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Arterial Stiffness: A focus on vascular calcification and its link to bone mineralization

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Abstract

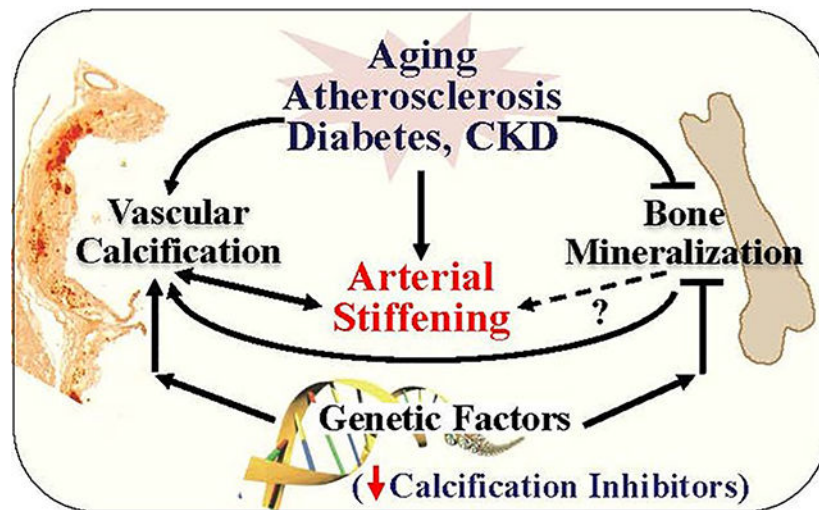
This review focuses on the association between vascular calcification and arterial stiffness, highlighting the important genetic factors, systemic and local microenvironmental signals, and underlying signaling pathways and molecular regulators of vascular calcification. Elevated oxidative stress appears to be a common pro-calcification factor that induces osteogenic differentiation and calcification of vascular cells in a variety of disease conditions such as atherosclerosis, diabetes mellitus and chronic kidney disease (CKD). Thus, the role of oxidative stress and oxidative stress-regulated signals in vascular smooth muscle cells (VSMC) and their contributions to vascular calcification are highlighted. In relation to diabetes mellitus, the regulation of both hyperglycemia and increased protein glycosylation, by advanced glycation end products (AGEs) and *O*-GlcNAcylation, and its role in enhancing intracellular pathophysiological signaling that promotes osteogenic differentiation and calcification of VSMC are discussed. In the context of CKD, this review details the role of calcium and phosphate homeostasis, parathyroid hormone, and specific calcification inhibitors in regulating vascular calcification. In addition, the impact of these systemic and microenvironmental factors on respective intrinsic signaling pathways that promote osteogenic differentiation and calcification of VSMC and osteoblasts are compared and contrasted, aiming to dissect the commonalities and distinctions that underlie the paradoxical vascular-bone mineralization disorders in aging and diseases.

Graphical Abstract

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DISCLOSURES

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Keywords

Vascular calcification; arterial stiffness; atherosclerosis; diabetes mellitus; chronic kidney disease

INTRODUCTION

Arterial stiffening is an important determinant of vascular aging and independently predicts cardiovascular events, including left ventricular hypertrophy, myocardial infarction, hypertension, atherosclerosis and stroke, as well as cardiovascular complications in diabetic mellitus and chronic kidney disease (CKD)¹⁻⁹. As stiffening of the large elastic arteries progresses with aging, it accelerates the development of cardiovascular diseases. Epidemiological studies have demonstrated an independent prognostic link of aortic pulse wave velocity (PWV), the indicator for arterial stiffness, with cardiovascular outcomes in older adults and patients with diabetes mellitus and hypertension^{7, 8, 10}. On the other hand, vascular stiffening is further exacerbated by metabolic syndromes, diabetes mellitus, obesity, CKD, and cardiovascular diseases including atherosclerosis and hypertension¹¹. Accordingly, pharmacological interventions that target these conditions, such as advanced glycation end-product (AGE) crosslink breaker, angiotensin-converting enzyme (ACE) inhibitors, and statins, are emerging as potential candidates to reduce arterial stiffness, although no selective and highly effective treatment for arterial stiffness has been identified^{10, 12-14}.

This is partly due to the lack of comprehensive and precise understanding of the mechanisms responsible for arterial stiffening. Nonetheless, it is well documented that arterial stiffening is linked to the structural and functional changes of the large elastic arteries with aging and during the development of cardiovascular diseases. As summarized by previous reviews, remodeling of extracellular matrix (ECM), including elastin degradation and excessive collagen deposition and glycosylation, is critical in regulating arterial stiffness and the development of cardiovascular diseases¹⁵⁻¹⁹. At the cellular level, while endothelial cells, fibroblasts and inflammatory cells all contribute to arterial stiffening, vascular smooth

muscle cells (VSMC) are essential to maintain vascular homeostasis in the arterial system, including the elastic arteries such as aorta, muscular arteries such as coronary and femoral arteries, as well as arterioles. Increased VSMC proliferation, migration, senescence and mineralization play predominant roles in age-related medial and intimal thickening and arterial stiffening as well as pathogenesis of cardiovascular diseases⁴. In particular, VSMC in the tunica media of the elastic arteries regulate vascular tone, thus VSMC dysfunction leads to impaired vascular homeostasis with aging and/or the development of major cardiovascular diseases.

VASCULAR CALCIFICATION AND ARTERIAL STIFFNESS

Intrinsic stiffness of VSMC is aggravated with aging²⁰. In addition, VSMC regulate arterial stiffness by overproducing various ECM components, such as collagen and elastin, which provide biomechanical, structural integrity and signaling regulation of the ECM to maintain vascular homeostasis under normal healthy conditions^{16, 21}. Furthermore, VSMC undergo osteogenic differentiation and mineralization in response to a variety of stimuli, which drives vascular calcification. Both vascular calcification and remodeling of ECM directly mediate arterial stiffening⁴. The remodeling of ECM in turn promotes VSMC calcification, which further accelerates vascular stiffening. For example, increased expression of matrix proteases, such as calpain-1 and matrix metalloproteinase 2 (MMP2), has been identified in aged human aortic wall and atherosclerotic lesions²². Studies with cultured VSMC *in vitro* and carotid artery rings *ex vivo* have shown that increased calpain-1 and MMP2 promote VSMC calcification, elastin degradation, alkaline phosphatase (ALP) activation, collagen production; and reduce expression of calcification inhibitors, such as osteopontin and osteonectin²². Emerging studies have linked vascular calcification to reduced arterial elasticity, decreased vessel walls compliance, increased arterial stiffening and accelerated development of cardiovascular diseases^{23–25}.

By definition, there are two major types of vascular calcification: medial calcification and intimal calcification. Medial arterial calcification, also called Mönckeberg sclerosis, is localized mainly in the tunica media that contains VSMC and elastic tissues. Medial arterial calcification exists in aging arteries in patients with diabetes mellitus and CKD, which often occurs independent of atherosclerosis^{26, 27}. Intimal arterial calcification, on the other hand, is present in atherosclerotic lesions in the tunica intima, which is often associated with hyperlipidemia, macrophage infiltration and vascular inflammation^{28, 29}. Intimal arterial calcification occurs mostly in patients with atherosclerosis, but also is found in patients with diabetes mellitus and CKD²⁶. An early study examining over 540 aorta samples from human autopsies has revealed an increased incidence of medial and intimal calcification with aging³⁰. In patients with diabetes mellitus and CKD, increased calcification in the media is associated with arterial stiffening as well as an increased risk of cardiovascular morbidity and mortality^{26, 31}. In addition, recent studies have linked vascular calcification to formation, progression and rupture of aortic aneurysm^{32, 33}. Consistently, combining coronary artery calcium score with conventional risk factors for cardiovascular diseases significantly improves the clinical risk reclassification of cardiovascular outcomes, in cohorts in the Multi-Ethnic Study of Atherosclerosis, Dallas Heart Study, Prospective Army Coronary Calcium Project, and the Framingham Heart Study^{34–37}. These clinical observations have

supported a strong link between vascular calcification and arterial stiffening, with aging and multiple cardiovascular diseases. On the other hand, arterial stiffening in atherosclerosis, diabetes and CKD may further accelerate vascular calcification, creating a vicious cycle. Therefore, better understanding of molecular mechanisms underlying vascular calcification should lead to identifying potential targets and strategies to selectively block vascular calcification and calcification-associated adverse cardiovascular outcomes.

REGULATION OF VASCULAR CALCIFICATION

Vascular calcification is now recognized as an active osteogenic process of vascular cells, mainly VSMC, resembling osteoblast formation that is defined by the osteogenic differentiation, matrix maturation and matrix mineralization stages³⁸. It is regulated by multifaceted mechanisms underlying intracellular osteogenic differentiation and remodeling of extracellular matrix, involving genetic, (micro)environmental and cellular signaling modulators³⁹. Osteogenic differentiation and calcification of VSMC is a common feature existing in both medial and intimal arterial calcification. VSMC possesses phenotypic plasticity. In response to environmental changes with aging and cardiovascular diseases, VSMC undergo phenotypic transition from the contractile phenotype, characterized by the expression of SMC-specific contractile proteins such as smooth muscle α -actin (SMA), SM22 α and smooth muscle cell myosin heavy chain (SMMHC), to a synthetic phenotype as well as trans-differentiation into other cells of mesenchymal lineages, such as osteoblasts, chondrocytes and adipocytes. Osteogenic differentiation of VSMC features increased expression of bone markers and concurrently decreased expression of VSMC markers^{40–42}. Similar to the osteogenic differentiation of bone cells, increased expression of alkaline phosphatase (ALP) and matrix proteins, such as type 1 collagen (Col1) that marks the matrix maturation³⁸ was evident in vascular calcification. Extracellular matrix mineralization involves the formation and secretion of matrix vesicles (MVs), membrane-bound nanoparticles that carry and transport calcium/phosphate and matrix proteins, followed by nucleation of calcium phosphate crystals and aggregation on collagen fibers in the extracellular matrix²⁹. The ALP catalyzes the degradation of inorganic pyrophosphate (PP_i), a major inhibitor for amorphous calcium phosphate nucleation, hydroxyapatite aggregation and crystal growth⁴³, thus promoting hydroxyapatite deposition and matrix mineralization (Figure 1).

The importance of MVs in regulating cardiovascular calcification in atherosclerosis, diabetes mellitus and CKD has been documented^{44, 45}. MVs are evident in atherosclerotic lesions as well as in medial calcification^{46, 47}. *In vitro* studies with human VSMC have further illustrated the critical role of MVs in promoting vascular calcification in response to extracellular calcium and phosphate stress⁴⁸. In addition, many bone matrix proteins and key osteogenic regulators have been identified in calcified arteries and vascular cells, including Col I, matrix Gla protein (MGP), osteopontin (OPN), matrix metalloproteinases (MMP), bone morphogenetic protein-2 (BMP-2) and the master osteogenic transcription factor, Runt-related transcription factor-2 (Runx2)^{29, 49}. *In vitro* studies with VSMC have provided direct evidence for osteogenic differentiation and calcification of VSMC in response to extracellular stimuli, which features decreased expression of SMC marker genes and concurrent upregulation of bone markers²⁹. These multiple lines of evidence have supported

the concept that vascular calcification represents a regulated osteogenic differentiation and calcification of vascular cells. This review discusses the integrated regulation of vascular calcification that promotes arterial stiffening, focusing on genetic inhibitors as well as pro-osteogenic regulators associated with atherosclerosis, diabetes mellitus and CKD, including oxidative stress, hyperglycemia and dysregulation of calcium/phosphate.

Genetic determinants of vascular calcification

Multiple genetic regulators of vascular calcification have been identified using genome-wide association studies (GWAS) and single nucleotide polymorphism (SNP)-based analyses in human, as well as genetically modified animal models. These diverse regulators include matrix proteins such as MGP and OPN, circulating calcium binding protein, Fetuin-A, as well as regulators for phosphate and pyrophosphate metabolism, including ectonucleotidic pyrophosphatase/phosphodiesterase-1 (ENPP1), pyrophosphate extracellular transporter progressive ankylosis protein (ANK), ecto-5'-nucleotidase CD73 and the ATP-binding cassette transporter subtype 6 (ABCC6). Increased vascular calcification and corresponding arterial stiffness are observed in each monogenic mutation mapped to these genetic factors. The identification of these genetic factors supports the concept that “loss-of-inhibitors” promotes the pathogenesis of vascular calcification (Table 1).

MGP is a small secreted matrix protein, a member of the vitamin K-dependent protein family containing γ -carboxylated glutamate residues, and binds to calcium and calcified matrices^{50, 51}. In humans, *MGP* SNPs with defective MGP function are linked to arterial calcification and mutations in the gene encoding the human MGP cause Keutel syndrome^{52–54}. Consistently, genetic ablation of the *mgp* gene in mice induces severe ectopic calcification in the arteries and cartilage⁵⁵. Additionally, inhibition of vitamin K metabolism leads to under carboxylation of MGP and subsequent medial calcification of the arterial vessel wall⁵⁶, further indicating the importance of MGP production and activity in inhibiting vascular calcification. Another matrix protein, OPN, has been shown to inhibit deposition of hydroxyapatite crystals and calcification of VSMC similarly to PP_i ^{57,58}. *Opn* deficiency does not cause calcification in mice, but increases arterial medial calcification in the *Mgp*^{-/-} mice⁵⁹ and vascular stiffening in *LDLR*^{-/-} mice⁶⁰, suggesting an inducible role of OPN in inhibiting vascular calcification and stiffening.

The circulating calcium binding protein alpha2-Heremans-Schmid glycoprotein (AHSG, Fetuin-A) has been reported to be a potent circulating calcification inhibitor. Fetuin-A is a serum glycoprotein produced in the liver, and is capable of binding ~100 Ca^{2+} ions per molecule. It binds to clusters of amorphous calcium phosphate to form primary calciprotein particles, which maintain calcium phosphate in solution and prevent it from precipitating^{61, 62}. Fetuin-A polymorphisms with decreased serum fetuin-A levels in humans⁶³ are associated with increased arterial calcification and stiffness in patients with atherosclerosis, diabetes mellitus and CKD^{64–66}. Consistently, mice deficient in *fetuin-A* develop heavy and diffused soft tissue calcifications throughout the whole body, as well as in calcified intimal plaques upon vascular injury⁶⁷, supporting the critical role of fetuin-A as a major systemic inhibitor of vascular calcification.

The importance of phosphate and pyrophosphate metabolism in regulating vascular calcification is bolstered by the identification of several key regulators in the ATP metabolism network (Figure 2). ENPP1 is the ectoenzyme that hydrolyzes ATP at the cell membrane into AMP and pyrophosphate (PP_i), and the resulting PP_i is subsequently transported to the extracellular matrix via the membrane transporter, ANK^{68, 69}. As PP_i is the major inhibitor for hydroxyapatite formation and matrix mineralization, the ENPP1 deficiency is associated with generalized arterial calcification of infancy (GACI) in humans⁷⁰, a genetic defect with increased arterial calcification, particularly in patients with atherosclerosis, diabetes mellitus and CKD^{70–72}. Mice deficient in *enpp1* have reduced pyrophosphate level and increased aortic calcification⁷³. Similarly, mice with an *ank* mutation exhibit some defect in extracellular pyrophosphate and develop medial arterial calcification⁶⁹. Treatment of pyrophosphate analogs, bisphosphonates, have decreased arterial calcification and improved the overall survival of patients with GACI due to *ENPP1* mutations⁷⁴. Accordingly, deficiency of either ENPP1 or ANK results in decreased PP_i in the extracellular matrix and increased formation of hydroxyapatite crystals and matrix calcification, supporting the inhibitory role of both ENPP1 and ANK in vascular calcification.

Another enzyme in the ATP metabolism pathway, CD73 ectoenzyme encoded by the ecto-5'-nucleotidase gene *NT5E*, has also been linked to vascular calcification⁷⁵. CD73 is a cell membrane protein that catalyzes the generation of extracellular adenosine and inorganic phosphate (Pi) from AMP, which acts directly downstream of extracellular ATP degradation by ENPP1. CD73 deficiency in mice promotes thrombosis and inflammatory response in the vasculature⁷⁶. *NT5E* mutations in human cause arterial calcification due to deficiency of CD73 (ACDC). *NT5E* mutations increase accumulation of calcium phosphate crystals. This is likely due to decreased adenosine-enhanced activity of tissue non-specific alkaline phosphatase (TNAP), since adenosine supplementation reverses the increased TNAP activity in CD73-deficient cells⁷⁵.

In addition, deficiency in *ABCC6*, a gene encoding a member of the transmembrane ATP-binding cassette transport proteins, is associated with arterial calcification and pseudoxanthoma elasticum (PXE) ^{77,78}. The *Abcc6* deficiency in mice causes increased arterial calcification⁷⁹. Because of the similarity in arterial lesions found in the PXE patients and ACDC patients, it was suggested that PXE and ACDC share common pathogenic pathways, and ABCC6 may serve as an adenosine transporter⁸⁰. Overexpression of fetuin-A rescues vascular calcification in *Abcc6*^{-/-} mice⁸¹, supporting the notion that lack of calcification inhibitors may contribute to ABCC6 deficiency-increased vascular calcification. Jansen *et al* identified that plasma PP_i levels in the *Abcc6*^{-/-} mice were significantly reduced compared to those in wild-type mice, and demonstrated the important role of ABCC6-mediated ATP secretion and generation of extracellular PP_i⁸². By crossing *Abcc6*^{-/-} mice with the *Enpp1*^{-/-} and *Nt5e*^{-/-} mice, Ziegler *et al* validated the metabolic crosstalk among ENPP1, CD73, and ABCC6 as well as the important role of ABCC6 in mediating extracellular ATP metabolism⁸³. A bisphosphonate and an inhibitor for TNAP activity attenuates the development and progression of calcification in *Abcc6*^{-/-} mice⁸³, further validating the role of ABCC6-mediated phosphate metabolism in vascular calcification..

Altogether, monogenic deficiencies of ENPP1, CD73 or ABCC6 cause human diseases (GACI, ACDC, PXE) that share a common pathology of spontaneous, and premature onset of arterial calcification, in which the homeostasis of the phosphate/pyrophosphate metabolism is critical. In addition to pyrophosphate, matrix proteins that bind to calcium phosphate, such as MGP and OPN, as well as circulating calcium binding protein, fetuin-A, have also been recognized as potent inhibitors for vascular calcification. These genetic determinants highlight the importance of inhibition of matrix mineralization under physiological conditions in the vascular cells, as they act predominantly at the matrix mineralization stage by blocking accumulation of calcium phosphate, and formation and growth of hydroxyapatite crystals. The mechanistic insights gained from these rare genetic disorders have not only improved our understanding of the fundamental regulation of matrix mineralization in the development of vascular calcification; but also shed new lights on developing potential strategies to prevent and inhibit vascular calcification associated with specific genetic defects, and beyond.

Regulatory signals for vascular calcification

Despite the common osteogenic and mineralization process shared by bone and vascular cells, decreased bone mineralization (osteoporosis) occurs concurrently with increased vascular mineralization (vascular calcification) in human and mice associated with aging, atherosclerosis and CKD. These paradoxically opposite outcomes in bone and vascular niche indicate that distinct intrinsic signals driven by the tissue-specific microenvironmental cues govern the mineralization differently⁸⁴. Mounting clinical and experimental findings in the past two decades have uncovered various important microenvironmental conditions as well as major signaling pathways and molecular regulators for both vascular calcification and bone mineralization. Elevation of oxidative stress, hyperglycemia and dysregulated calcium and phosphate homeostasis promote vascular calcification and inhibit bone mineralization. The regulation of vascular calcification by these pro-calcification conditions, and their activation of the major signaling pathways as well as their impacts on bone mineralization (Figure 3) are discussed below.

Increased oxidative stress—Increased oxidative stress and oxidative stress-induced signaling are a common feature in aging, cardiovascular and metabolic diseases, representing a major driving factor for vascular calcification and stiffness^{4, 49, 85, 86}. The mechanisms underlying increased oxidative stress-induced vascular calcification in the atherosclerotic lesions are linked to oxidized lipids, vascular inflammation and matrix protein secretion^{49, 86–88}. Elevated production of hydrogen peroxide (H₂O₂) production by residential vascular cells and infiltrated cells activates signaling pathways that lead to increased expression of inflammatory cytokines and adhesion molecules, which in turn promote the generation of H₂O₂ and increase oxidative stress in atherosclerotic lesions⁸⁹. *In vitro* studies have further demonstrated a direct effect of H₂O₂ on promoting osteogenic differentiation and calcification of VSMC⁴² and calcifying bovine vascular cells⁸⁵.

Increased oxidative stress is also manifested in patients with diabetes mellitus and animal models of diabetes⁹⁰. High levels of blood sugar (hyperglycemia) in diabetes increases the accumulation of advanced glycation endproducts (AGEs), which bind to the receptor for

advanced glycation (RAGE) that activates inflammatory signals and increases oxidative stress^{60, 91, 92}. Increased AGEs/RAGE signaling promotes vascular calcification *in vitro* and *in vivo*^{92, 93}. In CKD patients and animals, aortic and systemic increase in oxidative stress is a key feature of the uremic state⁹⁴, which is highly associated with elevated vascular calcification⁹⁵. High phosphate, a characteristic of CKD, induces production of mitochondria-mediated ROS and promotes VSMC calcification, which can be attenuated by antioxidants that limit high phosphate-induced ROS⁹⁶. Inhibition of oxidative stress by antioxidants also slows down the progression of vascular calcification in uremic CKD animals, demonstrating a critical role of uremia-induced oxidative stress in regulating vascular calcification in CKD⁹⁴.

Of note, oxidative stress modulates osteogenic differentiation of vascular cells and bone cells in an opposite manner, inducing VSMC calcification while inhibiting differentiation and mineralization of osteoblasts^{42, 85}, which may explain the inverse correlation of bone and vascular mineralization mediated by the systemic oxidative stress that is often found with aging, cardiovascular diseases and CKD conditions. Inhibition of oxidative stress via enhancing antioxidant systems and suppressing NADPH oxidase activity by statins promoted bone formation thus inhibiting osteoporosis in aged and ovariectomized rats⁹⁷. In contrast, statins block vascular calcification in the LDLR^{-/-} mice⁹⁸. Consistently, *in vitro* studies have demonstrated that elevation of intracellular oxidative stress, by oxidized lipids and H₂O₂, inhibits osteogenic differentiation of bone cells but induces osteogenic differentiation and calcification of VSMC. These paradoxical mineralization outcomes are related to the opposite effects of oxidative stress on the expression of Runx2, a key osteogenic transcription factor^{42, 86, 99}. As Runx2 is the essential regulator for osteogenic differentiation and mineralization in both bone cells and VSMC^{42, 88, 100}, oxidative stress may distinctly activate or inhibit intrinsic signaling pathways in two different cell types that ultimately converge to Runx2 for differential regulation of osteogenic transition. Uncovering oxidative stress-induced intrinsic signals in both bone and vascular cells may help to understand the inverse pathological outcomes in skeletal and vascular mineralization, and develop precision therapy targeting vascular calcification.

Hyperglycemia and protein glycosylation—Similar to the effects of oxidative stress, hyperglycemia and protein glycosylation signals are also associated with opposite mineralization outcomes in the bone and vasculature. Increased oxidized lipids and glucose promotes non-enzymatic glycation and accumulation of AGEs. The AGEs and their receptor RAGE-regulated signaling axis contribute significantly to age-associated cardiovascular diseases, including increased vascular calcification in diabetes, atherosclerosis and CKD^{92, 101, 102}. In addition to promoting oxidative stress, increased vascular accumulation of AGEs facilitates the binding of AGEs to RAGE and thus activates vascular inflammatory and multiple pro-osteogenic signaling pathways¹⁰³. In cultured VSMC, AGEs induce the expression of RAGE, thus accelerating VSMC osteogenic differentiation and calcification¹⁰⁴. AGEs form irreversible cross-links of matrix collagen and elastin, which results in stiffer ECM and arterial stiffening¹⁰⁵. Non-enzymatic glycation of collagen renders their resistance to enzymatic degradation and their accumulation in the arterial wall^{106, 107}. AGEs-modified elastin has also been linked to increased calcium binding and

aortic media calcification¹⁰⁵. RAGE mediates P_i-induced VSMC calcification *in vitro*¹⁰⁸ and vascular calcification in the *enpp1*^{-/-} mice¹⁰⁹. On the other hand, the AGEs/RAGE signaling pathways mediate diabetic osteopathy via regulating bone remodeling¹¹⁰. In cell cultures, AGEs inhibit osteogenic differentiation of osteoblasts by downregulating Runx2¹¹¹. Consistently, RAGE knockout mice exhibit increased bone mass¹¹². Overall, increased AGEs/RAGE signaling axis promotes vascular calcification but inhibits bone mineralization.

Chronic elevation of protein O-linked β-N-acetylglucosamine modification (O-GlcNAcylation), by increased glucose metabolism via the hexosamine biosynthesis pathway, has emerged as an important regulator for the pathogenesis of diabetes mellitus and diabetic cardiovascular complications⁹⁰. O-GlcNAcylation is a dynamic posttranslational modification on serine or threonine residues of proteins by a single sugar moiety GlcNAc, which is different from the classic N-glycosylation and O-glycosylation⁹⁰. O-GlcNAcylation is reversibly catalyzed by the sugar-adding O-GlcNAc transferase (OGT) and the sugar-removing O-GlcNAcase (OGA). A SNP in the *MGEA5* gene encoding OGA is associated with type 2 diabetes in Mexican Americans¹¹³, supporting a potential role of upregulation of O-GlcNAcylation in the pathogenesis of diabetes mellitus. Diabetes-associated chronic hyperglycemia and oxidative stress both can increase protein O-GlcNAcylation¹¹⁴, which accelerates the development of cardiovascular complications in diabetes^{114, 115}. We have recently demonstrated a novel role of increased protein O-GlcNAcylation in promoting VSMC calcification *in vitro* and vascular calcification in diabetes mice *in vivo*, via upregulation of Runx2¹¹⁶. The *in vivo* function of O-GlcNAcylation in regulating the formation of bone cells, including osteoblasts and osteoclasts, awaits further investigation. Increased O-GlcNAcylation inhibits BMP2-induced osteogenic differentiation of C2C12 cells, due to the suppressed Runx2 transcription activity¹¹⁷. However, another study has shown that increasing O-GlcNAcylation by OGA inhibition enhances osteoblastic differentiation of MC3T3-E1¹¹⁸. These *in vitro* studies need to be validated *in vivo* in animal models. The global OGT and OGA deletion mice are lethal^{119, 120}, which precludes *in vivo* characterization of the role of O-GlcNAcylation in regulating bone and vascular mineralization. Therefore, developing animal models with tissue-specific alteration of O-GlcNAcylation levels is necessary to dissect and define a causative role of O-GlcNAcylation in regulating bone and vascular mineralization *in vivo*.

Dysregulation of calcium and phosphate homeostasis—Genetic studies in humans and animal models discussed above have clearly linked impaired calcium, phosphate and pyrophosphate homeostasis to vascular calcification. In patients with CKD, particularly dialysis patients, decreased fetuin-A and pyrophosphates as well as increased serum phosphate (hyperphosphatemia), parathyroid hormone (PTH, hyperparathyroidism) and fibroblast growth factor 23 (FGF23) all compromise homeostasis of calcium and phosphate and promote vascular calcification, leading to increased arterial stiffness and cardiovascular mortality in these impacted patients^{95, 121, 122}.

Reduced concentrations of plasma pyrophosphate and fetuin-A, inhibitors of calcium phosphate, are documented in hemodialysis patients^{121, 122}. Both pyrophosphate and fetuin-A directly inhibit inorganic phosphate-induced VSMC calcification, by blocking osteogenic

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differentiation of VSMC and formation of hydroxyapatite crystals⁸⁴. Studies in CKD patients and human VSMC have demonstrated the uptake of the serum fetuin-A by VSMC and its secretion into ECM via matrix vesicles, thus inhibiting matrix vesicles-mediated nucleation of calcium phosphate¹²³. Despite the similar action of pyrophosphate and fetuin-A on bone and VSMC calcification, the vascular-bone mineralization paradox in CKD patients and mouse models remains. As noted in the pyrophosphate deficient *enpp1*^{-/-} mice, decreased bone density coexists with increased vascular calcification¹²⁴. On the other hand, the pyrophosphate analogs bisphosphonates inhibit vascular calcification in CKD patients and animal models of CKD^{125, 126}, suggesting that bone and VSMC may respond differently to the changes of extracellular PP_i. A recent study investigating phosphate-induced calcification has revealed that cultured VSMC exhibit much higher total NPP activity (generating PP_i) than osteoblasts. In contrast, osteoblasts possess over 100-fold higher TNAP activity (hydrolyzing PP_i and generating P_i) than calcifying VSMC¹²⁷. These differences in the basal PP_i/P_i metabolism in the two cell types may account for different levels of reactions between VSMC and bone cells in response to changes of PP_i/P_i ratios in their respective local microenvironment. Consistently, a TNAP inhibitor markedly inhibits bone formation in a concentration-dependent manner, but has no effect on VSMC calcification at the highest concentration used¹²⁷. In addition, ENPP1 appears to be necessary for early osteoblast differentiation via a mechanism independent of its enzymatic activity and generation of extracellular inorganic PP_i¹²⁸, which may contribute to the bone defects observed in the *enpp1*^{-/-} mice.

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Increased serum phosphate and PTH concentrations in CKD are associated with increased arterial calcification as well as decreased bone mineralization in hemodialysis patients and animal models of CKD^{129, 130}. Hyperparathyroidism induces release of serum phosphate and calcium from bone, the primary reservoir for calcium and phosphate that maintains systemic mineral homeostasis, thus resulting in bone mineral defects in CKD. Additionally, PTH has also been shown to induce the expression of RANKL, the master regulator for osteoclast formation, in osteoblasts; thus promoting osteoclast-mediated bone resorption that accelerates bone remodeling and high turnover of bone, the most common form of metabolic bone disorders in CKD¹³¹. Hyperparathyroidism-induced impaired phosphate excretion in CKD patients leads to hyperphosphatemia, which further stimulates PTH secretion¹³². Vitamin D analogues are used to reduce hyperparathyroidism, but the treatment has a side effect, often resulting in increased serum calcium and phosphate, and concurrently increased vascular calcification¹³³. In animals, vitamin D treatment induces medial arterial calcification¹³⁴.

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Hyperphosphatemia is one of the major factors that promotes the development of vascular calcification in patients with CKD, and increases the risk of cardiovascular events and mortality¹³². Inorganic pyrophosphate, transported via sodium-dependent P_i cotransporter, Pit-1, upregulates Runx2 and induces osteogenic differentiation and calcification of VSMC¹³⁵. Treatment of CKD patients with non-calcium-containing phosphate blocker delays progression of vascular calcification¹³⁶; whereas calcium-containing phosphate blockers accelerate the development of vascular calcification in CKD patients¹³⁷. Animal studies also demonstrate that both calcium containing and non-calcium-containing phosphate blockers exhibit similar effects on reducing phosphate levels in CKD mice, but

produce different results. Only non-calcium-containing phosphate blocker reduces calcium-phosphate and PTH levels, and inhibits progression of vascular calcification¹³⁸, suggesting an added role for calcium in promoting vascular calcification independent of phosphate levels.

The bone cells-derived FGF23 is an important regulator of PTH secretion, vitamin D metabolism and serum phosphate¹³⁹. Progressive increase in FGF23 is considered as a biomarker for CKD. Animals with deficiency in either *fgf23* or its co-receptor *klotho* exhibit increased vascular calcification and decreased bone mineral density concurrently, supporting an opposite regulatory role of FGF23/KLOTHO axis in bone and vascular mineralization^{140, 141}. Hyperphosphatemia and increased serum level of vitamin D are demonstrated in both *fgf23* and *klotho* deficient mice^{140, 141}, indicating that impaired calcium phosphate metabolism may contribute to FGF23/KLOTHO-mediated vascular calcification. Direct effects of high phosphate, and high calcium with or without high phosphate, on promoting VSMC calcification have been linked to multiple integrated cellular events, including increases in secretion of calcium phosphate-loaded matrix vesicles, apoptosis, and augmentation of osteogenic differentiation of VSMC^{135, 142–144}. Better understanding of how the different underlying mechanisms converge to modulate osteogenic differentiation and mineralization in bone and VSMC should advance our understanding of the bone-vascular paradox.

Pro-osteogenic signaling for VSMC calcification—In addition to regulating the essential matrix mineralization process in the ECM, systemic and local environmental changes differentially induce and activate intrinsic signaling pathways in vascular cells that promote osteogenic differentiation and expression of matrix proteins, leading to pathogenic calcification in the vasculature. Similar to osteoblast cells, upregulation of the osteogenic transcription factor, Runx2, is the hallmark for osteogenic differentiation of VSMC¹⁴⁵. Over the past two decades, numerous important signaling pathways and integrated regulatory mechanisms have been identified that define the transition of VSMC into “bone-like” cells, featuring decreased expression of SMC markers and increased expression of bone markers, including the master osteogenic transcription factor Runx2¹⁴⁶. A few key regulatory pathways are briefly highlighted below.

Upregulation of Runx2 is associated with calcification of multiple vascular cells, including calcifying vascular cells (CVC), VSMC, endothelial cells (via mesenchymal transition) and vascular progenitor cells^{42, 143, 147, 148}. A wide variety of stimuli, such as oxidative-stress, high phosphate, calcium and uremia, increase expression and activation of Runx2 and downregulate VSMC markers, inducing VSMC osteogenic differentiation and calcification^{42, 44, 60, 143, 149}. Using gain- and loss-of-function genetic approaches in VSMC and SMC-specific Runx2 ablation mice, we and others have determined an essential role of Runx2 in promoting vascular calcification and increasing production of matrix proteins. In contrast to the Runx2 null mice, which exhibit impaired early bone formation and neonatal lethality¹⁵⁰, SMC-specific Runx2 deficiency does not affect normal mouse development and arterial function^{88, 151}. However, the Runx2 deficiency attenuates VSMC calcification *in vitro* as well as intimal and medial arterial calcification in mouse models of atherosclerosis

and CKD^{42, 88, 151}. Accordingly, upregulation of Runx2 is a key determinant for VSMC calcification *in vitro* and vascular calcification *in vivo*.

Multiple pro-osteogenic signaling pathways are known to induce Runx2 upregulation and Runx2-mediated osteogenic function in VSMC, including the bone morphogenetic proteins (BMP), the msh homeobox 2 (Msx-2) as well as protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) signaling pathways. We have recently demonstrated that activation of p38 MAPK promotes VSMC calcification *in vitro* and vascular calcification *in vivo*¹⁵². The MAPK pathway increases phosphorylation of Runx2 on residues within the C-terminal proline-serine-threonine-rich domain, which may enhance binding of Runx2 to other co-factors, such as the coregulatory Smad proteins, thus enhancing osteoblast differentiation^{153, 154}. In addition, phosphorylation of Runx2 may also initiate epigenetic changes in the chromatin structure that are necessary for chromatin decondensation and increased transcription¹⁵⁵. In VSMC, p38 knockout inhibits Runx2 transactivity and calcification, supporting the role p38 MAPK in regulating Runx2 osteogenic function in VSMC¹⁵². Activation of the p38 MAPK signaling pathway by local stimuli, such as oxidative stress, extracellular matrix, mechanical loading, and BMP, may also interface with other signaling pathways such as Wnt¹⁵⁵, which further promotes upregulation of Runx2 and osteogenic differentiation of VSMC.

Activation of BMP signalling by lipid, glucose metabolism and inflammatory cytokines induces upregulation of Runx2 and osteogenic differentiation of VSMC^{156, 157}. The important regulation of the BMP-Msx2-Wnt signaling axis in promoting vascular calcification has been demonstrated, particularly in the context of diabetes. Msx2 promotes vascular calcification via Wnt signaling¹⁵⁸. SMC-specific ablation of Msx1 and Msx2 attenuates atherosclerotic calcification and aortic stiffness in diabetic mice^{60, 159}. Similarly, mice deficient in the *Msx2* gene manifest defects in skull ossification and a marked reduction in bone formation associated with decreases in osteoblast numbers¹⁶⁰. Furthermore, both Runx2 and Msx2 repress myocardin-mediated VSMC differentiation and enhance osteogenic differentiation, via direct binding to the SMC-specific transcription factors, serum response factor and myocardin^{161, 162}. Accordingly, upregulation of Runx2 and Msx2 in VSMC not only directly promotes osteogenic differentiation, but also inhibits the expression of VSMC markers genes that facilitates osteogenic differentiation and calcification.

Using cultured VSMC and animal models of diabetes and atherosclerosis, we have demonstrated that activation of AKT signaling pathway plays a major role in upregulating Runx2 and promoting VSMC calcification *in vitro* and vascular calcification *in vivo*^{42, 88, 95, 116, 163}. Oxidative stress activates multiple signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)/AKT¹⁶⁴. Inhibition of AKT activation blocks oxidative stress-induced upregulation of Runx2 and osteogenic differentiation of VSMC⁴². Furthermore, activation of AKT/FoxO1/3 signaling axis inhibits ubiquitination-mediated Runx2 degradation, leading to Runx2 upregulation and VSMC calcification^{42, 163}. In osteoblasts, AKT signaling is also critical for osteoblast proliferation and Runx2-dependent differentiation¹⁶⁵. Activation of the PI3K/AKT/FOXO1 signaling mediates 1 α ,25-Dihydroxyvitamin D₃-induced osteoblast differentiation and bone formation¹⁶⁶. However, in

contrast to activating PI3K/AKT signaling in VSMC, increased oxidative stress inhibits AKT and Wnt signaling-mediated proliferation and differentiation of osteoblasts¹⁶⁷; and increased activation of AKT blocks oxidative stress-induced osteoblast apoptosis¹⁶⁸. Therefore, although activation of PI3K/AKT/FOXO1 signaling axis promotes proliferation, survival and osteogenic differentiation signals similarly in bone and VSMC cells; its opposite outcomes in osteoblasts and VSMC may partially explain the differential mineralization seen in bone and VSMC.

In an animal model of diabetes, direct modification by *O*-GlcNAcylation is important for AKT activation, AKT-induced Runx2 upregulation as well as osteogenic differentiation and calcification of VSMC¹¹⁶. Chronic hyperglycemia increased *O*-GlcNAcylation in the vasculature of diabetic mice; and increased *O*-GlcNAcylation-mediated activation of AKT and upregulation of Runx2 promote vascular calcification and vascular stiffness in the diabetes mice¹¹⁶. *O*-GlcNAc modification of Runx2 has been reported in bone marrow mesenchymal stem cells¹⁶⁹, but the functional consequence of such modification remains unclear. Further investigations to determine whether Runx2 is directly modified by *O*-GlcNAcylation in VSMC and how *O*-GlcNAcylation of Runx2 may contribute to its osteogenic function will provide new molecular insights into Runx2-regulated osteogenic differentiation and mineralization of bone and vascular cells.

CONCLUSIONS AND PROSPECTIVE

Arterial stiffening and vascular calcification are emerging as markers of vascular aging and cardiovascular disease risk. Studies in humans and animal models of atherosclerosis, diabetic mellitus and CKD, in which vascular calcification is highly prevalent, have revealed a few important genetic determinants, systemic and local microenvironmental conditions, and intrinsic molecular regulators for vascular calcification. We have begun to appreciate the similar osteogenic differentiation and mineralization process shared by bone and vascular cells; and their distinct, often completely opposite responses and outcomes to the changes in their microenvironment. As a result, “soft tissues become hard while hard tissue become soft” are common pathologies associated with aging and metabolic disorders.

This review covers the contribution of the major disease-promoting factors, including oxidative stress, hyperglycemia and impaired calcium phosphate homeostasis, and their regulation of bone and vascular cells that account for the vascular-bone mineralization paradox. As bone is the major reservoir for calcium and phosphate minerals, increased release of serum phosphate and calcium from bone, by FGF23 deficiency and hyperparathyroidism, is responsible for decreased bone mineral density and subsequently induced vascular calcification in CKD. On the other hand, the loss of calcium and phosphate inhibitors, such as fetuin-A and pyrophosphate, promotes matrix mineralization of the vascular cells while reducing bone mineralization. The differences in the basal PP_i and P_i metabolism in bone and vascular cells and their response threshold to the changes of local PP_i and P_i is also responsible for the inverse mineralization seen in bone and vascular cells. Therefore, managing local and systemic PP_i and P_i levels may offer an effective strategy to mitigate the adverse mineral outcomes in both bone and vasculature.

It is encouraging that inhibition of oxidative stress and improving glucose and calcium phosphate metabolism are beneficial, at least partially, in reducing vascular calcification and increasing bone mineralization, notably in animal models. The cellular impact of these environmental stresses on VSMC is highlighted in this review, as VSMC are key to maintaining vascular homeostasis in all arterial system. The intrinsic stiffness and extracellular matrix remodeling of VSMC contribute to the osteogenic differentiation and calcification of VSMC. At the molecular level, upregulation of Runx2 is a major determinant for VSMC osteogenic differentiation and calcification. In atherosclerosis, diabetes mellitus and CKD, increased oxidized lipids, hyperglycemia and protein glycosylation, as well as high calcium and phosphate conditions all induce upregulation of Runx2, which is mediated via the activation of multiple signaling pathways, including BMP, Msx2/Wnt, MAPK and AKT signals. Of note, some of these signaling pathways are operating differently in bone cells. Many are inhibited by oxidative stress, hyperglycemia and high phosphate in osteoblasts, which leads to reduced bone mineralization.

To date, there is no effective and selective treatment for vascular calcification and arterial stiffness. Lessons learned from human and animal models have provided strong mechanistic explanations as to why some treatments, such as statins, ACE inhibitors, AGEs crosslink breaker, phosphate binders or pyrophosphate analogs, exhibit beneficial effects on vascular calcification and arterial stiffening^{10, 12–14}. Some of these treatments have also demonstrated positive impact on bone mineral density. For instance, *in vivo* animal studies showed that statins increased bone formation and inhibited osteoporosis via inhibiting oxidative stress⁹⁷. On the other hand, statins blocked vascular calcification⁹⁸. Recent studies found that treatment of CKD rats with the AGE crosslink breaker alagebrium decreased total AGEs amounts in bone but not aortas. However, alagebrium reduced aorta expression of RAGE and inhibited oxidative stress and aorta calcification, whereas did not improve bone mechanics¹⁷⁰, suggesting that specific matrix AGEs may regulate bone and arterial mineralization differently. The bone-resorbing bisphosphonates have generally shown beneficial effects on aortic and coronary calcification^{125, 126, 171}, while the effects may differ based on the type, potency, dosage and administration route of bisphosphonates. In addition, combination therapy such as statin plus bisphosphonate is significantly more effective in reducing atherosclerotic aortic plaques in hypercholesterolemic patients than either monotherapy¹⁷², supporting the use of mechanism-driven combination therapies for arterial stiffness in different underlying conditions. We have recently found that dietary potassium supplements effectively inhibit vascular calcification and arterial stiffness in the ApoE^{-/-} mice, which is associated with inhibition of Runx2 and VSMC calcification¹⁷³. Accordingly, introducing dietary approaches to lifestyle may help to prevent the development of vascular calcification and stiffening, thus improving vascular health.

Evidence from human, animal and cellular studies has advanced our knowledge on the development of vascular calcification and its links to arterial stiffening. A more comprehensive and precise understanding of the regulation of VSMC osteogenic differentiation, eg. identifying VSMC subpopulations and progenitor cells, and the key cellular events and regulators that mediate VSMC de-differentiation, will help to define useful biomarkers and structural changes for early detection and prevention of vascular calcification. Furthermore, since bone and vascular cells share similar mineralization

process, treatment strategies for vascular calcification should not ignore the potential impact on the bone, and vice versa. Further investigation to systemically compare vascular and bone cells in aging and vascular calcification-prevalent diseases may reveal cell type-specific regulatory network and/or disease-specific regulators. Uncovering the intrinsic cellular regulation of bone and vascular cells in responses to the systemic and local environmental changes should lead to identifying precise targets that are amendable for therapeutic interventions for vascular calcification, without affecting the skeletal system.

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ABBREVIATIONS

ABCC6	ATP-binding cassette transporter subtype 6
ACDC	arterial calcification due to deficiency of CD73
AGEs	advanced glycation end products
AKT	protein kinase B
ALP	alkaline phosphatase
ANK	ankylosis protein
ApoE	apolipoprotein E
ATP	adenosine triphosphate
BMP-2	bone morphogenetic protein-2
CKD	chronic kidney disease
Col 1	type 1 collagen
ECM	extracellular matrix
ENPP1	ectonucleotidase pyrophosphatase/phosphodiesterase-1
FGF23	fibroblast growth factor 23
GACI	generalized arterial calcification of infancy
GWAS	genome-wide association studies
H₂O₂	hydrogen peroxide
JNK	c-Jun N-terminal kinases

LDL	low-density lipoprotein
MAPK	mitogen-activated protein kinases
MGP	matrix Gla protein
MMP	matrix metalloproteinases
MVs	matrix vesicles
NADPH	nicotinamide adenine dinucleotide phosphate
NPP	nucleototide pyrophosphatase/phosphodiesterase-1
O-GlcNAcylation	O-linked β -N-acetylglucosamine modification
OPN	osteopontin
OxLDL	oxidized low-density lipoprotein
Pi	inorganic phosphate
PI3K	phosphatidyl inositol 3-kinase
PTH	parathyroid hormone
PWV	pulse wave velocity
PXE	pseudoxanthoma elasticum
RAGE	Receptor for advanced glycation end products
RANKL	receptor activator of nuclear factor kappa-B ligand
ROS	reactive oxygen species
Runx2	Runt-related transcription factor 2
SMA	smooth muscle α -actin
SMMHC	smooth muscle cell myosin heavy chain
SNP	single nucleotide polymorphism
TNAP	tissue non-specific alkaline phosphatase
VSMC	vascular smooth muscle cells

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HIGHLIGHTS

This review focuses on the contribution of vascular calcification to arterial stiffness. The important genetic factors, systemic and local microenvironmental signals, and underlying signaling pathways and regulatory molecules that promote vascular calcification-driven arterial stiffness are highlighted:

- Monogenic genetic mutations in humans, featuring dysregulation of PP_i/P_i metabolism and loss of inhibitors of calcium and phosphate, display vascular calcification and arterial stiffening,
- Elevation of oxidative stress, hyperglycemia and dysregulated calcium and phosphate homeostasis enhance vascular calcification and arterial stiffening,
- Activation of the major signaling pathways and their regulation on the master osteogenic transcription factor Runx2 in promotes osteogenic differentiation and calcification of VSMC,
- Differential regulation of intrinsic signaling pathways by local and systemic factors in bone and vasculature contributes to the vascular-bone mineral disorder paradox,
- Potential therapeutic strategies targeting molecular events that determines vascular calcification under different microenvironments.

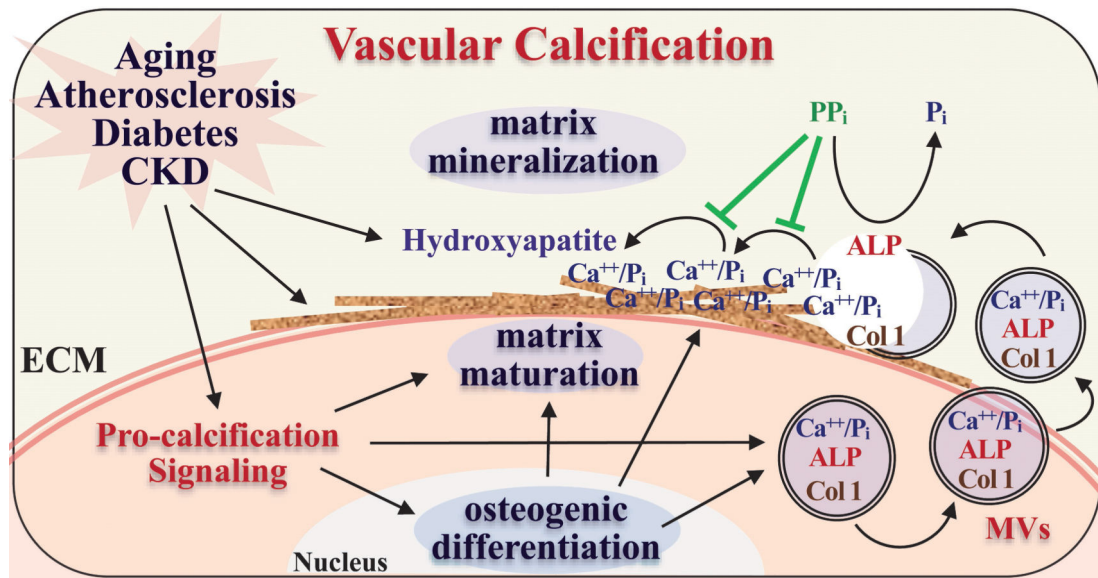


Figure 1.

Vascular calcification resembles the process of osteoblast differentiation and mineralization, involving osteogenic differentiation, matrix maturation and mineralization. Systemic and local microenvironmental conditions associated with atherosclerosis, diabetes mellitus, CKD and aging induce osteogenic differentiation of vascular cells and promote matrix remodeling and mineralization, leading to vascular calcification.

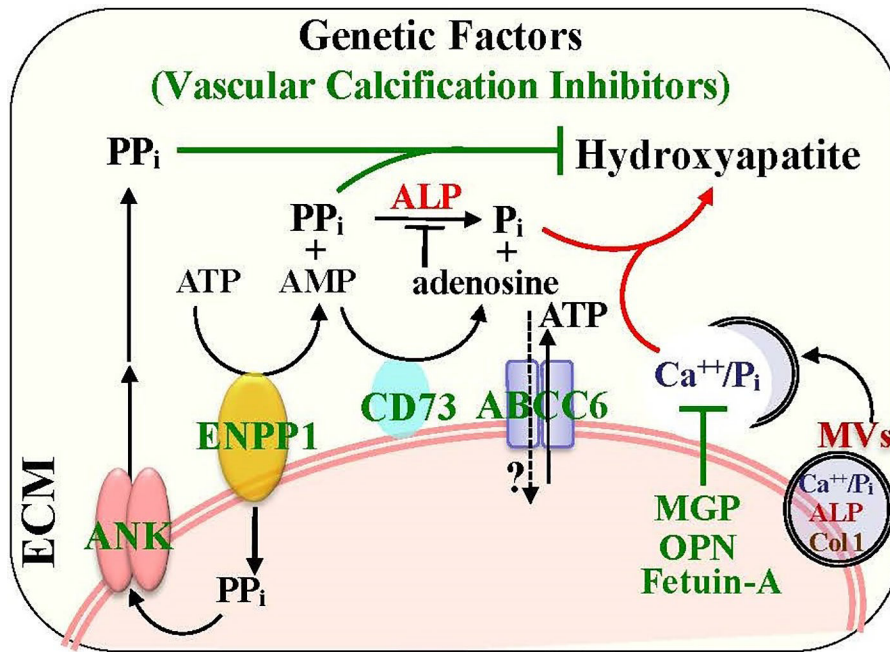


Figure 2.

Genetic and cellular determinants that regulate pyrophosphate and phosphate homeostasis and the formation of hydroxyapatite crystals in the ECM. The key enzymes (ENPP1, CD73 and ALP) and transporters (ANK and ABCC6) that mediate the ATP metabolic pathway and pyrophosphate catalysis are detailed. The functions of matrix vesicle (MV) that carry and transport calcium/phosphate (Ca⁺⁺/P_i) and matrix proteins to ECM for the formation of hydroxyapatite crystals and the secretion factors that are important for calcium binding and inhibition of the formation of hydroxyapatite crystals (MGP, OPN and Fetuin-A) are highlighted.

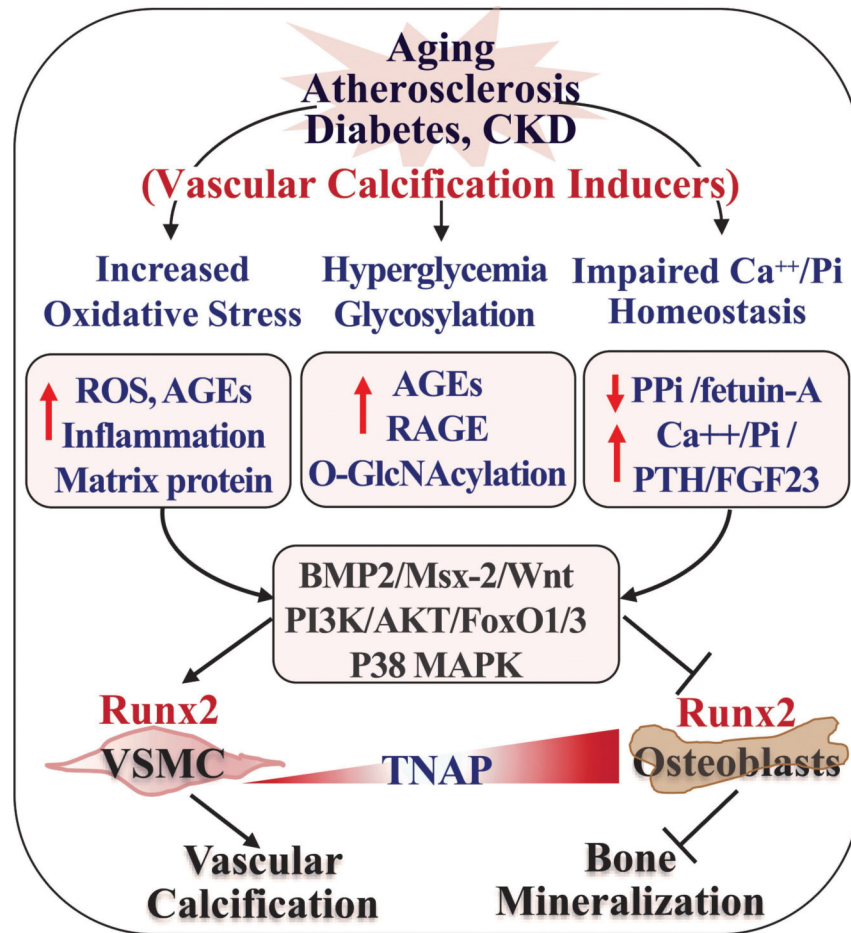


Figure 3. Differential responses to pathogenic inducers in vasculature and bone environment leads to opposite outcomes in mineralization of bone and vascular cells.

Aging and pathogenic conditions including atherosclerosis, diabetes and CKD are associated with systemic and tissue microenvironmental changes, such as increased oxidative stress, hyperglycemia, and impaired $\text{Ca}^{++}/\text{P}_i$ homeostasis. The difference in basal levels of TNAP, high in bone and low in VSMC, and their different responses to the pathologic conditions activate various intrinsic signaling cascades that eventually lead to opposite effects on the key osteogenic transcription factor Runx2, upregulating Runx2 in VSMC that promotes vascular calcification whereas inhibiting Runx2 in osteoblasts that decrease bone mineralization.

Table 1.

Genetic regulators of vascular calcification in mouse and human

Mechanisms	Gene Symbol	Mouse Models			Human Disease Correlation (SNPs / Mutations)
		Genotype	Vascular Phenotype	Bone Phenotype	
Calcium binding, inhibiting hydroxyapatite crystal formation	<i>MGP</i>	<i>Mgp</i> ^{-/-}	Arterial calcification	Cartilage calcification, Short stature, Osteopenia, fractures	Increased arterial calcification in men, Keutel syndrome
	<i>SPP1</i>	<i>Opn</i> ^{-/-}	Enhancing calcification of the mgp ^{-/-} mice, Aortic fibrosis and vascular stiffening in LDLR ^{-/-} mice;	Hypermineralized, Resistant to ovariectomy-induced bone resorption	
	<i>AHSG</i>	<i>Ahsg</i> ^{-/-}	Diffused arterial calcification, Calcified intimal plaques upon vascular injury		Alopecia-mental retardation syndrome 1, Increased arterial calcification and stiffness in patients with atherosclerosis, diabetes mellitus and CKD
Impaired phosphate and pyrophosphate homeostasis (ATP metabolism)	<i>ENPP1</i>	<i>ttw/tw (lipo-walking mouse)</i>	Aortic calcification in early life	Articular cartilage calcification, hyperostosis	Generalized arterial calcification of infancy 1, Increased aortic arch calcification in patients with type II diabetes, Cole disease, Autosomal recessive hypophosphatemic rickets 2
		<i>enpp1</i> ^{-/-}	Arterial calcification	Spine and peripheral joint fusion	
	<i>ANKH</i>	<i>ank/ank</i>	Arterial calcification	Arthritis, bone outgrowths, joint destruction	Cranio metaphyseal dysplasia, Chondrocalcinosis-2
	<i>NTSE</i>	<i>e-5'NT/CD73</i> ^{-/-}	Prothrombotic and proinflammatory phenotype of the vasculature		Arterial calcification due to deficiency of CD73 (ACDC); Calcification of joints and arteries
<i>ABCC6</i>	<i>abcc6</i> ^{-/-}	Myocardial calcification, Arterial and retinal calcification		Generalized Arterial Calcification of Infancy 2, Pseudoxanthoma Elasticum	