been shown to activate RUNX2 to promote calcification in human smooth muscle cells in vitro through production of type I collagen and alkaline phosphatase [12]. TNF- α can induce an MSX2-WNT- β -catenin axis in osteoblast-like precursors to promote calcification [10, 11]. RANKL activates NF- κ B to promote BMP4 expression and vascular smooth muscle cell calcification in vitro, and RANKL, BMP4 expression, and calcification co-localize in vascular plaques in vivo [14].

Early imaging of osteogenic activity has revealed an in vivo association between the recruitment of inflammatory cells like macrophages to plaque and early markers of calcium deposition, including a potential role for apoptotic matrix vesicles in early inflammatory activation and calcium nidus formation [5, 8, 15]. Matrix vesicles, small extracellular bodies derived from plasma membranes of osteogenic cells, are also thought to be involved in the mineralization of bone, yet their role in the nucleation of hydroxyapatite mineralization is incompletely elucidated [5]. Matrix vesicles are known to exhibit the proteins, matrix metalloproteinases, and phospholipids necessary for calcium mineralization and are abundant in sites of calcification. Moreover, the protein, sortilin, facilitates loading the calcium-mineralizing enzyme, alkaline phosphatase, into extracellular vesicles to promote mesenchymal calcification [16]. It is theorized that cell death may be the progenitor of matrix vesicle formation, and a prior study demonstrated that inhibiting apoptosis of vascular smooth muscle cells using caspase inhibitors also inhibits calcification [17]. Matrix vesicle-dependent mineralization is also influenced by extracellular calcium concentrations and is induced by calcium-dependent upregulation of phosphatidylserine and Anx6 complexes that appear to act as a nidus for mineral nucleation [18, 19].

Clinical Imaging of Coronary Calcification

Coronary artery calcium scoring (CACS) has emerged as a strong predictor of cardiovascular events, in asymptomatic primary prevention populations, yet its use in clinical practice has remained relatively low [20]. A major concern for adaptation of CACS has been radiation exposure, and in particular, the cancer-causing effects of radiation exposure remain a major concern for physicians and patients [21]. Several studies have

attributed a small increased lifetime risk of cancer attributable to CACS [22]. Developing standards that reduce radiation exposure along with improvements in technology and iterative reconstruction algorithms have potential to impact this concern, but careful validation will be required [20–22].

In intermediate risk groups, CACS has been demonstrated to be a more powerful tool for the assessment of relative risk than traditional risk factor-based algorithms like the Framingham Risk Score or inflammatory biomarkers [20, 23, 24]. Using the minimal threshold value of 130 Hounsfield units (HU), CACS defines the presence of calcium as determined by the product of calcified plaque area and calcium lesion density scale, thus identifying large areas of macrocalcification. Standardized categories are scores of <0 (absence of calcified plaque), 0–100, 101–400, and >400 and >1000 which are associated with increasing 10-year event rates of 1.1–1.7, 2.3–5.9, 12.8–16.4, 22.5–28.6, and 37 %, respectively. The absence of calcified plaque is associated with an extremely low event rate. For example, CAC score of 0 even in the presence of 3 risk factors is associated with a 5-year survival of 99 %. Studies of serial CAC have demonstrated rapid progression of vascular calcification to be associated with a poor prognosis, characterizing a CAD phenotype of aggressive calcification and increased risk of events [25, 26]. In sum, many studies have revealed a strong association between measures of calcium and increased cardiovascular risk, supporting the need to further develop the mechanistic relationship between calcium measures and plaque vulnerability.

As CACS is heavily influenced by both volume and density, a recent analysis from the MESA study illustrated the relative effects of each of these parameters independently. Calcium volume was associated with increased risk of cardiovascular events with HR 1.81 (95 % CI, 1.47–2.23) per SD, while calcium density was inversely associated with risk with HR 0.73 (95 % CI, 0.58–0.91) per SD [27•]. This demonstration that calcium density is inversely associated with cardiovascular risk suggested the possibility of greater plaque stability with increasing density, but the mechanisms regulating this newly identified association remain unknown.

Plaque density analysis illustrates the potential value in moving from general risk assessment of the patient to a more specific plaque vulnerability assessment, reflecting a paradigm shift from viewing calcification as simply a marker for atherosclerotic burden to understanding that unique characteristics of calcification within plaque may allow staging of vulnerability. The distinction of early or active calcification as a destabilizing process and late calcification as a more stable state has also been supported by histological studies [28, 29]. In fact, the use of confocal microscopy and microCT from autopsy samples of coronary arteries has implicated minute cellular-level microcalcifications in the rupture of thin fibrous capped plaques [28]. These findings were further supported by CT angiography (CTA), a clinical imaging technique with the power to provide some visualization and characterization of plaque morphologic features. In a small patient population presenting at the time of acute coronary syndrome (ACS), noncalcified, low attenuation plaques (HU <30) and minimal or “spotty” calcification (in addition to other features like positive remodeling) were significantly more frequent in ACS lesions [30]. In a follow-up small substudy of 1059 patients who underwent CT angiography, noncalcified low attenuation plaques were at

higher risk of developing ACS over a 2-year follow-up than lesions without these characteristics [31]. Moreover, in a subgroup analysis from the ROMICAT II trial, 472 patients presenting to the ED at the time of ACS with low HU plaque had a relative risk of ACS of 8-fold; spotty calcification carried a relative risk of ACS of 37-fold (Fig. 1) [32•].

Invasive imaging of calcification has the capability to provide detailed high-resolution anatomic and serial imaging required for accurate plaque characterization that is currently unattainable by clinical CT angiography. Optical coherence tomography (OCT), a gold standard of calcium detection, has the ability to measure calcium thickness, area, and volume, but does not provide biological information [33]. However, expanding the imaging from one exam to serial imaging of the same plaque to monitor ongoing mineral deposition as opposed to advanced or end-stage macrocalcification could provide important predictive value for targeted lesions. One site of calcification may represent different pathobiological processes than another site within the same artery (i.e., one calcified plaque may be more biologically active while another relatively quiescent). Micro-optical coherence tomography (mOCT) can even be used to detect early processes of atherosclerosis, such as endothelial dysfunction and inflammation. mOCT has been shown to provide visual images of subcellular features of atherogenesis with a resolution of 10 μm . mOCT is able to visualize endothelial cell disruption, leukocyte adhesion and influx into the intima, and even smooth muscle cell deposition of collagen matrix [34]. Microcalcifications located in the thin fibrous cap of early atherosclerotic plaques associated with the increased risk of plaque rupture can be detected by mOCT long before standard modalities are capable of detection. The major limitation of OCT in general remains its invasiveness, requiring a catheter-based approach which is less practical and associated with higher risk when considering serial imaging of individual patients.

Coronary intravascular ultrasound (IVUS) uses high frequencies to achieve adequate near-field resolution of the vessel wall for high-resolution imaging measurements of atheroma volume. Techniques measuring plaque calcification on IVUS have been well described [35]. Moreover, serial coronary IVUS has been pivotal in elucidating factors promoting the progression and regression of coronary atheroma [36]. A recent post-hoc analysis of 3495 patients from 8 prospective randomized trials demonstrated that independent of plaque lipid regression, statins appeared to promote coronary atheroma calcification, providing insight into how statins may be able to stabilize plaques beyond lipid-lowering effects [37•]. Ultimately, while the costs and versatility of ultrasound-based molecular imaging are appealing, wide-spread clinical applicability remains to be determined as it continues to be an invasive approach and quantification of calcium can be somewhat limited by acoustic shadowing.

Optical imaging with near-infrared fluorescence (NIRF) probes involves the intravenous administration of fluorophores capable of being visualized by excitation light in the near-infrared range (650–900 nm). Because of limited autofluorescence, NIRF imaging enables higher target-to-background ratios than other fluorophore-based modalities and results in increased sensitivity for in vivo imaging. One example of this is the visualization of augmented protease activity, using an injectable nanosensor agent, in macrophage-rich plaque to detect early stages of vascular inflammation [38]. Protease activity can serve as a

surrogate biomarker for active inflammatory activity in plaque, and as mentioned above, inflammation is critically associated with calcification. Other NIRF-based probes that measure calcium directly include a bisphosphonate derivative that binds with high affinity to hydroxyapatite [39]. NIRF excitation light has limited tissue penetration that may reduce its practical application to ex vivo imaging of tissue samples or catheter-based modalities.

A novel promising method to identify high-risk plaques involves MRI imaging of ultrasmall superparamagnetic particles of iron oxide (USPIOs). Administration of USPIOs prior to MRI demonstrated accumulation of USPIOs in macrophages predominantly in rupture-prone human atherosclerotic lesions. Pathology of lesions with high presence of USPIOs demonstrated intense clustering of the particles in macrophages on electron microscopy [40, 41]. As mentioned, macrophages are critically associated with sites of early calcification; however, whether these sites identified by USPIO imaging are associated with microcalcification remains to be established. Recently, the relationship between atherosclerotic plaque inflammation and plaque morphology was evaluated using a multimodality approach that included ^{18}F -FDG-PET/CT and MRI [42]. Morphology was characterized by combined use of CT and standard (T2-weighted and proton density weighted) MRI imaging, and the study revealed greater inflammation (as assessed by ^{18}F -FDG uptake) to be associated with low-density plaque. Use of MRI for direct imaging of calcification remains a challenge for the field. The lack of water protons in calcified tissue prevents MR signal intensity [43]. Calcium also shortens T1 times resulting in high-intensity T1-weighted MRI imaging parameters [44]. Despite this, several studies have demonstrated the potential feasibility of certain MRI sequences in elucidating calcium. The novel use of solid-state MRI of ^{31}P was applied to specifically detect the phosphorous nucleus of hydroxyapatite crystals associated with calcium mineralization and provided an early proof of principle [45]. Low signal intensity and long scan times represented challenges to this application that may be a barrier to broad clinical applicability. More recently, high-resolution MRI using a 3-T system and standard imaging sequenced effectively characterized vascular features like carotid artery calcification, using postmortem histology for validation and providing an important early step in developing high-resolution MRI as a plaque-characterizing tool [46].

Calcium deposition in neointimal lesions is clearly a common manifestation of atherosclerosis, and several imaging techniques are well adept at identifying structural manifestations of that process. However, anatomic imaging at a single point in time provides no information about the pathobiology of a plaque, its stage of disease, or the additional prognostic implications the information may hold. This has lead to interest in characterizing early stages of calcification metabolically by making use of the increased glycolytic activity of macrophages in atherosclerotic lesion through detection of ^{18}F -FDG uptake in the vessel wall by PET. As mentioned, inflammatory macrophages are intricately linked to vascular calcification [8–10]. ^{18}F -FDG competes with glucose for uptake into metabolically active cells (any cell, hence specificity can be a limiting issue) and is trapped inside cells upon phosphorylation. In carotid disease endarterectomy specimens, radiotracer uptake correlated well with the extent of macrophage infiltration, providing proof of principle [47].

There is evidence for reproducibility of radiotracer uptake over short periods of time, and importantly, the ^{18}F -FDG up-take was reduced in response to therapeutic intervention with statins, indicating the potential value of ^{18}F -FDG uptake as a marker of response to therapy in serial imaging analyses [48, 49]. One randomized trial demonstrated that therapy with statins resulted in significant and rapid dose-dependent reductions in ^{18}F -FDG uptake in the thoracic aorta and carotid arteries [42]. The prospect of using serial ^{18}F -FDG PET imaging to monitor plaque progression appears promising, but may be limited to ^{18}F -FDG imaging of carotid plaques. The assessment of coronary plaque in ^{18}F -FDG PET imaging is hindered by cardiac motion, the relatively small caliber of the vessels, and the high uptake of the tracer in the myocardium, overshadowing high-resolution visual interpretation of the small coronary arteries. Novel approaches to overcome this problem using calcium channel blockers or a fatty diet to significantly diminish myocardial uptake of tracer are under evaluation with the goal of making assessment of coronary ^{18}F -FDG more feasible [50, 51]. However, there is recent data that ^{18}F -FDG uptake does not correlate well with macrocalcification by CT or microcalcification as measured by ^{18}F -NaF uptake, raising concern about its specificity for staging inflammatory calcification [52–54]. A recent study demonstrated that polarization of macrophages may induce distinct metabolic profiles with respect to glycolysis versus oxidative phosphorylation, indicating that enhanced glucose uptake in active plaque may depend on the predominant macrophage phenotype, resulting in variability or inconsistency of ^{18}F -FDG uptake [55].

As mentioned, microcalcification, a feature associated with high-risk atheroma, can be detected with molecular imaging long before structural modalities (like CT) and can provide earlier evidence of calcific atherogenesis. Because of PET's low spatial resolution, merged PET-CT imaging provides critical improvement to localization of radiotracer uptake. ^{18}F -sodium fluoride (^{18}F -NaF)-based PET-CT imaging has demonstrated that radiotracer uptake correlates strongly with regions of microcalcification seen on histology or ex vivo microCT, providing insight into the state of evolution of calcified lesions [3, 25]. ^{18}F -NaF was initially FDA approved to detect metastatic bone lesions in prostate cancer and is thought to be incorporated into hydroxyapatite during the active stages of calcium mineralization [56]. Nearly half of the sites with macrocalcification did not demonstrate ^{18}F -NaF uptake, while some areas which lack CT identified calcium demonstrated high ^{18}F -NaF uptake, indicating that NaF avidity is not simply to any site of calcification (Fig. 2). In carotid artery atherosclerosis, it was demonstrated that ^{18}F -NaF uptake identified areas of nascent calcification, representing the final common pathway of precipitated micronodules but not upstream triggers of calcification such as activated macrophages or apoptosis [57•]. When defining microcalcification as calcium nodules $<50\text{ }\mu\text{m}$, and macrocalcification as calcium nodules $>50\text{ }\mu\text{m}$, the extent of ^{18}F -NaF uptake was highly concentrated in areas of microcalcification because of the limited ability of ^{18}F -NaF to penetrate sites of macrocalcification due to the well-organized, imperforable, and consequently smaller total surface area of the larger calcified nodules [57•, 58]. At the time of myocardial infarction presentation, ^{18}F -NaF PET-CT imaging demonstrated effectiveness in identifying and localizing ruptured plaque, although it remains unclear whether ^{18}F -NaF will have predictive value in identifying vulnerable plaques prior to event [57•, 59•].

The Role of Calcium Imaging in Personalized Medicine

Currently, there are no direct therapeutic interventions for vascular calcification; however, there are a growing number of therapeutic options becoming available for the treatment of atherosclerotic disease. The mainstay of event risk reduction for patients with CAD remains pharmacotherapy with HMG CoA reductase inhibitors (statins) to achieve reductions in LDL and in markers of systemic inflammation (e.g., high-sensitivity C-reactive protein (hs-CRP)) [49, 58, 60]. Statins can reduce risk by as much as 40 % [61]. However, a significant number of events occur despite adequate lowering of LDL or of hs-CRP, and fairly large numbers of patients must be treated to prevent small numbers of events, significantly adding to cost of the healthcare system [60–62]. Alternative preventive treatment options, including PCSK9 inhibitors, strong immunomodulatory agents like IL-1 β inhibitory antibody (canakinumab), and even preventive angioplasty, show promise for patients who may remain high risk despite adequate treatment with statins [63–67]. In the near future, delineation of these therapeutic options may require more sophisticated, individualized, and highly accurate risk assessment. This has led to increasing recognition that coupling a traditional risk assessment to a patient vulnerability bio-profile, using specific metabolic and structural information about plaque along with patient information (i.e., genetic, proteomic, metabolomic) may provide additional risk discrimination, truly moving cardiology toward an era of precision medicine [68–70]. Calcium-based molecular imaging has potential value in providing one key plaque risk component in this global vulnerability assessment.

Conclusions

Vascular calcification is a complex biological process that carries valuable information with regard to atherosclerotic plaque vulnerability. There has been a great resurgence of interest with regard to plaque characterization by CTA-based features, including low attenuation and spotty calcification, as well as ^{18}F -NaF PET-CT measures of microcalcification. Though the direct role of calcification in plaque vulnerability has not been clearly defined, there has been significant progress in defining the mechanisms of vascular calcification. Calcification as a biological process appears to be the convergence of a number of inflammatory-based signaling pathways that work to activate an osteogenic gene program in vascular cells, promoting an osteoblast-like phenotype in mesenchymal cells. We anticipate this field will continue to yield exciting new developments. Moreover, careful stratification of atherosclerotic plaque based on stage of calcification (not alone but in tandem with a full patient vulnerability profile) has potential value in providing additional information to risk assessment that may aid in complex decision-making regarding more aggressive medical therapy in the future.

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Fig. 1. Example of low HU and spotty calcification lesion in the proximal LAD during presentation for ACS

A sixty-seven-year-old man with a history of hypertension and dyslipidemia who presents with ACS after a recent normal myocardial perfusion study. **a** Normal myocardial perfusion imaging performed with exercise stress using 1-day ^{99m}Tc -Tetrofosmin rest-stress protocol. Patient had achieved 12 METs on the Bruce Protocol without ECG changes. *Upper panels*, stress. *Lower panels*, rest. **b** Coronary CT angiography followed by coronary angiogram performed at time of ACS presentation. *Left panel*, axial plane image denoting atherosclerotic lesion with low HU and spotty calcification in the proximal LAD. *Middle panels*, curved multiplanar reformatted (curved MPR) images at 90° from each other of the same lesion. *Right panel*, coronary angiogram demonstrating the high-grade stenosis associated with the proximal LAD lesion. The *yellow arrow* denotes the location of the same lesion in each of the panels

Fig. 2. Example of the preference of ^{18}F -NaF radiotracer uptake for coronary lesions with microcalcification over macrocalcification as detected by CT

a An 81-year-old man with subclinical CAD underwent ^{18}F -NaF PET-CT for detection of metastatic prostate cancer. Radiotracer uptake is evident in the RCA (*yellow arrow*) in an area without detectable macrocalcification by CT. **b** 72-year-old gentleman with established CAD underwent ^{18}F -NaF for detection of metastatic prostate cancer. Radiotracer uptake in the heavily calcified (by CT) area of RCA is not detectable above background. *Left panel*, ^{18}F -NaF PET. *Middle panel*, CT. *Right panel*, merged ^{18}F -NaF PET-CT