# Molecular Imaging Insights into Early Inflammatory Stages of Arterial and Aortic Valve Calcification

**Sophie E. P. New, Ph.D.** and **Elena Aikawa, M.D., Ph.D.**
Center for Excellence in Vascular Biology, Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 77 Avenue Louis Pasteur, NRB-741, Boston, MA 02115

Center for Interdisciplinary Cardiovascular Sciences, Brigham and Women's Hospital, 3 Blackfan Street, CLSB 17th Floor, Boston, MA 02115

## Abstract

Traditional imaging modalities such as computed tomography, although perfectly adept at identifying and quantifying advanced calcification, cannot detect the early stages of this disorder and offer limited insight into the mechanisms of mineral dysregulation. This review presents optical molecular imaging as a promising tool that simultaneously detects pathobiological processes associated with inflammation and early stages of calcification *in vivo* at the (sub)cellular levels.

Research into treatment of cardiovascular calcification is lacking, as shown by clinical trials that have failed to demonstrate the reduction of calcific aortic stenosis. Hence the need to elucidate the pathways that contribute to cardiovascular calcification and to develop new therapeutic strategies to prevent or reverse calcification has driven investigations into the use of molecular imaging. This review discusses studies that have used molecular imaging methods to advance knowledge of cardiovascular calcification, focusing in particular on the inflammation-dependent mechanisms of arterial and aortic valve calcification.

## Keywords

Aortic valve; atherosclerosis; inflammation; calcification; molecular imaging

## Cardiovascular Calcification – An Unresolved Medical Problem

Cardiovascular calcification, a disease of dysregulated mineral metabolism, is by no means a new dilemma. Indeed some reports date its existence as far back as the Ice Age.[1] Hypercholesterolemia, metabolic syndrome, end-stage renal disease, diabetes mellitus and increased age accelerate atherosclerosis and cardiovascular calcification. Ectopic mineralization mainly affects the aorta, coronary arteries, peripheral arteries, and aortic valves, with fully-formed bone observed in atherosclerotic plaques and stenotic aortic valves.[2] Once believed to be a passive degenerative disease, cardiovascular calcification is now recognized as an active process, with evidence suggesting that it follows a mechanism similar to that of bone formation. Age and lifestyle are still major factors however, thus the

rising average age of the population is accompanied by a growing burden of this disorder, translating into a large cost for society.[3-6]

### Arterial Calcification

Cardiovascular calcification, typically measured and quantified in patients using imaging modalities such as computed tomography (CT), serves as a marker for atherosclerotic coronary artery disease and is associated with increased cardiovascular events.[7] Coronary artery calcification scoring produced via the use of CT has been shown to predict future coronary heart disease events.[8, 9] Arterial microcalcifications located in the thin (< 65 μm) fibrous cap overlying the necrotic core of atherosclerotic plaques may cause microfractures, which can lead to acute thrombosis and even fatal myocardial infarction.[10-15] However, although evidence suggests that microcalcifications in thin fibrous caps increase the risk of plaque rupture, calcification remains a neglected pathology, and effective anti-calcification therapies are not available.

### Aortic Valve Calcification

Massing evidence suggests that valvular calcification possesses characteristics of arterial calcification.[16] Clinicopathological studies of human stenotic aortic valves have identified lesions similar to those in atherosclerotic plaques,[17, 18] and atherosclerotic-like lesions have been noted in the aortic valve leaflets of rabbit and mouse models of atherosclerosis.[19-23] Aortic valve stenosis and coronary atherosclerosis also share epidemiologic risk factors, further fueling recognition of their similarity.[16, 24, 25] Calcific aortic valve disease can range from mild valve thickening to severe calcification with impaired leaflet motion or aortic valve stenosis, the most common form of heart valve disease.[26] Thus calcification is a strong predictor of disease progression in patients with initially asymptomatic aortic stenosis.[27] Approximately 85,000 patients in the United States and 275,000 worldwide annually undergo valve replacement due to aortic valve stenosis[28, 29] – invasive and costly surgical intervention is the only effective treatment.[23, 30]

### Current Treatment Strategies

Current research is aimed at revealing the mechanisms involved in cardiovascular calcification in order to target specific pathways pharmacologically. Various therapeutic agents have been investigated — including statins, which have decreased osteoblastic differentiation and cell mineralization *in vitro*[31, 32] and prevented progression of macrophage burden and osteogenesis *in vivo*.[33, 34] However thus far, statin therapy has not proved beneficial in clinical trials.[3, 35] As no therapies are currently available to prevent or treat calcific disease progression, the Working Group on Calcific Aortic Stenosis of the National Heart, Lung, and Blood Institute (NHLBI) recently highlighted the importance of developing new imaging and therapeutic strategies to diagnose, prevent, and potentially reverse or delay the onset of the calcification process.[36] This review aims to establish underlying mechanisms of cardiovascular calcification as elucidated by *in vivo* molecular imaging, and demonstrate the prognostic value of optical near-infrared fluorescence (NIRF) imaging for the detection of specific changes associated with arterial calcification and aortic valve disease.

## The Evolving Molecular Imaging Approach

### Conventional Imaging Modalities for Detection of Calcification

The detection and quantification of advanced calcification in coronary arteries and aortic valves can be readily achieved by conventional diagnostic imaging techniques, including CT, intravascular ultrasonography (IVUS), transthoracic echocardiography and MRI.[4, 37] At present, IVUS is an excellent tool for detecting advanced calcification however calcium

volume is hard to quantify using these method due to acoustic shadowing.[4] It is clinically advantageous for diagnostic imaging techniques to be as noninvasive as possible to alleviate patient discomfort. Therefore noninvasive techniques such as CT and ultrasound are emerging as diagnostic contenders, particularly because they are more sensitive than other imaging modalities and can quantify calcium content. However, most imaging modalities have low spatial resolution and lack suitable molecular imaging agents, and thus cannot detect the earliest stages of calcification on cellular and molecular levels.

### Optical Molecular Imaging

While conventional imaging modalities proficiently visualize anatomic structures and macro changes, high-resolution optical imaging, particularly intravital fluorescence microscopy enables observation of processes on molecular and (sub)cellular levels. Novel molecular imaging technologies utilize targeted and activatable imaging agents for the *in vivo* detection of pro-inflammatory, pro-osteogenic, and proteolytic activity.[23, 38, 39] Chen et al. demonstrated the potential use of proteases as biomarkers for vulnerable plaques when probed with beacons[40]; we have since harnessed this technique to explore the pro-inflammatory mechanisms of cardiovascular calcification.[23, 34, 41, 42] Imaging agents utilize specific molecular or cellular processes to generate image contrast using high-resolution imaging technology. Advances in nanotechnology have yielded targeted imaging agents by chemically attaching an affinity ligand, such as an antibody or small molecule, to a fluorochrome or magnetic compound (e.g., bisphosphonate-conjugated fluorescent agent for the detection of hydroxyapatite[23, 34, 43-45], or cross-linked iron oxide fluorescent nanoparticles for the detection of macrophages[23, 34, 38]). Studies have also produced activatable imaging agents – chemically engineered substrates that interact with their targets (e.g., enzymes) and undergo a physicochemical change, resulting in signal amplification (e.g., protease-activatable imaging agents for the detection of matrix metalloproteinase or cathepsin activity).[23, 39, 41, 46]

Near-infrared fluorescence (NIRF; excitation in 650 to 900 nm wavelength) molecular imaging represents a useful platform for optical molecular imaging *in vivo*.[47, 48] Indeed near-infrared (NIR) light has the potential to penetrate tissues in the magnitude of centimeters rather than micrometers,[49] which makes NIRF imaging highly attractive, as it allows for greater depth sensing of a larger area of inflamed tissue or calcific lesion. In addition, fluorescence imaging in the NIR bandwidth offers reduced tissue autofluorescence.[48] Other imaging detection systems can also be used, depending on the resolution required. One such platform is fluorescence-mediated tomography (FMT), which can detect femtomole quantities of fluorochromes in whole animals with millimeter resolution.[39, 40]

Fluorescence molecular imaging is able to utilize two or more spectrally distinct imaging agents to visualize different biological processes simultaneously.[23, 34, 41, 42, 50] It can also be integrated with more conventional imaging techniques (e.g., magnetic resonance imaging (MRI) and CT) when using multimodality or multi-functional probes. A recently developed tri-modality iron oxide–based magnetic nanoparticle could be used for simultaneous NIRF, MRI, and positron emission tomography (PET) imaging of macrophages.[51] Therefore it may enable the attainment of both high imaging sensitivity (from NIRF) and high spatial resolution (from MRI).[52]

### Clinical Translation

For the successful treatment of calcification, we need to visualize the pathways involved in the earlier stages; studies that apply optical molecular imaging to the calcifying vasculo/valvulopathy or bone remodeling are therefore desired. We anticipate that clinical molecular

imaging approaches could provide new biological insights into human arterial osteogenesis far before the development of advanced calcification detected by current methods. To accomplish this goal clinical multimodal molecular imaging approaches will likely be required to detect and monitor the dynamic changes in inflammation/macrophages and osteogenesis/calcification in calcified aortic valves and atherosclerotic plaques in cardiovascular calcification.

Studies to improve molecular imaging methods, and increase their chances for clinical use, are ongoing. Clinical molecular imaging has already made significant headway into visualizing targets in larger vessels.[53] There is substantial evidence supporting the use of (18)F-fluorodeoxyglucose positron emisson tomography (FDG-PET) imaging for the evaluation of coronary artery disease.[54] Furthermore a novel intravascular NIRF catheter has been developed, which has been demonstrated to detect inflammation-associated protease activity in vessels the size of human coronary arteries in real time with an activatable-NIRF agent.[55] In addition more recently a new two-dimensional NIRF imaging catheter system based on rotational fiber design has been developed that will allow seamless integration of molecular imaging into the cardiac catheterization laboratory.[56] These advanced molecular imaging techniques do not only offer the potential to be sensitive diagnostic tools, but they also enable *in vivo* study of the mechanisms of atherosclerosis and cardiovascular calcification. For example the availability of a clinical intravascular NIRF catheter could accelerate the detection of high-risk plaques.[53] Due to the significant technological developments made in the field of molecular imaging over the past 2 decades, it is now deemed a clinically feasible diagnostic tool.[52]

## Imaging Identifies Underlying Molecular Mechanisms Involved in Early Aortic Valve Calcification

The mechanistic pathways involved in the development of calcific aortic valve disease remain largely unknown. Therefore the use of molecular imaging is thought advantageous to detect early molecular and functional abnormalities in aortic valves. Our recent research tested the hypothesis that molecular imaging can detect early changes in aortic valve disease, with positive outcomes.[23] We used a panel of distinct NIRF imaging agents to map endothelial cells, macrophages, proteolytic activity, and osteogenesis in the aortic valves of hypercholesterolemic apolipoprotein E–deficient (apoE$^{-/-}$) mice.

### Imaging of Valvular Endothelial Cell Activation

MRI and NIRF microscopy clearly demonstrated *ex vivo* that most of the VCAM-1-targeted agent[57] was distributed in the aortic valve leaflets near the attachment of the aortic root — a region known as the commissures — which was corroborated by immunohistochemical evidence using immunoreactive VCAM-1.[23] Increased expression of VCAM-1, ICAM-1, and E-selectin has been noted in surgically removed diseased heart valves,[58, 59] illustrating that injury to endothelial cells causes increased expression of adhesion molecules. The leaflets of the heart valves open and close at least $3 \times 10^9$ times over a single lifetime, therefore they have to endure a certain amount of 'wear and tear' due to the repetitive forces exerted upon them. During the cardiac cycle, the aortic valve leaflets are subjected to continual bending, shearing, and tensile and compressive stresses.[60] As the flexion area of the aortic valve leaflets near the commissures encounter the greatest amount of mechanical stress within the leaflet,[61] these areas might induce endothelial cell activation/injury, and thus the subsequent expression of adhesion molecules.

## Imaging of Macrophages and Proteolytic Activity

Elevated plasma lipids and other atherogenic factors may also induce valve endothelial cell activation. This results in an amplification cascade of events, including monocyte recruitment and subsequent macrophage accumulation within the extracellular matrix of the valve, as visualized by macrophage-targeted NIRF-conjugated iron nanoparticles. The use of NIRF protease-activatable probes provided direct evidence that valvular interstitial cells (in their activated form as myofibroblasts) and macrophages elaborate excessive levels of matrix metalloproteinases (e.g., collagenase-1/MMP-1, collagenase-3/MMP-13, gelatinase-A/MMP-2, and gelatinase-B/MMP-9) and cysteine endoproteases (cathepsins K and S), which corroborates with other studies.[41, 62-67] These proteolytic enzymes degrade collagen and elastin in the extracellular matrix, leading to vascular and valvular remodeling and subsequent structural changes. Activation of MMP-9 by osteopontin may play a role in aortic valve calcification[68] by initiating elastin degradation that could be a nidus for hydroxyapatite crystal formation.[69] Proteolytic activity not only affects the extracellular matrix, but also other substrates (e.g., IL-1β precursor, tissue factor pathway inhibitor), which may in turn enhance valve inflammation.[70, 71] Molecular imaging has thus enabled us to analyze proteolytic activity, which could provide diagnostic information on inflammation and matrix degradation and thereby predict risk of subclinical aortic valve stenosis.

## Imaging of Osteogenic Activity

The need to closely follow and evaluate changes once lesions are identified in patients with aortic valve thickening, which could eventually lead to aortic sclerosis, is evident due to the high morbidity and mortality rates reported.[72] The early stages of mineralization were observed using a bisphosphonate-conjugated imaging agent that binds to nanomolar concentrations of calcium hydroxyapatite complexes elaborated by valvular interstitial cells (e.g., myofibroblasts). Like all bisphosphonates, this non-cleavable pyrophosphate analog avidly binds calcium, thus accumulating at sites of active biomineralization and osteogenesis[44, 50] as detected by alkaline phosphatase activity. This calcium imaging agent can be excited at NIRF wave lengths that are spectrally distinctive from other imaging agents (e.g., macrophage-targeted or cathepsin-activatable agents), thus enabling simultaneous correlation of osteogenic activity with other biological processes. In our study immunohistochemical analysis of osteogenic markers was used to validate data produced via NIRF. Valvular interstitial myofibroblast-like cells were demonstrated to express osteopontin, osteocalcin, and osteogenic transcription factors such as Runx2 and Osterix, indicating the active regulation of mineralization prior to the development of end-stage calcification. Additionally, large populations of myofibroblast-like cells contained a cleaved form of Notch1,[23] which has been suggested to direct osteoblast differentiation.[73] Valve lesions have been identified in this study to possess features similar to atherosclerotic plaques, including endothelial cell activation, inflammation/macrophages, proteolytic activity, and osteogenesis — supporting the hypothesis that aortic stenosis and atherosclerosis share a similar pathogenesis.

## Inflammation-Dependent Mechanisms of Calcific Aortic Valve Disease

Although the cause of mineral dysregulation in early aortic valve disease requires further investigation, molecular imaging has enabled simultaneous visualization of the roles of various cells and enzymes in the early stages of mineralization *in vivo*, supporting the concept of inflammation-dependent development of calcific aortic valve disease. In summary, atherogenic factors and mechanical forces may activate valve endothelial cells and initiate recruitment of inflammatory monocytes/macrophages, which when activated produce a cocktail of pro-osteogenic cytokines, growth factors, and proteolytic enzymes. Extracellular matrix remodeling and thickening/stiffening of the leaflets due to proteolytic activity may result in valvular dysfunction and alterations of mechanical stresses across the

valve leaflet. The resulting change in flow patterns may further induce inflammation and the activation of fibroblasts into myofibroblasts, and subsequently into osteoblast-like cells through augmentation of the Runx2 pathway. The end result would be the deposition of calcium primarily in the regions of high mechanical stress and eventual immobilization of the aortic leaflets due to increased stiffening (Figure 1).

# Monitoring Changes in Osteogenic Activity during Atherosclerotic Plaque Progression and after Anti-inflammatory Treatment

### Monitoring Calcification and Inflammation in Living Animals

The limited knowledge regarding cardiovascular calcification has been blamed on the inability to spatially and temporally resolve and quantify the dynamic pro-osteogenic molecular mechanisms *in vivo*.[74] These limitations can be overcome using innovative molecular imaging tools to visualize and quantify components of inflammation, along with osteogenic activity associated with early-stage atherosclerosis.[34, 42] Though *in vitro* studies have suggested the potential role of inflammation in calcification,[75-79] few *in vivo* reports pursued this idea. We therefore employed molecular imaging to test the hypothesis *in vivo* that atherosclerotic plaque inflammation, determined as macrophage infiltration, triggers osteogenic activity, and that further reduction of inflammation may decrease arterial calcification. Nanoparticle technology was once again utilized to image macrophages and the calcium imaging agent used to image the osteogenic differentiation of smooth muscle cells and areas of active mineralization processes in the arteries[34, 42] — demonstrating the value of these imaging methods in analyzing calcific processes within both aortic valves and arteries. In this study, apoE$^{-/-}$ mice were fed an atherogenic, high-cholesterol diet supplemented with atorvastatin. For the first time intravital microscopy was performed sequentially on the carotid arteries of untreated mice and statin-treated cohort of mice at 20 weeks and 30 weeks of age. Macrophage number was found to increase in association with advanced osteogenic signal by the later time period, however this progression of macrophage burden and osteogenesis was prevented by anti-inflammatory statin therapy, which further supported our hypothesis that inflammation may trigger calcification.

### Arterial Calcification as an Inflammatory Disease

A series of groundbreaking *in vitro* studies by Demer's group demonstrated that macrophage-derived cytokines (e.g., IL-1β, IL-6, IL-8, TNF-α, IGF-1, and TGF-β) induce osteogenic differentiation and mineralization of vascular smooth muscle cells.[75-78] The results of these studies produced the theory that pro-inflammatory cytokines promote atherosclerosis-associated calcification by regulating the differentiation of calcifying vascular smooth muscle cells. Our *in vivo* molecular imaging studies corroborated previous reports and further linked macrophages with osteogenesis. Fluorescence reflectance imaging *ex vivo* elegantly visualized the real-time association of inflammation and early calcification.[34] Similar to macrophage and calcification signals noted in regions of high flexure and increased mechanical forces in the aortic valve, macrophage burden and osteogenic activity colocalized predominantly in proatherogenic regions of high mechanical stress, including the lesser curvature of the aortic arch, the aortic root, the innominate artery, the carotid bifurcation, and the aortic root (Figure 1). This evidence further supports the importance of macrophages in calcification.

The advancement of *in vivo* molecular imaging techniques has enabled us to further elaborate on the inflammation-dependent calcification paradigm (Figure 2), which can be split into three distinct phases: *initiation, propagation*, and *end-stage calcification*. We suggest that in the *initiation* phase, macrophage infiltration and inflammation precede calcification, and activated pro-inflammatory pathways induce osteogenic transformation of

vascular wall cells. This phase can be characterized by the expression of pro-osteogenic cytokines (e.g., IL-1β, IL-6, IL-8, TNF-α, IGF-1, and TGF-β) by macrophages and other inflammatory cells.[5, 80] In the *propagation* phase, vascular smooth muscle cells undergo osteogenic differentiation, characterized by the expression of transcription factors normally associated with differentiated osteoblasts and chondrocytes (e.g., Runx2, Osterix, and Sox 9)[23, 34, 81] and mineralization-regulating proteins (e.g., alkaline phosphatase, osteopontin, osteocalcin, osteonectin, collagen I and II, and bone morphogenic proteins).[82] In addition, in the absence of inhibitors, matrix vesicles released from living cells, and apoptotic bodies from dying macrophages and smooth muscle cells may provide a nidus for calcification.[83, 84] Indeed these events are associated with the generation of microcalcification. In our imaging study, these early stages of mineralization could also be seen using electron microscopy — matrix vesicles (30–300 nm) and apoptotic bodies (300–1000 nm) contained structures compatible with nanocrystals of hydroxyapatite.[34, 85] Additionally a recent study has demonstrated that apoptosis can be monitored in real-time using fluorescently-tagged Annexin A5 imaging agent.[86] If inflammation continues, as the plaque advances it will induce further formation of microcalcifications, fibrous cap thinning, and eventually plaque rupture. Using molecular imaging at this stage, we showed that inflammation and microcalcification evolved within close proximity and overlapped at border regions, and suggested that plaque ruptures may occur in these adjacent areas.[34]

The deposition of hydroxyapatite progresses quickly,[34] and microcalcifications evoke additional pro-inflammatory responses from macrophages, demonstrating that a positive feedback loop of calcification and inflammation drives disease progression.[87, 88] Moreover, the onset of microcalcifications is an added complication, as they may cause plaque rupture as a result of debonding, leading to acute clinical events as predicted by Vengrenyuk and colleagues.[13, 15, 89] It is hypothesized however, that reducing inflammation through anti-inflammatory therapy at the early stages could retard subsequent osteogenesis and stabilize the plaque until further inflammatory events are initiated.[34, 42, 50, 84] As reversing or halting the mineralization process in later stages of calcific disorders may be more difficult, the final of the three phases — *end-stage calcification* — is classically viewed as irreversible. This final phase is associated with advanced tissue mineralization and reduced inflammation, and can be readily detected by conventional imaging approaches (e.g., CT).[90]

## Detecting Elastolysis-triggered Calcification in Chronic Renal Disease

Cardiovascular disease is the most common cause of death in individuals with chronic renal disease (CRD).[91-93] In addition to the classic risk factors mentioned earlier, patients with CRD have hyperphosphatemia, an independent risk factor for cardiovascular death.[94, 95] While cardiovascular disease in the general population is associated with older age, lesions of cardiovascular calcification have been reported in dialysis patients of a much younger age,[95, 96] confirming that cardiovascular calcification is not simply a degenerative disorder. This issue, in our mind, also heightens the necessity for research into this area. We have already highlighted the implication of proteolytic enzymes expressed by activated macrophages in atherosclerotic plaque progression and aortic valve disease.[62, 97-100] Although the contribution of cathepsins to the pathogenesis of atherosclerosis and aortic stenosis has been previously established, our study using optical molecular imaging produced direct *in vivo* evidence for the role of cathepsin S, one of the most potent elastases, in accelerating arterial and valvular calcification in mice with atherosclerosis and CRD induced by 5/6 nephrectomy – a 2-step CRD-promoting procedure that is known to aggravate atherosclerosis and intimal calcification in apoE$^{-/-}$ mice.[41,101]

### Role of Elastin Degradation in Calcification

Elastolytic cathepsin S is abundantly expressed by macrophages and smooth muscle cells in atheroma.[102] Previous studies have suggested that the structure of the elastic fibers allows them to withstand proteolytic degradation.[103, 104] But during the progression of atherosclerosis, an imbalance in the regulation of cathepsins may enable proteases such as cathepsin S to degrade elastin.[69, 105] Elastolysis induced by inflammation in the atherosclerotic plaques may promote the release of biologically active, soluble elastin-derived peptides that trigger the osteogenic differentiation of smooth muscle cells.[41, 106, 107] These elastin-derived peptides act as biologically active molecules known as matrikines or elastokines, which can regulate cell processes such as migration and proliferation, as well as the release of bone-regulating proteins from myofibroblasts or smooth muscle cells, resulting in calcific lesions.[106-110]

### Imaging Elastolytic Activity

Our study utilized a new protease-activatable imaging agent that was specific for cathepsin S.[41, 111] Molecular imaging detected increased cathepsin S and osteogenic activities in CRD mice, whereas calcification was decreased in atherosclerotic plaques and aortic valves in mice lacking cathepsin S activity. These results were corroborated using optical projection tomography and quantitative histology, and provided the first direct *in vivo* evidence for the role of cathepsin S in calcification.

We have previously shown that proteolytic activity, in the form of cathepsins (cathepsin S and cathepsin K) and MMPs (MMP-2 and MMP-9), plays a role in valvular diseases.[62] Therefore, as mature aortic valves also have an elastin-rich structure [100] and the ability to develop lesions similar to those of atherosclerotic plaques,[23, 34] we proposed that similar mechanisms of cathepsin S-associated elastin degradation contribute to the development of calcific aortic valve disease. In the early stages of aortic valve and artery calcification, macrophage-derived elastolytic enzymes participate in the degradation of elastin matrix. This remodeling of the matrix leads to proliferation of vascular smooth muscle cells or valvular myofibroblasts, causing lesion formation and growth. The elastolysis-triggered release of biologically active peptides attracts more macrophages, which in turn produce more proteolytic enzymes, promoting further expansion of the lesion. These biologically active peptides promote osteogenic differentiation of the cells. Patients with CRD have the additional complication of hyperphosphatemia, a mineral imbalance leading to phosphate-induced release of matrix vesicles and apoptosis — which, in turn, accelerates calcification of vascular smooth muscle cells or valvular myofibroblasts.[41, 84, 112]

## Inverse Correlation of Arterial and Aortic Valve Calcification with Osteoporotic Bone Remodeling: A Role for Inflammation

Clinical studies have suggested associations between cardiovascular calcification, atherosclerosis, CRD, and osteoporosis.[113,114] Although this link initially was thought to be age-related, epidemiological evidence has demonstrated an age-independent correlation between bone mineral density (BMD) and cardiovascular events.[115-117] These studies noted a reduction in BMD along with arterial calcification in humans; this was corroborated by mouse studies that further demonstrated that atherosclerosis susceptibility corresponds with reduced bone mineralization.[118, 119] Limited studies suggest a mechanism behind this seemingly paradoxical event — a recent literature review, for example, discussed the possibility that osteoporosis and cardiovascular calcification are tissue-specific responses to chronic inflammation.[2] However the precise nature of the reciprocal regulation of arterial calcification, calcific aortic valve disease, and bone osteogenesis remains unknown.

### Arterial, Valvular and Bone Mineralization have Shared Pro-inflammatory Mechanisms

In our recent study[42] the relationship between cardiovascular calcification (arterial and valvular) and long bone remodeling (cortical and trabecular) was simultaneously quantified for the first time in mice with hyperlipidemia and with CRD using optical molecular imaging and high-resolution 3D micro-CT.[41] We hypothesized that cardiovascular calcification progresses with inflammation and inversely correlates with BMD. The study sought to provide mechanistic evidence on the role of inflammation in calcification and osteoporosis. Our results on the opposing effects of inflammation in cardiovascular organs (soft tissues) and in bone agree with previous reports. This study provided new insight into the relationship between osteoporosis and cardiovascular calcification, and suggested shared inflammatory mechanisms of ectopic calcification and bone osteolysis. Directly comparing macrophage burden and progression of osteogenic changes via NIRF imaging in each region of the same animals *in vivo* and *ex vivo*, enabled us to discover that bone osteogenic activity and BMD decrease as atherosclerosis and calcific aortic valve disease develop, and that the degree of cardiovascular calcification correlates directly with loss of BMD. Molecular imaging identified strong inflammatory activity in arteries, aortic valves, and long bones of mice with atherosclerosis and CRD, demonstrating that inflammation at these three locations is related probably via systemic or circulating inflammatory cues.

Osteoporosis was associated epidemiologically with atherosclerosis and hyperlipidemia many decades ago.[120] More recently, a clinical study reported that stenotic coronary narrowing was more prevalent among women with low BMD,[121] while preclinical studies have suggested that hyperlipidemia promotes cardiovascular calcification and reduces BMD via increased bone resorption.[118] Other evidence suggests that osteoporosis may contribute to cardiovascular calcification by releasing biochemical factors, such as increased amounts of circulating phosphate and calcium and decreased amounts of parathyroid hormone, which promote osteogenesis and mineralization of the arterial wall and aortic valve.[122, 123] This evidence agrees with results from studies showing that agents that block bone resorption in animals also block vascular calcification.[124] Biphosphonates, used in the management of osteoporosis, have been associated with decreased cardiovascular calcification in elderly subjects and increased prevalence of valvular, aortic and coronary arterial calcification in younger women with subclinical cardiovascular disease.[125] The reduction of cardiovascular calcification in elderly women may be due to decreased cholesterol levels and pro-inflammatory cytokines, or alternatively due to declining bone resorption and subsequent decrease of circulating calcium phosphate. Additional studies are needed to elucidate the beneficial effect of biphosphonates on bone metabolism in elderly patients; however further evaluation of unfavorable effects in younger women is warranted.

The use of molecular imaging in our imaging study has strongly suggested that systemic and local inflammation, seemingly paradoxically, drives both cardiovascular calcification and bone loss. In simplified terms, inflammation causes differential tissue responses that result in "hardening" of soft tissue and "softening" of hard tissue,[126] but it is unclear whether the pathways are similar. It has been suggested that inflammation in cardiovascular and bone regions may act through the NF-κB-RANKL pathway, but whether this mechanism is utilized simultaneously for cardiovascular calcification and osteoporosis is uncertain. Future studies could use molecular imaging to elucidate these signaling pathways, as there are still many questions to answer.

## Conclusions

Both *in vitro* and clinical studies have suggested that a sequence of active osteogenic processes contributes to cardiovascular calcification, and that osteogenic activity is initiated by inflammation. State-of-the-art multimodality molecular imaging has provided the

opportunity to effectively visualize *in vivo* these different biological processes simultaneously, by using two spectrally distinct imaging agents, and thus substantiate this theory. The studies cited in this review have led us to the hypothesis that calcification of the artery and the aortic valve are mechanistically similar, and thus the same sophisticated imaging modalities can examine both processes (Figure 3). A key finding of our studies was that molecular imaging techniques can visualize atherosclerotic plaques and aortic valve lesions in the early stages,[23, 34, 41, 50] which are undetectable by conventional imaging modalities. However this review does not aim to lessen the value of studies using histopathology and conventional imaging modalities, but rather support the development of innovative imaging techniques to enable further exploration of the pathogenesis of cardiovascular calcification.

Clinical trials have failed to demonstrate the benefit of statin therapy in slowing the progression of valve calcification.[21] This may be due to the late implementation of the statins – after aortic valve calcification has progressed to the irreversible stage. Adjusting atherogenic factors and/or using pharmacological therapies that target pro-inflammatory pathways may impede or even halt the progression of cardiovascular calcification, when implemented during the early stages of calcification. For example, anti-inflammatory therapies or the preservation of elastin integrity — via the inhibition of elastolytic cathepsins such as cathepsin S — might prevent cardiovascular calcification and its complications when introduced early. In addition, macrophage- or smooth muscle cell-targeted silencing of pro-inflammatory or pro-osteogenic factors with siRNA may retard the progression of calcification. Combining optical imaging agents with anti-calcification drugs (e.g., bisphosphonate) within the targeted construct may provide a unique platform for specific imaging and therapy for preclinical models and for future clinical translation. Moreover, therapeutic interventions for calcification-prone patients with CRD could target inflammation, matrix degradation, or mineral imbalance. Further studies are required to establish the relationship between cardiovascular calcification and osteoporosis, to identify factors to target for the reciprocal regulation of these processes.

Molecular imaging and particularly optical imaging is anticipated to have the most impact on preclinical research, including identification of novel targets and mechanisms and evaluation of imaging tools in preclinical models. It is apparent that various limitations and issues need to be addressed before the molecular imaging approach will be used clinically. Despite this several studies are producing favorable results in regards to the clinical translation of this evolving modality. Molecular imaging techniques, which utilize radionuclides could be available for clinical use within the next 1-3 years, as many of the imaging probes have already been routinely used clinically.[52] The complementary use of targeted molecular imaging agents with MRI has demonstrated promising results in animal studies. In addition, molecular imaging agents are being considered for clinical trials.[50, 53] Indeed the use of cysteine protease-activatable NIRF agent, is already believed to be clinically favourable[55] the backbone of this imaging agent has been found to be safe in clinical trials[127] and the NIR fluorochromes are similar to those of an agent used widely in retinal angiography.[128] The development of promising clinically feasible technology (e.g. intravascular NIRF for coronary artery imaging[55,56]) is ongoing and each study leads us closer to the clinical translation of this imaging modality.

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## Abbreviations

| | |
|---|---|
| **apoE** | apoliprotein-E |
| **BMD** | bone mineral density |
| **CRD** | chronic renal disease |
| **CT** | computed tomography |
| **FMT** | fluorescence-mediated tomography |
| **IGF-1** | insulin-like growth factor 1 |
| **IL** | interleukin |
| **IVUS** | intravascular ultrasonography |
| **ICAM-1** | intercellular adhesion molecule 1 |
| **MMP** | matrix metalloproteinases |
| **MRI** | magnetic resonance imaging |
| **NF-kB** | nuclear factor kappa B |
| **NHLBI** | National Heart, Lung and Blood Institute |
| **NIR** | near-infrared |
| **NIRF** | near-infrared fluorescence |
| **PET** | positron emission tomography |
| **RANK** | receptor activator of nuclear factor kappa B |
| **RANKL** | RANK ligand |
| **Runx2** | runt-related transcription factor 2 |
| **TGF-β** | transforming growth factor beta |
| **TNF-α** | tumor necrosis factor alpha |
| **VCAM-1** | vascular cell adhesion molecule 1 |
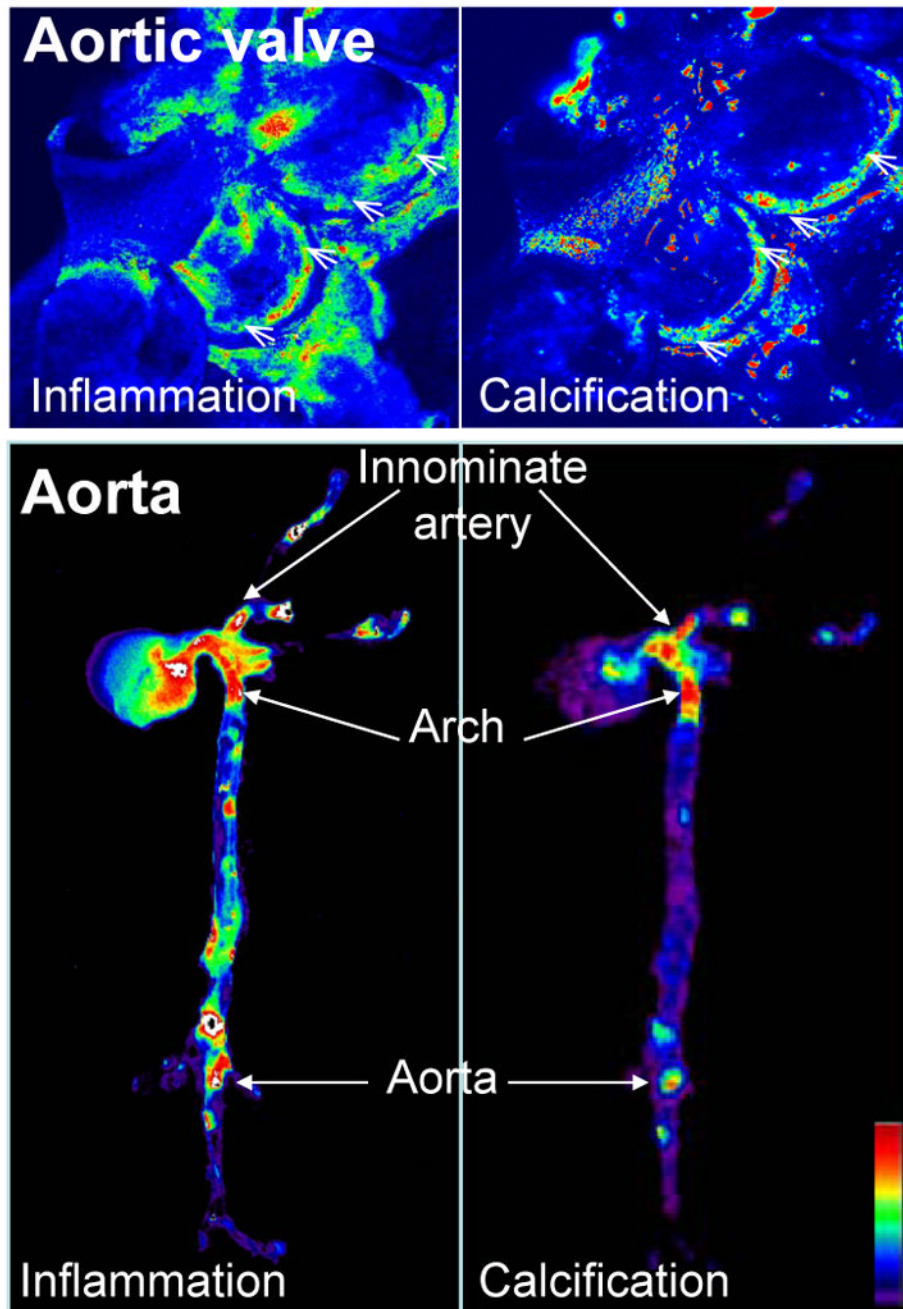
**Figure 1. Molecular imaging correlated inflammatory activity, defined as macrophage accumulation, with osteogenesis in the aortic valves and aortas of apoE$^{-/-}$ mice**
Mice were injected with magneto-fluorescent nanoparticles to visualize macrophage accumulation (left panels), and a spectrally distinct bisphosphonate-imaging agent that binds to nanomolar concentrations of hydroxyapatite to detect osteogenic activity (right panels). In the aortic valve (top) and in the aorta (bottom) molecular imaging detected that inflammatory and osteogenic activities colocalized in the areas of highest mechanical stresses at the aortic valve attachment (arrowheads) and at the atherosclerosis prone areas such as innominate artery, aortic arch, and abdominal aorta (arrows). Images were processed

using Osirix software. High signal intensities are shown in red-yellow-green. (Adapted with permission from Aikawa E. et al., *Circulation* 2009 and Aikawa E. et al., *Circulation* 2007).
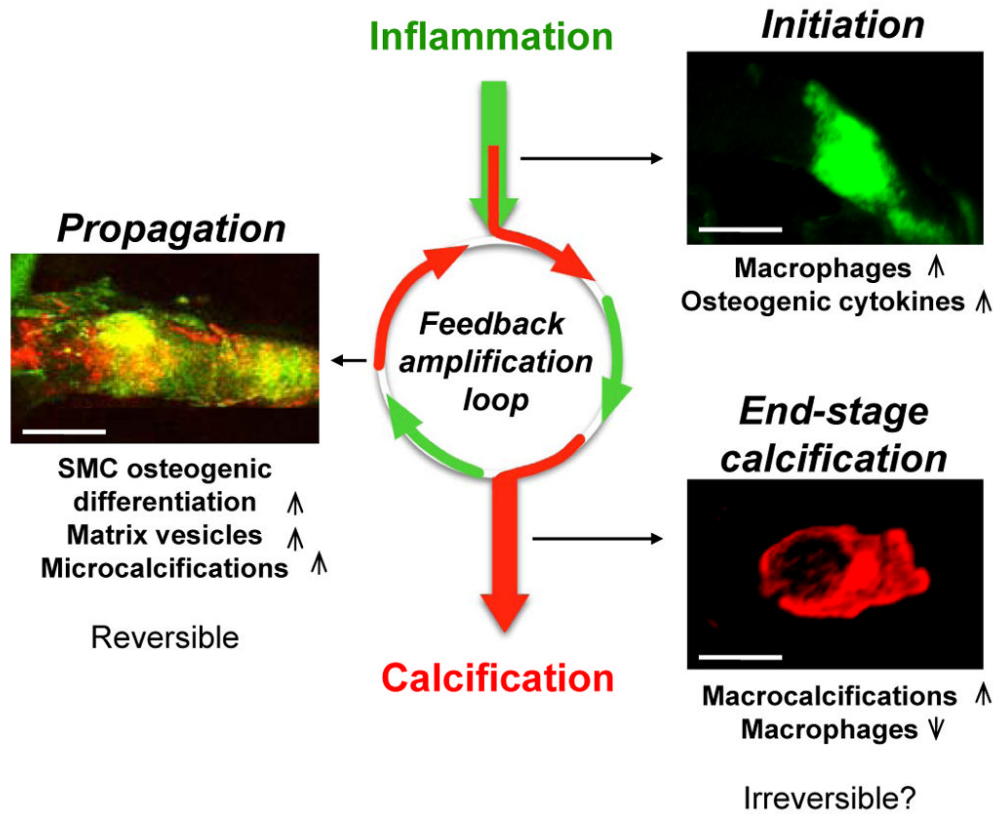
**Figure 2. The inflammation-dependent mechanism of calcification visualized by molecular imaging**

Sequential intravital fluorescence microscopy was used to observe changes in inflammation and osteogenesis in atherosclerotic arteries. Two spectrally distinct molecular imaging agents were administered through intravenous injection into apoE$^{-/-}$ mice and the common carotid artery was visualized: magneto-fluorescent nanoparticle to target macrophages (green), and a bisphosphonate-imaging agent to detect osteogenic activity/microcalcifications (red). Three different stages could be identified depending on atheroma progression in 20, 30 and 72 week-old apoE-/- mice feed high-fed diet. In the *initiation* phase – associated with increased macrophages and pro-osteogenic cytokines – only inflammation was observed (green). In the *propagation* phase – associated with osteogenic activity and generation of microcalcification – inflammation (green) and calcification (red) overlapped (yellow), suggesting that these two processes develop in parallel. Continued inflammation, in parallel with advancing plaque induces further formation of microcalcifications that provoke additional pro-inflammatory responses from macrophages suggesting that feedback amplification loop of calcification and inflammation drives disease progression. Reducing inflammation through anti-inflammatory therapy at this stage may retard osteogenesis and subsequent calcification. In the *end-stage* phase – associated with increased mineralization and decreased macrophages – macrocalcifications (red) were observed with limited inflammation. Reversing advanced calcification at this late stage is deemed difficult.
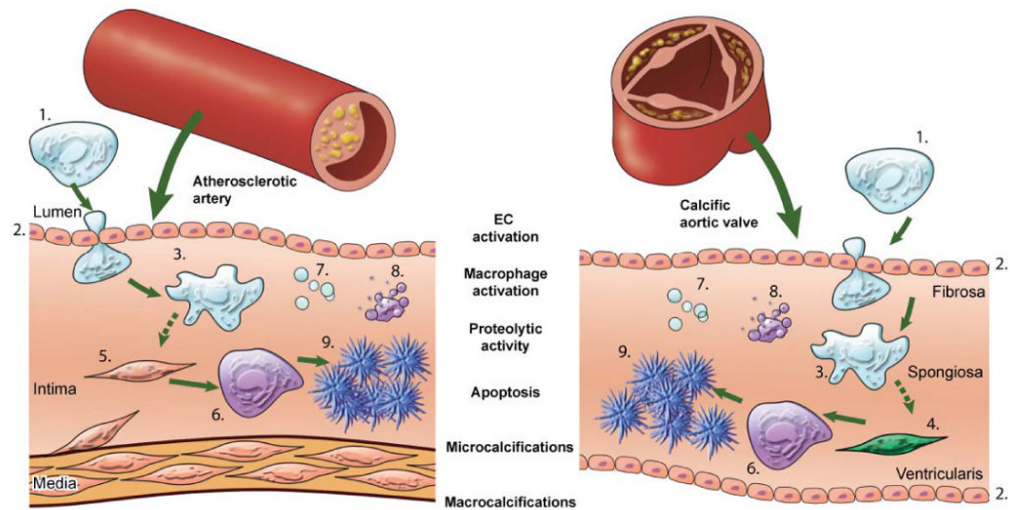
**Figure 3. Imaging shared molecular mechanisms underlying arterial and aortic valve calcification**

The studies cited in this review showed that calcification is an active process composed of a sequence of events, initiated by inflammation and resulting in mineralization. Pro-inflammatory monocytes (1) are recruited to a site via activated endothelial cells (EC) (2). The activation of EC causes increased expression of adhesion molecules, such as VCAM-1, which can be visualized by NIRF imaging using a VCAM-1-targeted agent. Subsequent macrophage (3) accumulation follows, which can be analyzed using NIRF macrophage-targeted nanoparticles. The release of proteolytic enzymes — including matrix metalloproteases and cathepsins — by macrophages, which stimulates the differentiation of myofibroblasts (4) and smooth muscle cells (5) into osteoblasts, can be visualized by molecular imaging using activatable imaging agents. Osteogenic activity in the form of osteoblast (6) formation and microcalcifications associated with generation of calcified matrix vesicles (7) can be identified by bisphosphonate-conjugated imaging agent, while apoptotic bodies (8) can be detected by fluorescently-tagged Annexin A5 imaging probe. Macrocalcifications (9) can be readily detected by molecular imaging and conventional imaging techniques. This schematic visualizes the theory that calcification follows similar processes in both the artery and the aortic valve, and summarizes how NIRF imaging can visualize dynamic sequences of calcification process.