Recent Highlights of ATVB
Calcification
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The presence of calcification in atherosclerotic arteries has long been recognized, but the mechanisms of calcification remain poorly understood. Risk factors of atherosclerosis, such as age, sex, hyperlipidemia, diabetes mellitus, hypertension, left ventricular hypertrophy, and smoking, have been shown to predict future coronary events. Traditionally, parameters known to be of predictive value were included in conventional risk scores such as the Framingham risk score and helped to divide patients into high risk, intermediate risk, and low risk of future cardiovascular events. Also, many studies have shown that calcification is accelerated in the presence of diabetes mellitus and chronic renal failure. Since the introduction of computed tomography (CT), a noninvasive technology with the ability to not only determine the extent of coronary narrowing but also determine the extent of vessel wall thickening, remodeling, and, above all, the presence or absence of calcification was lacking. Agatston et al devised a coronary artery calcification (CAC) scoring scheme, and others have shown calcium score to be a better predictor of future events than the Framingham risk index alone. There is also a close relationship between the presence of CAC and atherosclerotic plaque burden, with angiographic studies showing high sensitivity but poor specificity of CAC score for predicting obstructive disease. Physical activity is known to decrease cardiovascular mortality; however, there is no relationship between the extent of coronary calcification as assessed by CT in individuals engaged in moderate exercise. Allison et al showed that calcification in different vascular beds was predictive of different outcomes. Coronary calcification was predictive of cardiovascular disease mortality, whereas calcification in thoracic aorta, carotid arteries, and iliac arteries were predictive of total mortality when followed for 7.8 years. At the same time, histopathologic studies indicate that heavily calcified plaques are stable plaques and unlikely to lead to coronary events, whereas the vulnerable plaque tends to be either noncalcified or with only mild to moderate calcification, suggesting that calcification may exert a protective effect. These studies indicate that calcification is a complex, organized, and highly regulated process. Demer and Tinut have suggested that we divide calcification into 3 categories, that is, inflammatory (atherosclerotic, mostly intimal), metabolic (chronic kidney disease [CKD] and diabetes mellitus, mostly medial), and genetic background (pseudoxanthoma elasticum, generalized arterial calcification of infancy, arterial calcifications attributable to deficiency in CD73, and Marfan syndrome, mostly medial), although admitting that this may be an oversimplification. We will discuss the mechanisms of intimal atherosclerotic and valvular calcification, as well as calcification secondary to metabolic disorders because of some similarity in risk factors and clinical relevance.

Mechanisms of Intimal Calcification (Atherosclerosis)
Atherosclerosis is an inflammatory disease requiring recruitment of leukocytes into the vessel wall, which is dependent on various chemokines and adhesion molecules (eg, monocyte chemoattractant protein 1 and granulocyte-macrophage colony-stimulating factor) responsible for their transendothelial migration (eg, vascular cell adhesion molecule 1, intercellular adhesion molecule 1, E-selectin). The activation of endothelial cells and the expression of various proteins are regulated via nuclear factor-κB, which is responsible for the homing of inflammatory cells into the vessel wall and maintenance of chronic inflammation. It has been suggested by in vitro and in vivo studies that mechanisms modulating calcification are highly regulated and involve smooth muscle cell apoptosis, osteochondrogenic differentiation, and matrix vesicle release. The receptor activator of nuclear factor-κB ligand (RANKL) system consists of a triad of proteins: the membrane-bound RANK, its ligand RANKL, and the decoy receptor osteoprotegerin, which regulate osteoclast differentiation via RANK activation. Although osteoblasts secrete soluble RANKL, which results in RANK activation in osteoclasts, osteoprotegerin antagonizes the effect of RANKL via competitive inhibition and provides a negative feedback loop for osteoblasts in regulating bone metabolism. Consequently, inactivation of osteoprotegerin in mice results in severe osteoporosis. The RANKL has been implicated as a procalcification agent for smooth muscle cells (SMCs). Also, bone marrow–derived macrophage/SMC cocultures treated with RANKL further enhance SMC calcification compared with SMC alone, which is thought to be mediated via interleukin-6 and tumor necrosis factor-α. Osteoprotegerin deficiency in apolipoprotein E–deficient (ApoE−/−) mice results in increased atherosclerotic lesion size and calcification, introducing a link between bone homeostasis and its inactivation, resulting in severe osteoporosis and vascular calcification in the course of atherosclerosis progression. Important evidence is further derived from studies in ApoE−/−osteoprotegerin (OPG)−/− mice transplanted with ApoE−/−OPG−/− bone marrow, which developed smaller atherosclerotic lesions and deposited less calcium in the innominate artery than that of ApoE−/−OPG−/− mice transplanted with ApoE−/−OPG+/− bone marrow. Inhibition

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of bone morphogenetic protein by feeding a small molecule inhibitor of bone morphogenetic protein type I receptor kinase LDN-193189 in low-density lipoprotein–deficient mice fed a high cholesterol diet for 20 weeks resulted in the development of less atherosclerosis and calcification compared with placebo. Bone morphogenetic proteins have been shown to play an important role in vessel wall inflammation in the presence of laminar and oscillatory flow.

Abdominal aortic calcification has been related to bone loss in women but not in men. Estrogen is known to regulate bone metabolism by inducing osteoclast apoptosis and osteoblast expression of osteoprotegerin and inhibits atherosclerosis progression. Support of this notion comes from experimental studies that show that osteoprotegerin-deficient mice develop severe osteoporosis from a marked increase in osteoclast activity, with two thirds of the animals developing vascular medial calcification. RANKL plays an important role in aortic calcification, which is likely to be of relevance for the acceleration of aortic calcification in postmenopausal women. Estrogen-deficient ApoE−/− mice fed a high-cholesterol diet showed increased expression of RANKL, RANK, and osteopontin and developed vessel wall calcification and osteoporosis, whereas estrogen replacement inhibited both these effects with an increase in matrix Gla protein (MGP) mRNA expression. These studies showed that RANKL induces osteogenesis by acting on 2 important molecules: bone morphogenetic protein-2, an inducer of calcification, and MGP; an inhibitor of calcification.

Because vascular calcification is independently associated with hypertension in epidemiological studies and RANKL is essential for calcification, it is important to understand that RANKL is an activator of the renin–angiotensin system. However, infusion of angiotensin II (100 ng/kg per minute) in ovariectomized ApoE−/− mice significantly increased the expression of RANKL and calcification in vivo, whereas administration of angiotensin II receptor blocker (olmesartan, 3 mg/kg per day) decreased the calcification and expression of bone markers, which introduce a molecular feedback between endocrinology pathways and vascular calcification in the pathogenesis of hypertension. Cross-talk between the renin–angiotensin II and RANKL system has been suggested to work in a vicious cycle to promote vascular calcification in atherosclerosis.

Mechanisms of Aortic Valve Calcification
Calcification also affects the aortic valve, and its prevalence is estimated to be as high as 25% to 30% in patients aged >65 years. It has been suggested that there are many commonalities between vascular and aortic valve calcification, especially with respect to inflammation. A recent epidemiological study, which included 2212 participants (aged 45–81 years), revealed a close association between hepatic steatosis and aortic valve sclerosis. Plasma osteopontin levels have been used for the identification of asymptomatic individuals with either aortic valve sclerosis or calcification. With the development of new mouse models of aortic valve calcification induced via wire injury, the proliferative valves showed an increased production of reactive oxygen species and inflammation within 4 weeks, thus forwarding the theory that aortic valve calcification is the result of response to injury and that this model may provide another tool to better understanding of valve calcification.

The activation of valvular interstitial cells and their transdifferentiation into osteoblast-like cells have been suggested to be important mechanisms of valve calcification. Several studies have reported that Notch 1 promotes osteoblast differentiation. Zeng et al showed that Notch 1 enhanced osteogenic differentiation through extracellular signal–regulated protein kinase 1/2 and nuclear factor-kB activation. Recent studies support the concept that reactive oxygen species, likely produced by inflammatory cells, accelerate calcification through pro-osteogenic and profibrotic signaling cascades of vascular smooth muscle cells in vitro. Reactive oxygen species act on the valve cusp microstructure through the activation of the DNA damage response mechanism, inducing valvular interstitial cells to adopt an osteogenic phenotype with expression of markers such as osteopontin, osteonec tin, and transcription factors, runt-related transcription factor 2 and muscle segment homeobox 2.

Wnt signaling is important for all bone types, that is, osteoblasts that form bone, osteoclasts that resorb bone, and osteocytes that maintain bone; therefore, it is likely important in both arterial and valve calcification. The Wnt pathway is historically divided into 3 major systems: Wnt–β-catenin pathway, the Wnt–planter polarity pathway, and the Wnt–calcium pathway. In aortic endothelial cell cultures, overexpression of the Wnt antagonist dickkopf-1 suppressed endothelial cell differentiation, inducing a mineralizing myofibroblast phenotype. Myofibroblast and osteogenic marker genes, including Msx2, type I collagen, Oxs, runt-related transcription factor 2, and alkaline phosphate are upregulated, whereas Wnt7b and Msx2 signals preserve endothelial phenotype.

We know that vascular endothelial cells perform antithrombotic and thrombotic functions; however, little is known about the valve endothelial cells. Balaoning et al showed that in valve endothelial cells, levels of hemostatic proteins such as plasminogen activator inhibitor 1 are higher in elderly than in the young and adult pigs. Using an in vitro model, they confirmed that von Willebrand factor in culture significantly increased valvular interstitial cell nodule formation and calcification. Endoplasmic reticulum (ER) stress has been linked to a variety of conditions, including diabetes mellitus, obesity, atherosclerosis, and inflammatory conditions. Animal models of aortic valve calcification have shown marked induction of ER stress. In cultured valvular interstitial cells, Cai et al found that oxidized low-density lipoprotein caused ER stress in a cytosolic [Ca2+]−–dependent manner. Also, oxidized low-density lipoprotein promoted osteoblastic differentiation via ER stress, whereas tauroursodeoxycholic acid or 4-phenyl butyric acid—both inhibitors of ER stress—suppressed oxidized low-density lipoprotein–induced osteoblastic differentiation in valvular interstitial cells.

Mechanisms of Calcification in CKD and Diabetes Mellitus
CKD and diabetes mellitus are strongly associated with accelerated calcification, which occurs not only in the intima but also in the media. Vascular calcification of the media can occur
because of varying causes, with Monckeberg’s medial calcification being the most common variant and is characterized by localization of calcium in the media of the arteries of the extremities and is commonly associated with type 2 diabetes mellitus and CKD. \(^{50}\) From the Multi-Ethnic Study of Atherosclerosis (MESA) study of 2,795 participants followed for 2.4 years, the incidence of calcification as determined by cardiac CT correlated with impairment of glomerular filtration rate estimated by cystatin-c and that part of this association was mediated by lipid phenotype comprising triglyceride-rich lipoproteins. \(^{51}\) One of the major risk factors for vascular calcification, especially in the setting of uremia and hyperphosphatemia, is warfarin treatment, an anticoagulant commonly used in patients with risk of embolism. Warfarin has been correlated with the presence of cardiovascular calcification in humans and animal models. \(^{52}\) MGP is a vitamin K–dependent protein, and warfarin inhibits \(\gamma\)-carboxylation of MGP, thus promoting vascular calcification. Warfarin treatment of DBA/2 wild-type mice induced significant calcification that resulted in a decrease in MGP mRNA expression, whereas the inactive expression of MGP increased. \(^{53}\) Transglutaminase 2, a calcium-dependent enzyme than can cross-link nearly all extracellular matrix components, is a potent regulator of osteochondrogenic differentiation. Warfarin treatment of rats and MGP \(^{-/-}\) mice demonstrates vascular calcification and an increase in transglutaminase 2 protein that acts through the activation of \(\beta\)-catenin. \(^{54}\) Similarly, vitamin D receptor antagonists show increased calcification in uremic animals and is thought to be because of an increase in circulating calcium or phosphate levels. However, Lomashvili et al. \(^{55}\) used vitamin D receptor–null mice and showed that treatment with calcitriol that activates vitamin D receptor promoted calcification through a systemic effect and not through a direct vascular action.

Epidemiological studies of asymptomatic individuals with type 2 diabetes mellitus have shown that an increase in CAC score by CT (Agatston score >100 U) and an abnormal myocardial perfusion scintigraphy were potent predictors of coronary events within 2.2 years compared with those with CAC score <100 AU. \(^{56}\) In 2009, the MESA study determined the correlation of hemoglobin A1C (HbA1C) and CAC by CT and showed that although a small but positive association exists between HbA1C and carotid intimal medial thickness, this association between HbA1C and CAC was only observed in asymptomatic women. \(^{57}\) However, recently Chang et al. \(^{58}\) showed that higher HbA1C levels were associated with a modest but independent increase in CAC in healthy men and women, thus showing that a higher HbA1C score even in asymptomatic individuals was a modest predictor of subclinical coronary atherosclerosis. Both in humans and in animals, advanced glycation end products have been implicated in calcification. Advanced glycation end products enhance vascular calcification in vascular SMCs through its receptor (RAGE [receptors for advanced glycation end-products]). \(^{59}\)

**Conclusions**

Arterial calcification in asymptomatic individuals has now been well demonstrated to be a predictor of future cardiac events and deaths. It is therefore important not only to understand the mechanisms of vascular (intimal and medial) and aortic valve calcification but also to further understand how risk factors such as diabetes mellitus and CKD result in acceleration of calcification. Treatments besides statins must be sought to prevent this worldwide continued increase in atherosclerosis, which is expected to affect >30% of the population. Increasing the basic understanding of calcification in man as well as further refinement of animal models in time will help conquer this epidemic of atherosclerosis and calcification.

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**Disclosures**

None.

**References**

1. Wilson PW, D’Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor catego-
4. Lamprea-Montealegre JA, McClelland RL, Astor BC, Matsushita K, Shlipak M, de Boer IH, Sklizko M. Chronic kidney disease, plasma lipopro-
6. Agatston AS, Janowitz WR, Hildner FJ, Zius NR, Vielmonte M, Jr, Detrano R. Quantification of coronary artery calcium using ultrafast com-
   sis is highly correlated with atherosclerotic plaque burden in humans: a histologic study of 78 coronary artery segments using nondecalcifying methodology. \(\textit{J Am Coll Cardiol}\). 1998;31:126–133.
10. Allison MA, Hsi S, Wassel CL, Morgan C, Ix JH, Wright CM, Criqui MH. Calcified atherosclerosis in different vascular beds and the risk of mortal-
    ity. \(\textit{Arterioscler Thromb Vasc Biol}\). 2012;32:140–146.
11. Otsuka F, Sakakura K, Yabuki K, Joner M, Virmani R. Has our under-
13. Simionescu M. Implications of early structural-functional changes in the endo-
    binding protein mediates the proangiogenic or proinflammatory activity of gremlin. \(\textit{Arterioscler Thromb Vasc Biol}\). 2014;34:136–145.
16. Clarke MC, Littlewood TD, Figg N, Maguire JJ, Davenport AP, Goddard M, Bennett MR. Chronic apoptosis of vascular smooth muscle cells acceler-