Giving Calcification Its Due: Recognition of a Diverse Disease
A First Attempt to Standardize the Field
Joshua D. Hutcheson,* Mark C. Blaser,* Elena Aikawa

Cardiovascular calcification is a growing burden and a leading predictor of and contributor to acute cardiovascular events. Arterial calcification associates with a 4-fold increase in cardiovascular events, and patients with aortic valve calcification have a 5-year event-free survival rate of only 26%,1,2 worse than that of many cancers. Despite massive healthcare costs and extensive research efforts, effective therapeutic strategies remain elusive. Cardiovascular calcification, often generalized as one disease, is in reality a multifaceted disorder that occurs in diverse milieus as a result of multiple, interacting pathogenic processes. Herein, we explore these variables and propose a set of guidelines for studying cardiovascular calcification.

Variability Between Tissues
Although vascular and valvular calcification share several risk factors, only ≈25% to 50% of patients with aortic valve calcification also present with significant coronary artery disease,3 suggesting that common risk factors initiate divergent disease processes.

Vascular Versus Valvular Calcification
Differences in prevalence are not unexpected, particularly when these 2 tissues are closely examined. Unlike the collagen and elastin-rich medial layer of the vasculature, the aortic valve has a complex trilayered architecture consisting of collagen, elastin, and proteoglycans. Calcific nodules occur almost exclusively on the stiffer, collagen-rich aortic side of the valve. Calcification takes the form of either hyperphosphatemic medial mineralization or inflammatory-driven intimal calcification, and although these discrete forms may coexist, they tend to be mechanistically distinct. Only 10% to 13% of valves and vasculature exhibit mature bone formation, while the remainder is dystrophic mineralization.4 The resident cell populations responsible for maintaining these tissue structures are also dissimilar: instead of vascular smooth muscle cells, valve interstitial cells populate the aortic valve. Valve interstitial cells are normally a quiescent fibroblastic population that may undergo myofibrogenesis, osteogenesis, and chondrogenesis during disease, thereby adding complexity to valvular calcification. The valve is particularly sensitive to biomechanical stimuli—calcific nodule formation occurs in vitro when valve interstitial cells are plated on stiffer substrates, pathological levels of stretch can drive osteogenesis, and myofibrogenic processes have key roles in the induction of calcification.5 Behavior of valvular endothelial cells differs dramatically from that of vascular endothelial cells in response to flow, and valve interstitial cells regulate pathological endothelial-to-mesenchymal transition of valvular endothelial cells toward an osteogenic phenotype.6 To date, animal models of valvular calcification have mimicked those used for vascular calcification on the assumption that the shared risk factors present in humans translate to mice. Hyperlipidemic (Ldlr−/−, Apoe−/−) or hyperphosphatemic (Klotho−/−, vitamin D overload) or renal dysfunction models (5/6 nephrectomy) are common, but the time courses required for valve calcification are longer than those needed for vascular mineralization. This could indicate that the valve has a greater intrinsic resistance to calcific insults or that current models of vascular calcification miss component(s) necessary for efficient valve disease development. Combinatorial models (Apoe−/−+5/6 nephrectomy) and direct wire injury of the valve hold promise for more rapid calcification.7

Diversity in Calcification Initiators
In addition to varying cellular and spatial contributors across cardiovascular tissue beds, calcification can initiate from various stimuli. Different stimuli lead to differences in timing of mineral deposition, underlying cellular and molecular responses, and potentially, the type of mineral deposited. In vivo calcification arises from 2 major driving forces: serum hyperphosphatemia and chronic inflammation. These 2 conditions can produce different pathological features and clinical outcomes and are studied using different models.

Hyperphosphatemia, Chronic Kidney Disease, and Medial Calcification
Hyperphosphatemia associated with chronic kidney disease or Monckeberg’s syndrome leads to rapid vascular wall and aortic valve calcification. Histopathologic analyses of arteries from these patients reveal gross, aligned mineral deposits.
between elastin fibers within the medial layer. Mineralization often appears independent of inflammation and plaque formation. Similarly, as opposed to decades-long fibrotic remodeling that accompanies aortic valve calcification in the inflammation-driven case, valve leaflets undergo rapid mineralization in hyperphosphatemic patients, which hardens the tissues, increasing systemic resistance and producing a large cardiac burden, leading to heart failure (Figure).

In vitro models of hyperphosphatemia use direct stimulation with extracellular phosphate. Under these conditions, smooth muscle cells and macrophages undergo calcification after only hours/days of stimulation. The extracellular phosphate produces a variety of cellular responses, but osteogenic differentiation and the increased expression of tissue nonspecific alkaline phosphatase (TNAP) do not serve as an initial trigger for mineral deposition. Recent studies have shown that the phosphate leads to mineral nucleation within extracellular vesicles (EVs) from smooth muscle cells and macrophages. Though these EVs share functional similarities to bone matrix vesicles, they arise from different cellular processes and contain a distinct proteomic profile. The exact location of mineral nucleation remains unclear, but evidence suggests endocytosed phosphate complexes with protein mediators of mineral growth (eg, fetuin-A) inside the cell. Subsequent nascent mineral release from the cell occurs in EVs from exosomal origins.

The accelerated rate of mineralization observed in the in vitro models is mirrored in animal models of hyperphosphatemia. These models reduce kidney function or increase dietary phosphates and perturb phosphate homeostasis (eg, Klotho). Rapid mineralization is also observed in patients with hyperphosphatemia, and the complications (eg, kidney failure) that cause calcification are well known. Rapid development of mineralization in these patients with easily identifiable clinical risk factors and adequate research models offer hope that a universal drug therapy could be identified and tested in standard clinical trials. This stands in stark contrast with the more complex clinical situation in inflammation-driven remodeling.

**Figure. Mechanisms of Calcification.** Various mineralization modes may coexist within a tissue. Hyperphosphatemia leads to rapid tissue calcification via extracellular vesicle (EV)–mediated mineral nucleation and osteogenesis. Inflammation-mediated calcification may be driven by osteochondral differentiation of resident smooth muscle cells (SMCs)/valve interstitial cells (VICs), leading to loading of tissue nonspecific alkaline phosphatase (TNAP) into EVs, producing free phosphate. Alternatively, inflammation may stimulate osteogenic differentiation or drive cell death. Microcalcifications in the fibrous cap may potentiate rupture, and macrocalcification may stabilize atherosclerotic plaques but are responsible for impairment of valve opening/closure.
Inflammation-Driven Calcific Remodeling

Cardiovascular calcification most commonly occurs in inflamed plaques characterized by lipid deposition. As such, hyperlipidemic animal models serve as common experimental platforms to study this type of calcification. Because this calcification follows a large inflammatory response, we use the term inflammation-driven calcification. Inflammation inhibition prevents calcification in mice, and proinflammatory stimuli exacerbate calcification through promotion of both cell death/apoptosis and osteogenic pathways in vascular and valvular cells. Notably, osteogenic mechanisms depend on the increased expression and activity of TNAP to convert phosphate sources (eg, ATP) into free phosphate for mineral nucleation. In contrast to the TNAP-independent phosphate recycling observed in the EVs of cells under hyperphosphatemic conditions, osteogenic transitions cause loading of active TNAP in EVs. Though active cellular processes mediate EV phosphate trafficking and mineral deposition, cellular differentiation and phosphate generation serve as rate-limiting steps, resulting in a slower mineralization process in inflammation-driven calcification. The underlying mechanism through which inflammation induces calcific responses is not a simple unidirectional causal relationship. Rather, calcification serves as an end point to inflammation-driven remodeling. Because of their anti-inflammatory action, statins may prevent calcification if given prior to the onset of mineralization, but once the remodeling has begun, statins accelerate the onset of calcification and may also directly stimulate osteogenic responses.

Calcification in plaques appears first as spherical or ellipsoidal microcalcifications that form from the aggregation and fusion of calcifying EVs. These microcalcifications then merge to form larger macrocalcifications that may stabilize atherosclerotic plaques but disrupt aortic valve function. Microcalcifications in fibrous cap ranging from 5 to 30 μm in diameter can result in a large increase in local tissue stress, potentiating plaque rupture. The various cellular interactions and the importance of morphology in inflammation-driven calcification make the potential therapeutic strategies complex. Further complicating matter is that patients exhibit clinical symptoms after gross remodeling severely affects tissue function or plaque rupture-induced vascular occlusion causes a myocardial infarction or stroke. Even if a patient exhibits significant risk factors, current clinical imaging modalities cannot visualize early calcification. These points underscore the need to develop a holistic understanding of each type of calcification. In the end, calcific mineral forms; however, the targetable processes that lead to mineralization may vary widely. By recognizing this point, we can begin to develop diagnosis protocols to understand the root causes of mineralization and treatment strategies best suited to each case.

Study Design Requirements for Cardiovascular Calcification

Confusion often results from the myriad of methods capable of producing calcification. Because calcium is pervasive in biological fluids and phosphate serves as a common stimulus in molecular signal transduction, slight shifts in ion homeostasis can cause mineralization. Therefore, demonstrating that an experimental perturbation can result in the formation of calcific mineral in vitro or in vivo does not necessarily indicate clinically relevant calcification. Results must be tested for generality before the field accepts a paradigm-shifting conclusion. As a first effort to standardize the field, we propose that the following 10 requirements should be addressed in all studies of cardiovascular calcification.

1. Discuss whether in vivo models recapitulate hyperphosphatemic, inflammatory, or both calcification triggers.
2. In vitro and in vivo models used in the same study should model the same calcific processes.
3. Take care when translating studies of vascular calcification to the aortic valve (and vice-versa).
4. Comment on model relevancy to pathogenesis in the tissue bed of interest.
5. Analyze cell phenotypes within models to indicate presence or absence of osteogenic processes (eg, Runx2 nuclear translocation, TNAP) and cell death/apoptosis (eg, TUNEL staining).
6. Indicate phosphate sources (eg, direct phosphate stimulation or TNAP substrates, such as β-glycerophosphate) for in vitro studies.
7. Address significance of each component used to induce calcification in cell culture models (eg, cytokines, insulin, dexamethasone) to avoid nonpathophysiological mineralization pathways.
8. Avoid over-reliance on nonspecific calcium and phosphate tracers and dyes.
9. Provide spectroscopic information on mineral composition for comparison to human pathological specimens.
10. Confirm the relevance of animal models to human disease.

Although all studies may not have the resources to address each point experimentally, the major study findings should be discussed in the context of the study limitations. By keeping results in the context of the exact type of calcification (eg, hyperlipidemic or inflammation-driven) being modeled, future studies are better able to build on findings and potentially expedite treatments for appropriate patient populations.

Sources of Funding

This work is supported by National Institutes of Health (NIH) grants R01HL114805 and R01HL109506 to E.A.

Disclosures

None.

References


Giving Calcification Its Due: Recognition of a Diverse Disease: A First Attempt to Standardize the Field
Joshua D. Hutcheson, Mark C. Blaser and Elena Aikawa

Circ Res. 2017;120:270-273
doi: 10.1161/CIRCRESAHA.116.310060
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2017 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/120/2/270

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/