Bidirectional Translation in Cardiovascular Calcification

Cynthia St. Hilaire, Marcel Liberman, Jordan D. Miller, on behalf of the Early Career Committee

Over the past decade, we have witnessed an explosion of fundamental research aimed at understanding mechanisms contributing to cardiovascular calcification. As highlighted in recent reviews, numerous animal models and patient group studies have lent key insights into mechanisms and processes underlying pathological remodeling of soft tissues, including activation of signaling cascades related to bone morphogenetic proteins, Wnt/β-catenin, matrix γ-carboxyglutamate (Gla)-protein (MGP), transforming growth factor (TGF)-β, and various downstream targets. Although there are compelling data supporting the biological importance of these pathways, harnessing these mechanisms for the development of therapeutics has not yet been realized. Many pathways play an integral role in bone homeostasis, making systemic targeting a nonviable therapeutic approach.

In this Recent Highlights focused on cardiovascular calcification, we have drawn from the pool of recent publications in ATVB and other leading journals that focus on genetic and non-genetic upstream modulators of ectopic calcification pathways, and we posit that interventions aimed at reducing their impact may be more readily translated to clinical therapies for patients. A greater understanding of the key local and systemic cofactors, initiators, and outcomes will create a complementary approach to advancing both science and medicine. We further argue that identification of biomarkers that are prognostic not only for the presence of vascular calcification (VC) but also for the rate of progression of VC will be instrumental in the early identification and appropriate management of patients in the future.

Insights From Conditions of Genetic Predisposition

Unlike metastatic calcification—caused by elevated levels of calcium in the blood—cardiovascular calcification is most often attributed to injury or maladaptive cellular responses to stress. Although in vitro studies and genetically modified model organisms can serve as useful platforms to understand the biology of disease phenotypes, the reality is that >85% of drugs stemming from these kinds of studies fail in clinical trials. Moving forward, translational research strategies must leverage observations and discoveries from multiple contexts—for example, basic biology, clinical observations, genetic predispositions—to identify novel druggable pathways that will not negatively affect bone homeostasis.

Early studies of vascular smooth muscle cell (VSMC) phenotypes identified MGP as being highly expressed in contractile states. MGP plays a significant role in bone remodeling through the modulation of bone morphogenic protein (BMP) signaling, and knockout mice have demonstrated that MGP is essential for keeping ectopic mineralization at bay. Keutel Syndrome (OMIM 245150) is an autosomal recessive disease resulting from mutations in the MGP gene, which underscores the translational potential of harnessing this protein in VC.

A similar molecule, Gla-rich protein, was recently found in calcified human vessels and valves colocalized in matrix vesicles alongside MGP and fetuin-A. In addition to its role as a vitamin K-mediated calcium “sink,” MGP was identified as a regulator of TGF-β/Wnt crosstalk. MGP-deficiency led to TGF-β-mediated loss of Wnt16-dependent signaling and subsequent loss of the VSMC contractile state. Although known to modulate a variety of cellular processes, Wnt16-dependent modulation of VSMC phenotype is novel, and the therapeutic potential of this pathway should be further explored not only in the context of Keutel syndrome, but in other congenital and acquired VC disease states.

The largest group of genetic diseases presenting with VC stem from inborn errors in genes modulating phosphate homeostasis and extracellular purine metabolism and identify novel mechanisms that have potential to be targeted in therapeutic strategies. Here, we highlight the utility of screening multiple genetic conditions to gain insight into mechanisms underlying cardiovascular calcification using observations from 4 genetic conditions as a platform to illustrate this approach.

First, in early adulthood, idiopathic Basal Ganglia Calcification (or Fahr’s disease; OMIM 213600) patients exhibit devastating neuropsychiatric symptoms stemming from calcification of brain vasculature, driven by inactivating mutations in the inorganic phosphate transporter SLC20A2/Pit2. PDGFRI-b or PDGF-B (the latter 2 drive expression of SLC20A2/Pit2). Second, in contrast, pyrophosphate—which potently inhibits hydroxyapatite formation—is dramatically reduced in patients with homozygous mutations in the enzyme ectonucleotide pyrophosphatase/phosphodiesterase 1 and results in Generalized Arterial Calcification of Infancy (OMIM 208000). These infants exhibit extensive medial dysplasia...
and calcification of the internal elastic lamina of large arteries and die within months of birth because of heart failure. Third, pseudoxanthoma elasticum (OMIM 264800), is a multisystem disorder with calcification along elastic fibers of the skin and vasculature, where mutations in the ABCC6 transporter are thought to limit ATP movement to the extracellular space. A small number of patients exhibiting pseudoxanthoma elasticum phenotypes have also been found to harbor mutations in ENPP1, highlighting the variable presentation of disease phenotypes. Finally, patients with Arterial Calcification due to Deficiency of CD73 (OMIM 211800) harbor mutations in the gene encoding for the extracellular enzyme CD73, which is downstream of ENPP1 and converts extracellular AMP to adenosine and inorganic phosphate. Arterial Calcification due to Deficiency of CD73 patients develop late-onset medial VC and peri-articular calcification around small joints in part via an increase in tissue nonspecific alkaline phosphatase, which bolsters the hypothesis that Pi/pyrophosphate homeostasis is a major regulator of ectopic calcification. Collectively, what were originally considered to be separate disorders may be defined as a series of conditions operating across a syndromic spectrum, with a relative reduction in extracellular pyrophosphate/increase in Pi as a key point of convergence.

Although the successful management of Generalized Arterial Calcification of Infancy patients with the first-generation pyrophosphate mimetic etidronate has been hailed as a major therapeutic success, its use must be closely monitored because severe rickets—a condition in which hypophosphatemia results in weakened bones, high fracture risk, and stunted bone growth—can be a devastating side effect.

In a slightly different context, genetic mutations that are associated with congenital aortic valve malformations greatly increase the risk of valvular calcification and stenosis. Although it is difficult to untangle the proportional impact of gene mutations relative to the mechanical effects of a congenital valve defect in humans, experimental model systems have been critical in providing insights into these processes. With regards to gene mutations, Notch1 and SMAD6 (aortic valve disease; OMIM 109730 and 614823, respectively) have been implicated in the embryological development and subsequent late-life calcification of bicuspid valves. Importantly, in vivo studies found that Notch1-mediated valvular calcification is dystrophic, illustrating that therapeutic strategies targeting osteogenic pathways may be futile in this pathology. Other gene mutations implicated in bicuspid valve formation (eg, GATA5 mutations) also seem to elicit dysregulation of Notch1 signaling, although additional investigation into the precise developmental mechanisms regulating the penetrance and phenotypic heterogeneity of such mutations is essential.

Insights From Nonsyndromic Acquisition

In contrast to diseases with a defined genetic origin and aggressive early-life onset, acquired diseases are generally thought to initiate and progress because of physiological, behavioral, or environmental risk factors combined with the presence of a permissive genetic or epigenetic landscape. Importantly, increasing age is a risk factor for a litany of chronic diseases and is associated with accumulation of DNA damage, epigenetic changes promoting genomic instability, and accumulation of senescent cells. In line with the latter, DNA damage accelerates cardiovascular calcification in vitro, and age-associated reductions in SIRT1 promote osteogenic differentiation of VSMCs in vivo and in vitro.

Intrinsic with aging are hemodynamic changes that often correlate with cardiovascular calcification. More specifically, age-associated increases in vascular stiffness and pulsatility can increase shear forces on both the heart valves and vessels and are strongly associated with VC. In addition to changes in physical forces exerted on the aging cardiovascular system, hemostatic dysregulation can alter gene expression in endothelial cells, inducing the release of the thrombotic factor von Willebrand factor, which has been shown to promote calcification in vitro. Further evidence for the significance of blood–vascular wall interactions comes from a mouse model of aortic valve disease in which platelet-derived TGF-β contributed to valvular fibrosis and calcification. Whether the osteogenic effect of platelet-derived TGF-β is because of the effects of TGF-β itself, or because of the recruitment of circulating mesenchymal stem cells to sites of vascular/valvular injury, has yet to be determined, but remains an exciting area of investigation.

Compelling clinical and experimental evidence illustrates that hyperglycemia is a major risk factor that, per se, stimulates VC and potentiates ectopic mineralization in the cardiovascular system. The pathobiology and microtopographical patterns of VC in diabetes mellitus suggest that it is not simply a risk factor that accelerates development and expansion of atherosclerotic plaques, but instead drives a unique VC phenotype. More specifically, a study comparing 60 amputees with peripheral arterial disease found that diabetes mellitus was associated with an increased severity of medial VC, which was distinct from atheroma-linked intimal VC. Hyperlipidemic mice with obesity and type 2 diabetes mellitus develop significant medial arterial and valvular calcification that was associated with upregulation of homeodomain transcription factors MSX1 and MSX2. From a more mechanistic perspective, hyperglycemia-driven upregulation of the paracrine inflammatory cytokine TNF-α promotes sustained Wnt/β-catenin signaling via MSX2 activity, activating a milieu of downstream osteochondrogenic genes culminating with medial calcification. Importantly, BMP signaling seems to be a critical intermediary in this pathway because overexpression of MGP effectively rescues the VC phenotype. Furthermore, hyperglycemia drives modification and activation of proteins, such as protein kinase B (PKB), also known as Akt, via O-linked N-acetylgalactosamine, which is important for downstream osteochondrogenic signaling and VSMC transdifferentiation. In line with these findings, specific deletion of phosphatase and tensin homolog resulted in sustained activation of AKT and increased phosphorylation of FOXO1/3, promoting VSMC calcification via blocking Runx2 ubiquitination.

Leveraging Biomarkers to Guide Intervention

Biomarkers are likely to play 3 critical roles in the field of cardiovascular calcification. First, biomarkers may predict...
high risk of developing clinically significant cardiovascular calcification, allowing for early intervention in subsets of patients. Second, biomarkers could identify patients with rapidly progressing cardiovascular calcification, an important patient subpopulation because the “typical” rate of progression in the general population is too variable and slow to effectively test in a Phase III trial. Finally, identification of labile biomarkers that can provide an early index of response to treatment will be important as a surrogate outcome in clinical trials and will facilitate making decisions at key “go/no-go” milestones throughout the process of advancing from biological discovery to clinical trials. Because no drugs have made it through Phase III testing for cardiovascular calcification to date, the ensuing sections focus on the first 2 classes of biomarkers.

Studies of patients with chronic kidney disease have provided great insights into biomarkers for cardiovascular calcification because this patient population has an exceptionally high risk of developing vascular and valvular calcification. Perhaps one of the strongest biomarkers involved in the pathogenesis of cardiovascular calcification in patients with chronic kidney disease is Fibroblast Growth Factor-23 (FGF-23). Although increases in circulating FGF-23 levels were first identified as being predictive of cardiac hypertrophy and carotid atherosclerosis, FGF-23 quickly rose to prominence in chronic kidney disease (CKD) as a bone-derived hormone that plays an instrumental role in regulating phosphate levels. A secondary target of FGF23 is the parathyroid gland, where it is thought to stimulate expression of klotho protein. Vitamin D–induced klotho expression in VSMCs is a critical context allowing FGF-23 to exert an anti-osteoergic effect in cardiovascular tissues. Neutralization of FGF-23 attenuates deleterious bone phenotypes in CKD while reciprocally accelerating cardiovascular calcification. Thus, circulating FGF-23 has great utility as a biomarker, but the context-dependence of its signaling makes FGF-23 exceptionally difficult to harness therapeutically. CKD patients are also prone to develop VC due in part to systemic and local aberrations in mineral homeostasis, with the former being a measurable biomarker and potential therapeutic target in this population. Finally, advanced glycated end products can accumulate in CKD patients, which can be visualized noninvasively via skin autofluorescence and correlate with VC in CKD.

Recent studies leveraging high-throughput lipid metabolomics have profoundly influenced our understanding of biomarkers and targets in cardiovascular disease by identifying a large number of molecules predictive of human diseases. One example of this in the field of cardiovascular calcification was the identification of apolipoprotein C-III levels as a predictor of cardiometabolic phenotypes and coronary artery calcification. More specifically, loss-of-function mutations were predictive of lower triglyceride levels, and increases in ApoC-III levels were associated with increased triglyceride levels and increased cardiovascular risk.

Although it is known that overt metabolic dysfunction is predictive of cardiovascular disease and mortality, recent work using cardiac computed tomography suggests that increases in glycohemoglobin A1C levels in euglycemic patients is associated with coronary artery calcification. Furthermore, in a cohort of over 41 thousand healthy young and middle-aged euthyroid men and women, low levels of normal free thyroxin and thyroid-stimulating hormone were associated with a higher prevalence of coronary calcification.

Noninvasive central arterial hemodynamics can also predict risk of VC. Arterial stiffness, a hallmark of aging, is characterized by changes in extracellular matrix, such as degradation of elastin and accumulation of collagen. In a community-based sample without cardiovascular disease, both higher carotid–femoral pulse wave velocity and central pulse pressure were associated with greater thoracic aortic calcification and abdominal aortic calcification, whereas higher augmentation index was associated with abdominal aortic calcification detected by cardiac multidetector computed tomography. Critically, carotid–femoral pulse wave velocity was the strongest correlate of all calcification measures in multivariable-adjusted models. In line with these findings, carotid pulse wave velocity predicted carotid plaque calcification and hemorrhage independent of changes in plaque lipid content, suggesting that these associations are not simply correlations because of disease severity.

Finally, there is a unique class of molecules that blurs the lines between biomarker, risk factor, and iatrogenic disease. For example, warfarin is widely used to anticoagulate patients with high risk of thrombosis/thromboembolism, but it also impairs Vitamin K–dependent γ-carboxylation of MGP, which has an inhibitory effect on VC. Experimentally, treatment with warfarin is associated with increased VC in animals and in humans. Clinically, recent studies reported increased vascular mineralization in mammograms from warfarin-treated women. Importantly, this is not solely a pathology of drug side effects because a threonine to alanine (Thr83Ala) polymorphism in MGP confers increased risk of coronary VC progression. Emerging data suggesting sequence variations in the gene(s) encoding vitamin K epoxide reductase complex subunit 1, the enzymatic target of warfarin, may also lend insight into high risk subpopulations of coronary VC progression and poorer survival rates. Although reports are mixed on the utility of phylloquinone (Vitamin K1) supplementation as a countermeasure to these pharmacological/biological side effects (which has been shown to both decrease or increase risk of coronary artery calcification), a deeper understanding of interactions between drugs, genetics, and cardiovascular calcification will likely lead to novel potential therapeutic targets to slow progression of VC or counter significant side effects related to anticoagulation therapy. Observations from these patient populations provide a firm rationale and foundation for the concept of personalized medicine, in which select patients may benefit from nonvitamin K–related anticoagulants and the genotype-guided dosing of warfarin.

Conclusions
The characterization of one’s research as translational or spanning from bench-to-bedside is becoming increasingly prevalent in the scientific community, being driven in part by the imperatives of major funding agencies and the general impatience of the public for cures stemming from the scientific
community. Reverse translation, or the strategy of leveraging mechanistic insights from rare diseases as a means to identify novel or underappreciated therapeutic targets, is emerging as a viable approach to drive basic/discovery research.

Recent studies, including those published in ATVB and highlighted herein, have greatly improved our understanding of the initiation and progression of VC and the potential utility of biomarkers, but there is still much to be learned. Moving forward with well-defined, actionable strategies to identify and develop therapeutics to treat cardiovascular calcification is of paramount importance in the field. Given the relatively slow rate of progression of cardiovascular calcification, early proof-of-concept trials must not only identify high-risk populations with aggressive disease, but also allow for completion of such studies in a reasonable period of time. The discovery of biomarkers that correlate with VC levels or are predictive of the rate of progression of VC will help to enroll appropriate patient pools for these studies and facilitate identification of vulnerable patients before the development of symptoms associated with VC. Leveraging rare diseases or unique acquired contexts that promote rapid progression of ectopic calcification in humans will likely prove to be a viable strategy for accelerated drug development in phase II studies; however, the generalizability of such findings to various patient populations remains unclear.

In closing, recent discoveries in the field of cardiovascular calcification suggest that the continued pursuit of fundamental/discovery-based research will be instrumental to advancing our understanding of mechanisms that contribute to cardiovascular calcification (and critically, how they differ from skeletal/orthotopic ossification). Reciprocally, continued efforts in clinical investigation that aim to identify unique patient subsets with aggressive disease or syndromes that are associated with cardiovascular calcification will be essential in advancing the field and identifying patient populations for clinical trials. In the long term, sustained interaction and collaboration between basic/discovery scientists and their clinical counterparts will be critical for pushing concepts from the bench to the clinic and accelerated development of novel therapeutics (which includes empowering basic/discovery scientists to push concepts forward and become engaged with their clinical counterparts and vice versa). Collectively, such efforts will accelerate the discovery, translation, and implementation of novel therapeutics for the growing patient population having complications related to cardiovascular calcification.

Sources of Funding
This work is supported by the University of Pittsburgh School of Medicine Heart and Vascular Institute (HVI), the Pittsburgh Heart, Lung, and Blood Vascular Medicine Institute (VMI), and the National Institutes of Health K22 HL117917 (Dr St. Hilaire); the Fundacao de Amparo a Pesquisa do Estado de Sao Paulo 2010/07455-5 and 2013/09611-2 (Dr Liberman); and the National Institutes of Health R01 HL111121 and UH3 TR000954 (Dr Miller).

Disclosures
None.

References


Casadesús-Bushrow AE, Bhat JF, Levin AM, Sheedy PF II, Turner ST, Boerswinkel E, Lin X, Kardia SL, Peysar PA. Matrix gla protein polymorphism is associated with increased coronary artery calcification


**Key Words:** aortic valve disease ■ arteriosclerosis ■ calcification ■ calcium-binding proteins ■ vascular biology ■ vascular disease ■ vascular remodeling ■ vascular smooth muscle

---

**Technical Note:**

The text above contains references to various studies related to the progression of aortic valve disease, calcification, and other related topics. The references include studies on the association between sequence variation in vitamin K epoxide reductase gene and outcome in chronic kidney disease, vitamin K supplementation and coronary artery calcium progression, phylloquinone concentrations and vascular calcification risk, and a randomized trial of genotype-guided dosing of warfarin. The key words are provided to indicate the focus of the text, which includes aortic valve disease, arteriosclerosis, calcification, calcium-binding proteins, vascular biology, vascular disease, vascular remodeling, and vascular smooth muscle.