13 Identification of Early Pathological Events in Calcific Aortic Valve Disease by Molecular Imaging

Eduardo Martínez-Martínez and Elena Aikawa

Introduction

The aberrant mineralization of soft tissues (i.e., ectopic calcification) recently has been associated with various chronic and degenerative conditions in humans. Cardiovascular tissue, particularly vasculature and valves, are among the peripheral tissues affected by the pathological deposition of calcium phosphate in connective tissue [1]. Although whether ectopic calcification originates from the same causes in different soft tissues is still unclear, the mineralization process seems to be triggered by chronic inflammatory conditions. This relationship has been especially demonstrated in the cardiovascular system, where macrophage infiltration and subsequent release of proteolytic enzymes and cytokines precedes the transformation of vascular smooth muscle cells and valve interstitial cells (VICs) into osteoblast-like cells. Over the last two decades, cardiovascular calcification has gradually gained the attention of more research groups with the acknowledgement that calcification constitutes an independent risk factor for cardiovascular morbidity and mortality [2–7]. Moreover, the prevalence of arterial calcification and calcific aortic valve disease (CAVD) is expected to increase, due to aging worldwide population. An estimated 2.1 million patients in Europe and 3.5 million patients in North America will suffer from severe CAVD by 2025 and 2050, respectively [8]. Therefore, a better understanding of the mechanisms underlying the initiation of CAVD will lead to the development of novel diagnostic and therapeutic methods to improve patients' quality of life.

Common epidemiological risk factors such as age, hypercholesterolemia, metabolic syndrome, chronic renal disease, and diabetes mellitus have been identified for atherosclerosis and CAVD [5, 9, 10]. Nevertheless, different molecular mechanisms may participate in arterial and aortic valve calcification, as suggested by the lack of benefit of statin therapy on the reduction of valve calcification [11, 12]. Indeed, we now recognize that aortic valve calcification represents a late stage of a progressive disease that alters the anatomy and functionality of the aortic valve [13, 14]. In a healthy valve, a layer of endothelium encloses three distinguishable layers of connective tissue: fibrosa, spongiosa, and ventricularis. Heart valve disease begins with microstructural changes involving a degenerative remodeling of the fibrosa layer. This stage of the disease, characterized by a mild thickening of the valve without overt changes on blood outflow from the left ventricle, is known as aortic sclerosis. A subset of patients develops aortic stenosis, characterized by the presence of macroscopically calcific lesions and limited motion of the valve leaflets. Stiffening of the aortic valve results in major

E. Martínez-Martínez • E. Aikawa, MD, PhD (✉)
Division of Cardiovascular Medicine,
Center for Excellence in Vascular Biology,
Brigham and Women's Hospital,
Harvard Medical School, 77 Avenue Louis Pasteur,
NRB-741, Boston, MA 02115, USA
e-mail: eaikawa@partners.org

N.M. Rajamannan (ed.), *Molecular Biology of Valvular Heart Disease*,
DOI 10.1007/978-1-4471-6350-3_13,

hemodynamic changes and remodeling of the left ventricle that can lead to fatal myocardial infarction in these patients [14].

Currently, the only available treatment options for aortic stenosis are surgical or transcatheter valve replacements [15]. But these interventions are intended for the end stage of the disease, and generally result in a poor prognosis. Furthermore, pharmacological therapies—including statins, mineralocorticoid receptor antagonists, and bisphosphonates—have not been effective in clinical practice [12, 16–18]. Two interrelated challenges thus need to be addressed by CAVD investigators. First, we must identify molecular targets to stop the progression of CAVD at different stages of the disease; and second, we should endeavor to generate new non-invasive methods of diagnosis that allow the visualization and quantification of early events of CAVD. The technology available in current clinical practice, such as intravascular ultrasonography (IVUS), transthoracic echocardiography (TTE), computed tomography (CT), or magnetic resonance imaging (MRI), fails to detect microcalcifications and to measure other key cellular events that are crucial to detect before valve damage is irreversible.

Molecular imaging, an emerging modality, can be used to visualize molecular processes in vivo [19, 20]. This approach has been extremely useful in several biomedical areas, including cardiovascular research. The design of fluorescent probes to target specific molecules or to monitor certain enzyme activity have helped researchers to understand biological processes simultaneously in real time at cellular and molecular levels. This chapter aims to provide a review of the imaging studies that have been crucial to our understanding of the early stages of CAVD, and to give an overview of the most recent advancements with potential to be translated to the clinic.

Imaging of Aortic Valve Disease

The notion that inflammation plays a fundamental role in the development of cardiovascular calcification is quite recent [13, 14]. To a large extent, studies using fluorescence-based imaging techniques support the paradigm that an identifiable sequence of cellular events triggers cardiovascular calcification [21–24]. The most frequent imaging modalities used for this purpose are fluorescence reflectance imaging (FRI) and intravital confocal microscopy (IVM). These platforms offer great versatility, as two or more fluorochromes can be employed in the same experiment to visualize two different biological processes simultaneously. The macroscopic resolution of FRI makes this platform ideal for imaging entire organs, while IVM is more suitable for obtaining information at the microscopic level. Both imaging systems have been used complementarily with a variety of targeted and activatable imaging agents. The targeted agents consist of an affinity ligand (antibody or small molecule) conjugated to a fluorochrome or magnetic compound [25–27]. The activatable agents must be chemically modified, commonly by an enzyme, in order to emit fluorescence. For in vivo applications, the near-infrared region of the light spectrum (600–900 nm) is preferred over visible or infrared light. The use of near-infrared fluorescence (NIRF) probes enables imaging from deeper regions of the tissue, avoids photon absorption, and increases the signal-to-noise ratio. Through the imaging of a battery of NIRF agents, we have been able to monitor over time the main cellular events leading to valve calcification: (1) endothelial activation, (2) macrophage infiltration, (3) extracellular matrix degradation, and (4) osteogenic activity in the aortic valves of hypercholesterolemic apolipoprotein E–deficient ($apoE^{-/-}$) mice [21–24].

Monitoring of Endothelial Activation and Inflammation In Vivo

In response to atherogenic factors, endothelial cells express a variety of adhesion and chemotactic molecules that initiate a local inflammatory response [28–31]. Furthermore, the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin increases in the valvular endothelium

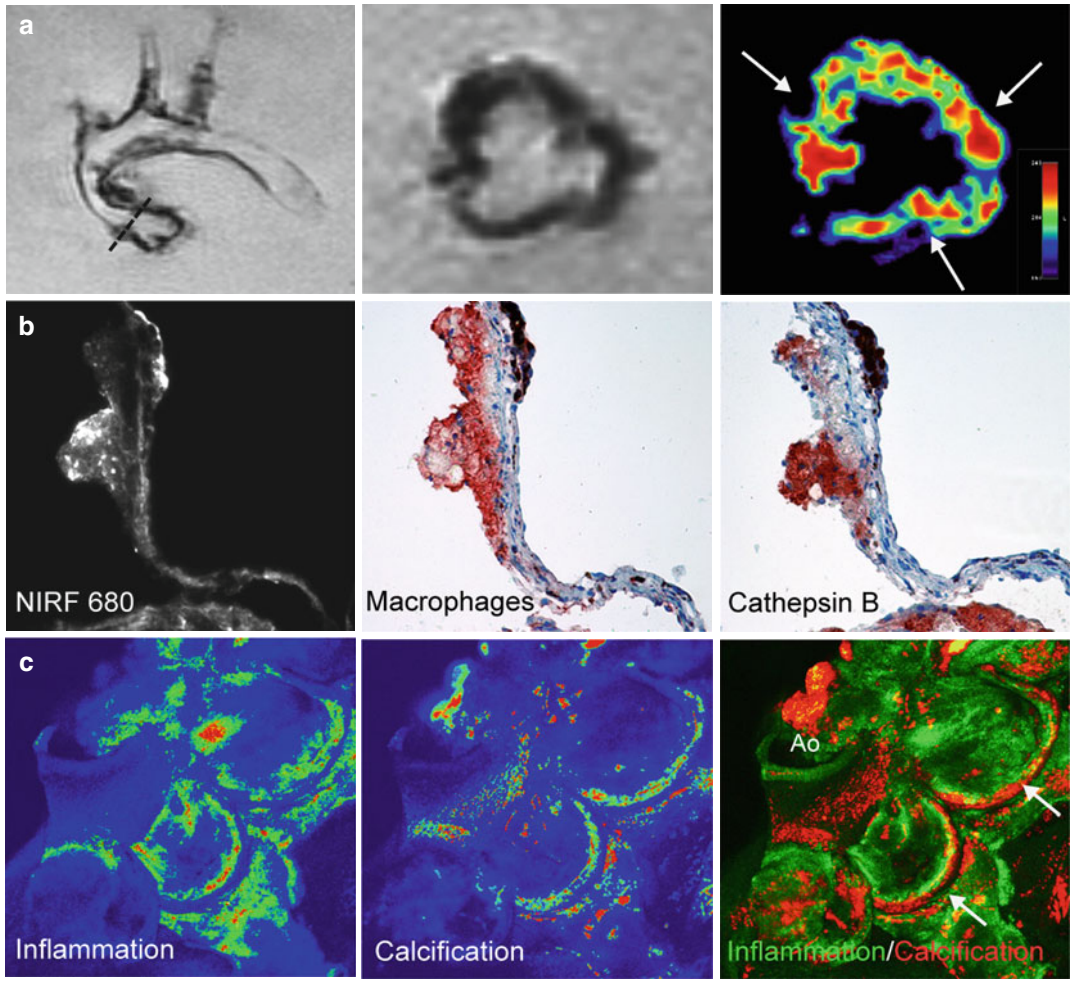

Fig. 13.1 Visualization of early events of aortic valve calcification through molecular imaging. (**a**), Endothelial cell activation occurs in the commissures of diseased aortic valves. (*Left*) Ex vivo MRI showing the long-axis view of the aortic arch and root. *Dotted line* depicts slice position of short-axis view (*middle*). Dark areas in the middle panel denotes where the nanoparticles were taken up by endothelial cells. (*Right*) Color-coded signal intensities of short-axis view show focused uptake of molecular agent in commissures (*arrows*). (**b**), NIRF microscopy with a protease-activatable agent for cathepsin activity (*left*). Immunochemistry colocalized NIRF signals with macrophages (Mac3) expressing cathepsin B. (**c**), In the aortic valve of atherogenic mice, the inflammatory activity correlates with osteogenesis as visualized by ex vivo IVM. (*Left*) Macrophage accumulation measured by accumulation of magnetofluorescent nanoparticles. (*Middle*) Bisphosphonate-imaging agent binds to nanomolar concentrations of hydroxyapatite to detects osteogenic activity. Molecular imaging allow inflammatory and osteogenic activities colocalized in the areas of highest mechanical stress at the aortic valve attachment (*arrows*) (Adapted from Aikawa et al. [21–23])

of diseased valves [31, 32]. Thus, adhesion molecules also may reflect an injury response of valvular endothelial cells. In support of this possibility, we found by MRI and NIRF microscopy that VCAM-1 is mainly distributed in the aortic valve leaflet near the region known as the commissure [23] (Fig. 13.1). This region is where the valve leaflet is attached to the aortic root, and is subjected to the greatest amount of mechanical stress during the cardiac cycle [33]. Interestingly, endothelial activation reflected by the expression of VCAM-1 has been found in an ex vivo model that recreates shear stress of the bicuspid aortic valve [34].

Through the use of magnetofluorescent nanoparticles, it was possible to determine that macrophages accumulate in regions of the leaflet that present endothelial cell activation [22, 23]. The NIRF signal from the nanoparticles was mainly located in the thickened regions of the valve containing abundant Mac3 immunoreactive cells. This finding suggested that macrophages are involved in the remodeling of the extracellular matrix (ECM) of the valve. Remarkably, the presence of macrophages determines how rapidly the aortic stenosis progresses [35]. Another consequence of macrophage accumulation is that the released cytokines, such as TGF-β, IL-1β, and TNF-α, induce the activation of VICs into myofibroblasts [36–38]. In our experiments, we have noticed a close spatial relationship between these cell types that needs further investigation. In this context, a pathological interaction between macrophages and VICs may lead to thickening and dysfunction of the valve through an aberrant remodeling of its architecture. A consideration about the interpretation of imaging experiments with polysaccharide-coated magnetic nanoparticles should be noted: a recent study found that valvular myofibroblasts also can uptake magnetonanoparticles, opening the possibility that to some extent, other cell types could accumulate these nanoparticles [39]. This finding is especially important considering that the immune infiltrate in CAVD also includes lymphocytes, plasma cells, and mast cells [40–43]. We therefore must generate cell-specific imaging agents to achieve better insight into the functions of different types of immune cells. Experiments with new imaging agents will help to determine if the timing and presence of a particular immune cell type associate with the progression and severity of calcification.

Extracellular Matrix Remodeling Promotes Valve Calcification

The release of matrix-degrading proteinases has been proposed as a mechanism to explain the pathobiology of CAVD [44–46]. Valvular myofibroblasts and macrophages synthesize matrix metalloproteinases (MMPs; e.g., collagenase-1/MMP-1, collagenase-3/MMP-13, gelatinase-A/MMP-2, and gelatinase-B/MMP-9) and cysteine endoproteases (cathepsins B, K, and S) [47–50]. Using hypercholesterolemic ApoE$^{-/-}$ mice, we have shown that regions of the aortic valve enriched with valvular myofibroblasts and macrophages displayed activity of cathpesins B and K, as well as MMP-2 and MMP-9 [21, 23]. The implications of collagen and elastin degradation are not only structural, but also functional. ECM composition has been proposed to modulate VIC calcification and proliferation [51–53]. A reduction in the number of interaction sites with ECM or by-products of ECM degradation may facilitate VIC transdifferentiation into an osteoblastic-like phenotype. In agreement with this notion, we found that cathepsin S activity accelerates arterial and valvular calcification in mice with atherosclerosis and chronic renal disease (CRD) [21]. Using a protease-activatable imaging agent specific for cathepsin S, we used intravital microscopy to detect increased activity of this elastase in regions displaying ostegenic activity (Fig 13.1). CRD-induced calcification was clearly diminished in cathepsin S-deficient mice, even with a similar macrophage accumulation in the valve leaflets. In in vitro experiments with vascular smooth muscle cells, elastin-derived peptides significantly induced the expression of alkaline phosphatase. Taken together, these findings suggest that elastin degradation modulates the calcification potential of myofibroblasts. In this regard, the elastin degradation products—known as matrikines or elastokines—regulate myofibroblastic migration, proliferation, and production of bone-regulating proteins [53–55]. An additional repercussion of the enhanced proteolytic activity can be the activation of proinflammatory precursors deposited in the ECM, which enhances the inflammatory response [56, 57].

Visualization of Early Osteogenic Activity Preceding Valve Calcification

The possibility of detecting nanomolar concentrations of calcium hydroxyapatite crystals through in vivo molecular imaging experiments constitutes one of the greatest technical advancements that have revolutionized our understanding

of cardiovascular calcification. While conventional histological techniques for calcium deposit detection (e.g., Von Kossa and alizarin red stains) are only useful for staining advanced calcific lesions, a bisphosphonate-conjugated fluorescent agent (Osteosense 680/750) allows the localization of active sites of biomineralization in early stages of the calcification process. With the aid of multichannel NIRF microscopy, and the agent either injected into or applied directly on to a tissue section, we have associated osteogenic activity with precise proinflammatory events [21–23, 58]. In our in vivo and ex vivo experiments, osteogenic activity is spatially correlated with macrophage accumulation and proteolytic/elastolytic activity (Fig. 13.1) [21–23]. Of note, NIRF longitudinal studies in the same animals allowed us to monitor dynamic changes in inflammation and osteogenesis simultaneously in vivo. In the second stage of calcification (propagation), these two processes develop in close proximity, and calcification typically appears in areas preceded by macrophage accumulation [22]. Overall, these findings suggest that inflammation precedes calcification. NIRF imaging experiments have been extensively validated through complementary immunohistochemical analysis showing expression of osteopontin, osteocalcin, Runx2, and Notch1 by valvular myofibroblasts [23]. Our results reveal that valve calcification is a highly complex process triggered by inflammation, leading to the expression of osteogenic markers by valvular myofibroblasts. Moreover, these findings provide new insights into the initiation of early calcific lesions in aortic valves (Fig. 13.2). The initial deposition of hydroxyapatite crystals appears to be in the form of microcalcification. These discrete regions of mineralization may cause recurrent activation of an inflammatory response that would enhance ECM degradation and VIC differentiation. Crystal nucleation may start on the surface or lumen of extracellular vesicles released by myofibroblasts [22, 58–60]. The gradual aggregation of microcalcification could result in the large spherical particles composed of highly crystalline hydroxyapatite that recently have been observed in human aortic valves by electron scanning microscopy, even in the absence of macroscopic lesions [61].

Molecular imaging methods offer the great advantage for longitudinal studies to follow the evolution of a biological process over time in the same group of animals. This advantage has been exploited to test the hypothesis that inflammation precedes the events of calcification. Carotid arteries of $ApoE^{-/-}$ mice were imaged to quantify macrophage accumulation and osteogenesis after 10 weeks of a high-cholesterol diet. The carotid arteries showed clear signs of inflammation, but barely any signs of osteogenesis [22]. In a subgroup of these mice, the atherogenic diet was supplemented with atorvastatin and a second imaging session was performed in the same carotid artery region after 10 more weeks. Statin treatment prevented the progression of macrophage burden and osteogenesis, supporting the hypothesis that inflammation promotes osteogenic activity. Similar strategies have been used to find therapeutic targets that block progression of inflammation in the aortic valve [11, 12]. Recently, a role of Notch ligand Delta-like 4 (Dll4) has been proposed in macrophage-mediated inflammation underlying the pathogenesis of cardiometabolic disorders [62]. By ex vivo mapping experiments using FRI, aortic valve calcification was attenuated after blockade of Dll4-Notch signaling with an anti-Dll4 monoclonal antibody [63]. Future work is necessary to understand the mechanisms that promote accumulation of macrophages in the valve leaflets, as well as the signaling pathways involved in inducing the inflammatory response.

Epidemiological surveys have indicated an association among osteoporosis, atherosclerosis, CRD, and cardiovascular calcification [64–66]. In humans, arterial and aortic valve calcification correlates with decreased bone mineral density (BMD). In atherogenic mice, a high-fat diet induces osteoblastic apoptosis, which in turn reduces bone formation [67]. Using 3D micro-CT and optical molecular imaging, we found that in atherosclerotic and CRD mice, the degree of cardiovascular calcification relates inversely to BMD. Most importantly, the bones of these mice also showed signs of inflammatory activity, visualized as uptake of NIRF-conjugated iron nanoparticles. No definitive explanation currently exists for the seemingly disparate responses of cardiac and bone tissue to inflammation.

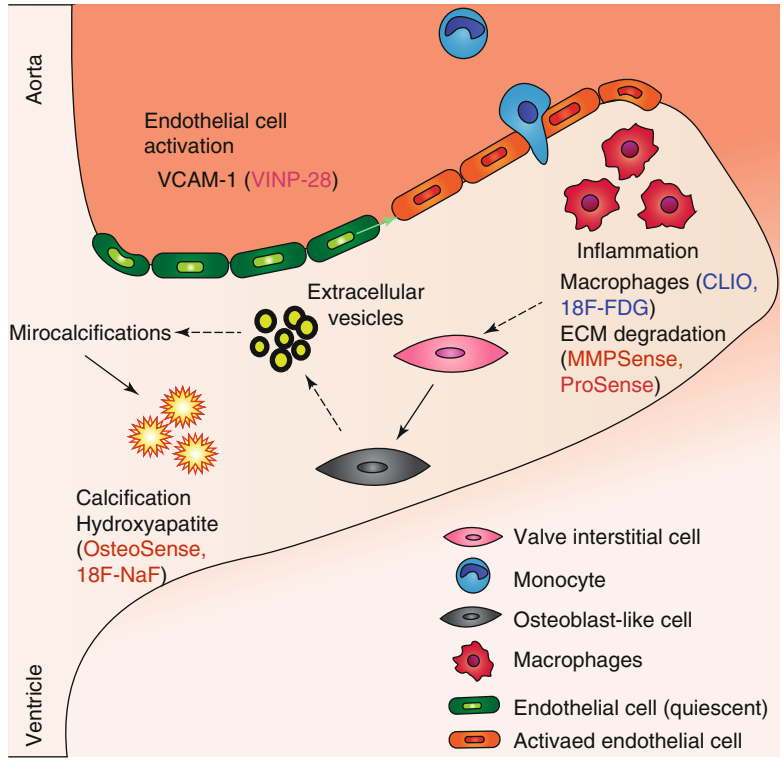

Fig 13.2 Major cellular events underlying calcific aortic valve disease as revealed by molecular imaging. Chronic inflammatory conditions lead to the activation of endothelial cells denoted by an increased expression of adhesion molecules, such as VCAM-1, which can be visualized by NIRF/MRI imaging with magnetofluorescent nanoparticles. The endothelium activation promotes the recruitment of circulating monocytes, which eventually results in macrophage accumulation in the valve interstitium. This process has been analyzed with NIRF macrophage-targeted nanoparticles (CLIO) and a radiolabeled glucose analogue (18F-FDG). The inflammatory process is associated with an extensive remodeling of the extracellular matrix triggered by the release of proteolytic enzymes. Specific probes for matrix metalloproteases and cathepsins have been developed for NIRF applications. Overall the inflammatory environment stimulates the differentiation of valvular myofibroblast into osteoblasts. These osteoblast-like cells release active vesicles capable of nucleating hydroxyapatite on their membranes. Microcalcifications can be identified by a bisphosphonate-conjugated imaging agent before macrospcopic lesions and valvular dysfunction are noted

Osteoporotic bones may release mineralization-promoting signals into the circulation that eventually impact the cardiovascular system [68]. Alternatively, osteoporosis and calcification have been proposed as tissue-specific responses to chronic inflammation [69].

Perspectives for Valve Imaging and Clinical Applications

Despite the enormous utility of IVM, FRI, and other imaging modalities to our understanding of the mechanisms underlying CAVD, these platforms present several disadvantages in their clinical application. Most of these modalities involve an invasive approach and have limited tissue penetration [20]. Considering that the detection of microcalcification formation represents an ideal time point for effective intervention, we need innovative imaging technologies with resolutions capable of their identification. Some promising recent efforts in this direction have combined the widely used technology available in the clinic with molecular imaging agents. Positron emission tomography (PET) combined with CT was used to assess in humans the degree of inflammation and calcification at different stages

of aortic valve disease. A glucose analogue, 18F-Fluorodeoxyglucose (18F-FDG)—uptaken by cells with high metabolic demands, such as macrophages—was used to target inflammatory activity [70, 71]. Sites of active mineralization were identified with 18F-Sodium fluoride (18F-NAF), which directly reacts with hydroxyapatite crystals [72–75]. The results of this study indicate that 18F-NAF allows the detection of regions of microcalcification and consolidated calcific lesions, thus representing a major advance for CAVD diagnostics. The 18F-FDG signal was observed in early stages of the disease, as would be expected from an inflammation marker, but it was also present in calcific regions. The latter may suggest that the incorporation of 18F-FDG may not only reflect inflammatory activity, as macrophages also exhibit increased glucose uptake in response to hypoxia [76]. Thus, additional experiments are needed to validate the use of glucose analogs as markers of inflammation in CAVD [77].

A new technology, micro-optical coherence tomography (μOCT), is one of the most promising approaches in terms of high resolution. This new imaging system allows the visualization of cellular and subcellular features with a resolution of less than 1 μm [78]. The cytoarchitecture of the atherosclerotic artery recently was studied using this technique; remarkably, cholesterol crystals, superficial calcium nodules, and microcalcifications were all visualized. Although conceived as an invasive catheter for its clinical application, μOCT has the potential for improving diagnosis by providing real-time information obtainable only with histological processes.

The advances over the past several years regarding in vivo and ex vivo imaging techniques have accelerated our understanding of cardiovascular calcification. New efforts are oriented to understand better the role of inflammation as an inducer of calcification. Clinical practice soon will benefit from the availability of imaging techniques that will guide the decision of eliminating risk factors from patients showing microcalcification. The need for innovative techniques suitable for the clinic will also aid in the evaluation of drugs with therapeutic potential for aortic valve calcification.

Acknowledgements This work was supported by a National Institute of Health grants (R01HL114805 and R01HL109506 to E.A.) EMM was a Research Fellow supported by Consejo Nacional de Ciencia y Tecnología (CONACYT; Estancias Postdoctorales y Sabáticas al extranjero: 175413) and Fundación México en Harvard, A.C. The authors thank Sara Karwacki for her editorial assistance.

References

1. Li Q, Uitto J. Mineralization/anti-mineralization networks in the skin and vascular connective tissues. Am J Pathol. 2013;183:10–8.
2. Budoff MJ, Shaw LJ, Liu ST, et al. Long-term prognosis associated with coronary calcification: observations from a registry of 25,253 patients. J Am Coll Cardiol. 2007;49:1860–70.
3. Abedin M, Tintut Y, Demer LL. Vascular calcification: mechanisms and clinical ramifications. Arterioscler Thromb Vasc Biol. 2004;24:1161–70.
4. Katz R, Budoff MJ, Takasu J, et al. Relationship of metabolic syndrome with incident aortic valve calcium and aortic valve calcium progression: the Multi-Ethnic Study of Atherosclerosis (MESA). Diabetes. 2009;58:813–9.
5. Nasir K, Katz R, Al-Mallah M, et al. Relationship of aortic valve calcification with coronary artery calcium severity: the Multi-Ethnic Study of Atherosclerosis (MESA). J Cardiovasc Comput Tomogr. 2010;4:41–6.
6. Takasu J, Budoff MJ, O'brien KD, et al. Relationship between coronary artery and descending thoracic aortic calcification as detected by computed tomography: the Multi-Ethnic Study of Atherosclerosis. Atherosclerosis. 2009;204:440–6.
7. Kelly-Arnold A, Maldonado N, Laudier D, et al. Revised microcalcification hypothesis for fibrous cap rupture in human coronary arteries. Proc Natl Acad Sci U S A. 2013;110:10741–6.
8. Osnabrugge RL, Mylotte D, Head SJ, et al. Aortic stenosis in the elderly: disease prevalence and number of candidates for transcatheter aortic valve replacement: a meta-analysis and modeling study. J Am Coll Cardiol. 2013;62:1002–12.
9. Stewart BF, Siscovick D, Lind BK, et al. Clinical factors associated with calcific aortic valve disease. Cardiovascular Health Study. J Am Coll Cardiol. 1997;29:630–4.
10. Allison MA, Cheung P, Criqui MH, et al. Mitral and aortic annular calcification are highly associated with systemic calcified atherosclerosis. Circulation. 2006;113:861–6.
11. Cowell SJ, Newby DE, Prescott RJ, et al. A randomized trial of intensive lipid-lowering therapy in calcific aortic stenosis. N Engl J Med. 2005;352:2389–97.
12. Rossebo AB, Pedersen TR, Boman K, et al. Intensive lipid lowering with simvastatin and ezetimibe in aortic stenosis. N Engl J Med. 2008;359:1343–56.

13. Rajamannan NM, Evans FJ, Aikawa E, et al. Calcific aortic valve disease: not simply a degenerative process a review and agenda for research from the National Heart and Lung and Blood Institute Aortic Stenosis Working Group. Circulation. 2011;124:1783–91.
14. Otto CM. Calcific aortic stenosis – time to look more closely at the valve. N Engl J Med. 2008;359:1395–8.
15. Clark MA, Duhay FG, Thompson AK, et al. Clinical and economic outcomes after surgical aortic valve replacement in Medicare patients. Risk Manag Healthc Policy. 2012;5:117–26.
16. Jaffe IZ, Tintut Y, Newfell BG, et al. Mineralocorticoid receptor activation promotes vascular cell calcification. Arterioscler Thromb Vasc Biol. 2007;27:799–805.
17. Gkizas S, Koumoundourou D, Sirinian X, et al. Aldosterone receptor blockade inhibits degenerative processes in the early stage of calcific aortic stenosis. Eur J Pharmacol. 2010;642:107–12.
18. Elmariah S, Delaney JA, O'brien KD, et al. Bisphosphonate use and prevalence of valvular and vascular calcification in Women MESA (The Multi-Ethnic Study of Atherosclerosis). J Am Coll Cardiol. 2010;56:1752–9.
19. Pittet MJ, Weissleder R. Intravital imaging. Cell. 2011;147:983–91.
20. Weissleder R, Ntziachristos V. Shedding light onto live molecular targets. Nat Med. 2003;9:123–8.
21. Aikawa E, Aikawa M, Libby P, et al. Arterial and aortic valve calcification abolished by elastolytic cathepsin S deficiency in chronic renal disease. Circulation. 2009;119:1785–94.
22. Aikawa E, Nahrendorf M, Figueiredo JL, et al. Osteogenesis associates with inflammation in early-stage atherosclerosis evaluated by molecular imaging in vivo. Circulation. 2007;116:2841–50.
23. Aikawa E, Nahrendorf M, Sosnovik D, et al. Multimodality molecular imaging identifies proteolytic and osteogenic activities in early aortic valve disease. Circulation. 2007;115:377–86.
24. Hjortnaes J, Butcher J, Figueiredo JL, et al. Arterial and aortic valve calcification inversely correlates with osteoporotic bone remodelling: a role for inflammation. Eur Heart J. 2010;31:1975–84.
25. Zaheer A, Lenkinski RE, Mahmood A, et al. In vivo near-infrared fluorescence imaging of osteoblastic activity. Nat Biotechnol. 2001;19:1148–54.
26. Zaheer A, Murshed M, De Grand AM, et al. Optical imaging of hydroxyapatite in the calcified vasculature of transgenic animals. Arterioscler Thromb Vasc Biol. 2006;26:1132–6.
27. Kozloff KM, Volakis LI, Marini JC, et al. Near-infrared fluorescent probe traces bisphosphonate delivery and retention in vivo. J Bone Miner Res. 2010;25:1748–58.
28. Cybulsky MI, Gimbrone Jr MA. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science. 1991;251:788–91.
29. Nakashima Y, Raines EW, Plump AS, et al. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. Arterioscler Thromb Vasc Biol. 1998;18:842–51.
30. Obrien KD, Allen MD, Mcdonald TO, et al. Vascular cell-adhesion molecule-1 is expressed in human coronary atherosclerotic plaques – implications for the mode of progression of advanced coronary atherosclerosis. J Clin Invest. 1993;92:945–51.
31. Nahrendorf M, Jaffer FA, Kelly KA, et al. Noninvasive vascular cell adhesion molecule-1 imaging identifies inflammatory activation of cells in atherosclerosis. Circulation. 2006;114:1504–11.
32. Ghaisas NK, Foley JB, O'briain DS, et al. Adhesion molecules in nonrheumatic aortic valve disease: endothelial expression, serum levels and effects of valve replacement. J Am Coll Cardiol. 2000;36:2257–62.
33. Thubrikar MJ, Aouad J, Nolan SP. Patterns of calcific deposits in operatively excised stenotic or purely regurgitant aortic valves and their relation to mechanical stress. Am J Cardiol. 1986;58:304–8.
34. Sun L, Chandra S, Sucosky P. Ex vivo evidence for the contribution of hemodynamic shear stress abnormalities to the early pathogenesis of calcific bicuspid aortic valve disease. PLoS One. 2012;7:e48843.
35. Akahori H, Tsujino T, Naito Y, et al. Intraleaflet haemorrhage is associated with rapid progression of degenerative aortic valve stenosis. Eur Heart J. 2011;32:888–96.
36. Yu Z, Seya K, Daitoku K, et al. Tumor necrosis factor-α accelerates the calcification of human aortic valve interstitial cells obtained from patients with calcific aortic valve stenosis via the BMP2-Dlx5 pathway. J Pharmacol Exp Ther. 2011;337:16–23.
37. Clark-Greuel JN, Connolly JM, Sorichillo E, et al. Transforming growth factor-beta1 mechanisms in aortic valve calcification: increased alkaline phosphatase and related events. Ann Thorac Surg. 2007;83:946–53.
38. Cushing MC, Mariner PD, Liao JT, et al. Fibroblast growth factor represses Smad-mediated myofibroblast activation in aortic valvular interstitial cells. FASEB J. 2008;22:1769–77.
39. Hamilton AM, Rogers KA, Belisle AJ, et al. Early identification of aortic valve sclerosis using iron oxide enhanced MRI. J Magn Reson Imaging. 2010;31:110–6.
40. Šteiner I, Krbal L, Rozkoš T, et al. Calcific aortic valve stenosis: immunohistochemical analysis of inflammatory infiltrate. Pathol Res Pract. 2012;208:231–4.
41. Wallby L, Janerot-Sjoberg B, Steffensen T, et al. T lymphocyte infiltration in non-rheumatic aortic stenosis: a comparative descriptive study between tricuspid and bicuspid aortic valves. Heart. 2002;88:348–51.
42. Otto CM, Kuusisto J, Reichenbach DD, et al. Characterization of the early lesion of 'degenerative' valvular aortic stenosis. Histological and immunohistochemical studies. Circulation. 1994;90:844–53.
43. Olsson M, Dalsgaard CJ, Haegerstrand A, et al. Accumulation of T lymphocytes and expression of interleukin-2 receptors in nonrheumatic stenotic aortic valves. J Am Coll Cardiol. 1994;23:1162–70.

44. Rabkin E, Aikawa M, Stone JR, et al. Activated interstitial myofibroblasts express catabolic enzymes and mediate matrix remodeling in myxomatous heart valves. Circulation. 2001;104:2525–32.
45. Aikawa E, Whittaker P, Farber M, et al. Human semilunar cardiac valve remodeling by activated cells from fetus to adult: implications for postnatal adaptation, pathology, and tissue engineering. Circulation. 2006;113:1344–52.
46. Aikawa M, Rabkin E, Okada Y, et al. Lipid lowering by diet reduces matrix metalloproteinase activity and increases collagen content of rabbit atheroma: a potential mechanism of lesion stabilization. Circulation. 1998;97:2433–44.
47. Helske S, Syvaranta S, Lindstedt KA, et al. Increased expression of elastolytic cathepsins S, K, and V and their inhibitor cystatin C in stenotic aortic valves. Arterioscler Thromb Vasc Biol. 2006;26:1791–8.
48. Deguchi JO, Aikawa E, Libby P, et al. Matrix metalloproteinase-13/collagenase-3 deletion promotes collagen accumulation and organization in mouse atherosclerotic plaques. Circulation. 2005;112:2708–15.
49. Rabkin-Aikawa E, Aikawa M, Farber M, et al. Clinical pulmonary autograft valves: pathologic evidence of adaptive remodeling in the aortic site. J Thorac Cardiovasc Surg. 2004;128:552–61.
50. Rabkin-Aikawa E, Farber M, Aikawa M, et al. Dynamic and reversible changes of interstitial cell phenotype during remodeling of cardiac valves. J Heart Valve Dis. 2004;13:841–7.
51. Masters KS, Shah DN, Walker G, et al. Designing scaffolds for valvular interstitial cells: cell adhesion and function on naturally derived materials. J Biomed Mater Res A. 2004;71:172–80.
52. Rodriguez KJ, Masters KS. Regulation of valvular interstitial cell calcification by components of the extracellular matrix. J Biomed Mater Res A. 2009;90:1043–53.
53. Yip CY, Chen JH, Zhao R, et al. Calcification by valve interstitial cells is regulated by the stiffness of the extracellular matrix. Arterioscler Thromb Vasc Biol. 2009;29:936–42.
54. Simionescu A, Sirnionescu DT, Vyavahare NR. Osteogenic responses in fibroblasts activated by elastin degradation products and transforming growth factor-beta 1 – role of myofibroblasts in vascular calcification. Am J Pathol. 2007;171:116–23.
55. Jacob MP, Fulop T, Foris G, et al. Effect of elastin peptides on ion fluxes in mononuclear-cells, fibroblasts, and smooth-muscle cells. Proc Natl Acad Sci U S A. 1987;84:995–9.
56. Belaaouaj AA, Li AG, Wun TC, et al. Matrix metalloproteinases cleave tissue factor pathway inhibitor – effects on coagulation. J Biol Chem. 2000;275:27123–8.
57. Qin X, Corriere MA, Matrisian LM, et al. Matrix metalloproteinase inhibition attenuates aortic calcification. Arterioscler Thromb Vasc Biol. 2006;26:1510–6.
58. New SE, Goettsch C, Aikawa M, et al. Macrophage-derived matrix vesicles: an alternative novel mechanism for microcalcification in atherosclerotic plaques. Circ Res. 2013;113:72–7.
59. New SE, Aikawa E. Role of extracellular vesicles in de novo mineralization: an additional novel mechanism of cardiovascular calcification. Arterioscler Thromb Vasc Biol. 2013;33:1753–8.
60. Kapustin AN, Davies JD, Reynolds JL, et al. Calcium regulates key components of vascular smooth muscle cell-derived matrix vesicles to enhance mineralization. Circ Res. 2011;109:E1–U41.
61. Bertazzo S, Gentleman E, Cloyd KL, et al. Nano-analytical electron microscopy reveals fundamental insights into human cardiovascular tissue calcification. Nat Mater. 2013;12:576–83.
62. Fung E, Tang SM, Canner JP, et al. Delta-like 4 induces notch signaling in macrophages: implications for inflammation. Circulation. 2007;115:2948–56.
63. Fukuda D, Aikawa E, Swirski FK, et al. Notch ligand delta-like 4 blockade attenuates atherosclerosis and metabolic disorders. Proc Natl Acad Sci U S A. 2012;109:E1868–77.
64. Farhat GN, Cauley JA, Matthews KA, et al. Volumetric BMD and vascular calcification in middle-aged women: the study of women's health across the nation. J Bone Miner Res. 2006;21:1839–46.
65. Farhat GN, Strotmeyer ES, Newman AB, et al. Volumetric and areal bone mineral density measures are associated with cardiovascular disease in older men and women: the health, aging, and body composition study. Calcif Tissue Int. 2006;79:102–11.
66. Frost ML, Grella R, Millasseau SC, et al. Relationship of calcification of atherosclerotic plaque and arterial stiffness to bone mineral density and osteoprotegerin in postmenopausal women referred for osteoporosis screening. Calcif Tissue Int. 2008;83:112–20.
67. Hirasawa H, Tanaka S, Sakai A, et al. ApoE gene deficiency enhances the reduction of bone formation induced by a high-fat diet through the stimulation of p53-mediated apoptosis in osteoblastic cells. J Bone Miner Res. 2007;22:1020–30.
68. Tekin GO, Kekilli E, Yagmur J, et al. Evaluation of cardiovascular risk factors and bone mineral density in post menopausal women undergoing coronary angiography. Int J Cardiol. 2008;131:66–9.
69. Demer LL, Tintut Y. Mechanisms linking osteoporosis with cardiovascular calcification. Curr Osteoporos Rep. 2009;7:42–6.
70. Rudd JH, Warburton EA, Fryer TD, et al. Imaging atherosclerotic plaque inflammation with [18F]-fluorodeoxyglucose positron emission tomography. Circulation. 2002;105:2708–11.
71. Rudd JH, Narula J, Strauss HW, et al. Imaging atherosclerotic plaque inflammation by fluorodeoxyglucose with positron emission tomography: ready for prime time? J Am Coll Cardiol. 2010;55:2527–35.
72. Blau M, Ganatra R, Bender MA. 18F-fluoride for bone imaging. Semin Nucl Med. 1972;2:31–7.
73. Grant FD, Fahey FH, Packard AB, et al. Skeletal PET with 18F-fluoride: applying new technology to an old tracer. J Nucl Med. 2008;49:68–78.

74. Derlin T, Richter U, Bannas P, et al. Feasibility of 18F-sodium fluoride PET/CT for imaging of atherosclerotic plaque. J Nucl Med. 2010;51:862–5.
75. Derlin T, Wisotzki C, Richter U, et al. In vivo imaging of mineral deposition in carotid plaque using 18F-sodium fluoride PET/CT: correlation with atherogenic risk factors. J Nucl Med. 2011;52:362–8.
76. Folco EJ, Sheikine Y, Rocha VZ, et al. Hypoxia but not inflammation augments glucose uptake in human macrophages: implications for imaging atherosclerosis with 18fluorine-labeled 2-deoxy-D-glucose positron emission tomography. J Am Coll Cardiol. 2011;58:603–14.
77. Marincheva-Savcheva G, Subramanian S, Qadir S, et al. Imaging of the aortic valve using fluorodeoxyglucose positron emission tomography increased valvular fluorodeoxyglucose uptake in aortic stenosis. J Am Coll Cardiol. 2011;57:2507–15.
78. Liu L, Gardecki JA, Nadkarni SK, et al. Imaging the subcellular structure of human coronary atherosclerosis using micro-optical coherence tomography. Nat Med. 2011;17:1010–4.