Peripheral arterial disease (PAD) refers to occlusion or narrowing of the upper and lower extremity arteries, causing insufficient blood flow to the limbs. Patients with PAD often present with symptoms, such as claudication, ischemic rest pain, ischemic ulcerations, and limb loss. These patients are also highly predisposed to cardiovascular morbidity and mortality. PAD is underdiagnosed, and little is known about its pathogenesis, although it has generally been considered as secondary to atherosclerotic disease, which mainly affects lower limb vascularization. Indeed, the major treatment for PAD is based around revascularization therapies to bypass/alleviate stenosis and occlusions. However, recent literature has uncovered a role for vascular calcification not only as a major factor limiting treatment but also as a potential driver of PAD.3–5

Vascular calcification is a pathology characteristic of aging but is accelerated in chronic kidney disease (CKD), diabetes mellitus, and atherosclerosis. Although localized intimal calcification is a feature of atherosclerosis, extensive, widely disseminated medial calcification, often visible on plain X-ray, is a common presenting feature of lower extremity PAD.1 The prevalence of medial arterial calcification (MAC) in PAD is imprecisely reported because of the methods by which diagnosis is reached, as well as asymptomatic cases that are often unreported.2 Currently, the gold standard by which PAD is diagnosed is by noninvasive means via the ankle brachial pressure index and toe brachial pressure index measurements and subsequently confirmed by duplex scan and angiography which can be extended to computed tomography and magnetic resonance imaging. Accurate diagnosis can be challenging because of preexisting CKD or diabetes mellitus. For example, the effectiveness of the ankle brachial pressure index ratio in diagnosis of PAD for diabetic and CKD patients on dialysis is reduced when there is presence of MAC and peripheral neuropathy.6 Moreover, endovascular treatment strategies are often ineffective in patients with PAD and MAC, despite advances in recanalization techniques.7 Thus, there is much incentive for a paradigm shift and closer examination of the pathology of MAC in PAD to develop better therapies.2

**Types of Vascular Calcification and Pathological Outcomes**

Vascular calcification involves crystallization of calcium/phosphate in the form of hydroxyapatite in the extracellular matrix and occurs in both the intima and the media of the arterial wall via different disease etiologies. Intimal calcification is associated with atherosclerosis and forms in association with vascular smooth muscle cells (VSMCs), macrophages, and the necrotic lipid core. Rupture or erosion of atherosclerotic plaques can lead to obstruction in blood flow and suboptimal organ perfusion and is usually considered the major event leading to ischemia in PAD. It remains controversial whether calcification plays a role in promoting plaque rupture. Various models have been proposed to account for the association of intimal calcification with cardiovascular events, including a role for calcification in destabilizing the mechanical integrity of the plaque during hemodynamic stress, whereas more...
recent data suggest that small nanocrystals of calcification are more prevalent in unstable plaques and can promote macrophage inflammation, VSMC death, and fissuring of the fibrous cap.\(^8\) Whether this type of intimal nanocalcification is prevalent in PAD has not been studied.

In contrast, medial calcification occurs independently of atherosclerosis and is strongly associated with aging, CKD, and diabetes mellitus.\(^10\) Deposition of hydroxyapatite crystals occurs in the absence of inflammatory cells along concentric elastin fibers, directly abutting VSMCs. Medial calcification causes vascular stiffening and decreased compliance of the vessel. However, although the adverse effects of MAC in the aorta on stiffening, systolic blood pressure, and coronary perfusion are well studied, the pathological effect of MAC in other vascular beds is poorly understood. In PAD with MAC, the peripheral arterial system is stiffened. This is likely to have effects on localized blood flow, and there has also been some suggestion that it may exacerbate the effects of aortic stiffness and affect heart and brain health, but further investigations are required to define these mechanisms.\(^11\)

**What Does the Pathology of Calcification in PAD Tell Us About the Disease Process?**

Histological examination of arteries from patients with PAD confirm that MAC, as an entity distinct from atherosclerosis, is prevalent.\(^12\) The calcification present in the vessel media is most often dystrophic; however, the presence of real bone and chondrogenic metaplasia has also been reported, particularly in patients with diabetes mellitus.\(^13\) Anecdotal evidence from vascular surgeons suggests that these bone-like calcifications can actually protrude into the vessel lumen. MAC can extend from the small digital arteries of the toes up to the large tibial, popliteal, and femoral arteries of the leg. It is important to note that medial calcification alone is associated with increased cardiovascular mortality;\(^1\) thus, a key question is whether these medial calcifications can directly cause cardiovascular events, such as vessel occlusion, or whether ultimately they act indirectly to accelerate atherosclerosis or plaque rupture.

There are some evidence from other conditions characterized by MAC of small arteries that it may in some instances directly precipitate ischemic events. Calciphylaxis, a devastating complication of CKD and dialysis, is associated with medial calcification of the small arteries of the skin. Occlusion of these calcified arteries because of thrombus formation is thought to be the initiating factor in ischemia and onset of gangrene in the skin and underlying fatty tissue.\(^19\) How the occlusion is initiated is unknown, but it is plausible that similar processes could occur in PAD. To date, there have been no systematic clinical studies to determine whether MAC precedes or enables atherosclerosis development and rupture in PAD. Such studies are difficult to perform in older PAD patients because of overlapping risk factors for atherosclerosis and MAC, as well as the limitations of imaging technologies. However, a study in children with CKD showed that there was a correlation between the severity of MAC in abdominal arteries and carotid intima–media thickness.\(^20\) Although these measurements were performed in different vascular beds, they may hint that calcium loading of the vessel wall can lead to adverse remodeling, which may predispose to atherosclerosis. In support of this notion, an animal study showed that the initiation of calcification, using the anticoagulant warfarin, during the atherosclerotic process caused adverse remodeling/vulnerable plaque.\(^21\) Finally, it cannot be ruled out that increased calcification in either the intima or media in patients with PAD may cause plaque rupture directly or indirectly by affecting processes, such as hemodynamics or fibrous cap integrity as described earlier and summarized in Figure 1.

**Mechanisms of Vascular Calcification**

Currently, there are no treatments for vascular calcification. Understanding the mechanisms of calcification may help provide insights into potential therapies and also inform on how MAC may participate in the adverse outcomes of PAD.

Vascular calcification was long presumed to be a passive, degenerative process of calcium/phosphate precipitation. However, research findings over the past 2 decades have clearly illustrated that the pathogenesis of vascular calcification is active and cell-mediated, driven primarily by VSMCs.\(^10\) Several key events are necessary, and are likely to occur simultaneously, for VSMC-mediated calcification. These events are initiated when VSMCs become damaged and overburdened by hostile conditions in the microenvironment, causing them to lose essential defensive mechanisms and stimulating them to undergo a vicious cycle of cell death and transdifferentiation to promote an osteo/chondrogenic phenotype that can actively drive mineralization (Figure 2).

**Nidus Formation**

The initial phase of mineralization involves formation of nucleation sites for the deposition of hydroxyapatite crystals.
The nidus for mineralization is provided by calcium-rich, membrane-bound matrix vesicles released by living VSMCs, as well as apoptotic bodies released during programmed cell death. EM studies show localization of matrix vesicles close to elastin and collagen fibrils. Recent evidence in VSMCs suggests that an exosomal pathway is the major source of matrix vesicles in the vessel wall and that this pathway enables the loading of these exosomes with specific cargoes that act as inhibitors to block calcification, including matrix Gla protein and fetuin-A. However, prolonged exposure to procalcific conditions, such as mineral imbalance and dysregulation of calcium/phosphate homeostasis as occurs in CKD, leads to loss of inhibitors from these exosomes and exposure of phosphatidylserine and annexin on their surface to initiate mineralization.

Indeed, loss of inhibitors is key to the onset of calcification, and studies in both animals and man have highlighted that the loss of a single calcification inhibitor can initiate MAC. A healthy vessel wall is protected from calcification via production and secretion of these inhibitors, including the endogenous inhibitors matrix Gla protein and pyrophosphate. Animals lacking matrix Gla protein develop extensive calcification in the tunica media and cartilaginous metaplasia of the media of all elastic arteries, and this can lead to aortic rupture. Phosphate homeostasis also plays a key role in the inhibition of calcification. Pyrophosphate is a powerful inhibitor released from breakdown of adenosine triphosphate by the ENPP1-NTSE purine metabolic pathway. Mutations in ENPP1 and NTSE (coding for ectonucleotide pyrophosphatase phosphodiesterase I and 5′-nucleotidase, respectively) perturb this balance and lead to several human diseases, with frequent presentation of MAC in the lower limbs. Inactivating mutations in the ENPP1 gene causes generalized arterial calcification of infancy that typically results in neonatal fatality with systemic calcification, although mutations in the NTSE gene have been implicated in PAD. Although patients with NTSE mutations show some features of typical PAD, more clinical data are required to determine the exact nature of the calcific lesions they develop.

**Osteo/Chondrogenic Differentiation**

When challenged with a procalcific milieu, VSMCs undergo phenotypic transition characterized by a loss of contractile markers and increased expression of bone-related genes, such as bone sialoprotein, Msx2, osteocalcin, Cbfa1/Runx2, and BMP. Human peripheral arteries with medial calcification were shown to have decreased expression of calcification inhibitors and elevated levels of osteo/chondrogenic markers. Runx2 is often referred to as the master regulator mediating this phenotypic switch, not only by activating bone gene expression but also by suppressing myocardin/SMC, which regulates expression of SMC markers. Although the dynamics and mechanisms of this process remain unclear, it seems likely that the maintenance of normal VSMC contractile function is protective against calcification.

**Evidence for VSMC Premature Aging: Impetus for Medial Calcification in PAD**

Although much progress has been made in understanding the cell and molecular mechanisms driving calcification, our knowledge of the risk factors that drive MAC in PAD is incomplete. Although PAD shares traditional risk factors for CVD, including advanced age, diabetes mellitus, smoking, hypertension, and hyperlipidemia, MAC is generally considered an age-associated pathology. VSMCs have been shown to exhibit an aged phenotype in disease, as well as during serial passaging in vitro.

Most importantly, these aged VSMCs upregulate a variety of molecules that promote osteogenic differentiation, leading to the notion that premature aging of VSMCs may be a key driver of osteo/chondrogenic change and calcification in PAD. Importantly, aged and senescent VSMCs are also prevalent in vulnerable plaque,
suggesting that aging of VSMC may be a key link between MAC, atherosclerosis, and PAD.\textsuperscript{35}

**Cellular Senescence and Vascular Aging Links MAC and Atherosclerosis?**

One defining feature of aging is the accumulation of senescent cells. The concept of cellular senescence was first described by Hayflick in 1965 to refer to the finite replicative lifespan of human somatic cells in culture.\textsuperscript{36} Although senescence was initially ascribed to telomere attrition, it is now clear that a variety of factors, including DNA damage and oxidative stress, can also induce cellular senescence. In man, most studies report on vascular senescence associated with atherosclerosis.\textsuperscript{37} For example, VSMCs in atherosclerotic plaques display hallmarks of cellular senescence, such as upregulation of p16 and senescence-associated β-galactosidase activity.\textsuperscript{37}

VSMC senescence has also been reported in CKD patients, but its prevalence is unclear in PAD.\textsuperscript{33}

Recently, Liu et al reported that accumulation of persistent DNA damage triggers the DNA damage response in VSMCs. On sensing genomic insult, downstream kinase ataxia telangiectasia–mutated signaling is activated, and the cell is directed toward one of several fates—apoptosis, transient cell cycle arrest, or senescence.\textsuperscript{38} Importantly, in vitro VSMC senescence is often preceded by upregulation of p16 and followed by subsequent development of a senescence-associated secretory phenotype, characterized by release of a host of growth factors, proteases, and inflammatory cytokines, including BMP2, interleukin-6, and OPG that promote the osteogenic phenotype.\textsuperscript{33} Hence, the implications are that senescent VSMCs are able to act as a paracrine source, signaling osteogenic differentiation and inflammation to remote sites, while simultaneously mediating calcification locally. In the context of PAD with associated MAC, it is plausible that this senescence-associated secretory phenotype emanating from calcified VSMCs also affects cells in atherosclerotic plaques and may act to promote plaque progression and rupture. Reports that several inflammatory mediators, including interleukin-6, are increased in the circulation of patients with PAD and predict outcome provide some support for this hypothesis.\textsuperscript{39}

**Roles for Oxidative Stress and DNA Damage in VSMC Aging**

One of the major drivers of premature VSMC aging and DNA damage in PAD is likely to be oxidative stress.\textsuperscript{40} Oxidative stress primarily refers to an imbalance state between production of reactive oxygen species and a counteracting mechanism in cells. Importantly, diseases, such as diabetes mellitus and CKD, have been shown to be in a pro-oxidant state.\textsuperscript{41} For example, in diabetes mellitus, oxidative stress is generated as a result of chronic hyperglycemia exposure coupled with increased activity of superoxide-producing enzymes, and this is further exacerbated by the proinflammatory extracellular environment in disease.

One of the major cellular targets of reactive oxygen species is DNA, and exposure to elevated reactive oxygen species results in lesions, such as DNA breaks and point mutations, as well as compromised telomere integrity.\textsuperscript{42} Both telomere shortening and mitochondrial DNA damage have been linked to accelerated atherosclerosis.\textsuperscript{43,44} In addition, increased oxidative stress is also a prominent factor in the pathobiology of vascular calcification, whereas there is some evidence to suggest that increased DNA damage also affects VSMC contractility.\textsuperscript{45} In line with the hypothesis that elevated oxidative stress increases DNA damage, which hastens disease progression, significantly higher levels of 8-hydroxy-2-deoxy-guanosine, a known marker for oxidative stress-related DNA damage, have been reported in patients with diabetes mellitus and CKD.\textsuperscript{46,47}

To this end, however, DNA damage because of elevated oxidative stress has not been fully investigated in PAD.

**Nuclear Lamina Defects Accelerate VSMC Aging in MAC and Atherosclerosis**

Emerging evidence suggests that there may also be tissue-specific mechanisms of VSMC aging acting via nuclear lamina dysfunction and affecting VSMCs in both calcified and atherosclerotic lesions. Importantly, these mechanisms are specific and may be amenable to therapeutic intervention. Evidence for such mechanisms emerged from studies of the segmental aging disorder, Hutchinson-Gilford Progeria Syndrome, which is caused by a mutation in the LMNA gene, which encodes for nuclear lamins A/C.\textsuperscript{48} Children with Hutchinson-Gilford Progeria Syndrome display premature, accelerated aging with vascular calcification and VSMC attrition and usually succumb to cardiovascular complications during the second decade of life.\textsuperscript{49} Mouse models of Hutchinson-Gilford Progeria Syndrome develop progressive loss of VSMCs in the medial layer of large arteries and later develop MAC associated with defective pyrophosphate metabolism.\textsuperscript{49,50}

Lamins A/C are nuclear intermediate filament proteins that not only act as scaffolding proteins underlying the nuclear envelope, but also play regulatory roles in key signaling pathways via their interactions with a host of lamin-binding proteins.\textsuperscript{51} Nuclear lamins are translated as a precursor protein, prelamin A, which undergoes carboxylation and farnesylation steps, finally ending with proteolytic cleavage by the FACE1/Zmpste24 metalloproteinase to yield mature lamin A. Hutchinson-Gilford Progeria Syndrome patients harbor a de novo point mutation that deletes the FACE1 cleavage site, resulting in a permanently farnesylated protein form of prelamin A called progerin. The finding that both progerin and prelamin A can be detected in normally aged tissues, and most prominently in the vasculature, has generated immense interest in this progeroid model,\textsuperscript{52} in the hope that insights gleaned can be translated to physiological vascular aging.

Children with CKD display similarities to those seen in these progeroid syndromes. The lack of conventional risk factors spurred the notion that perhaps the uremic milieu in CKD accelerates the aging process. Interestingly, prelamin A has been found to accumulate in normal, senescent VSMCs and in calcified arteries from young patients with CKD, which coincidentally exhibited increased DNA damage and osteogenic differentiation.\textsuperscript{53,54} Downregulation of FACE1 expression, potentially because of enhanced oxidative stress, is postulated to be the cause of prelamin accumulation.\textsuperscript{54} Studies using vessel rings from CKD children and treated with H2O2...
show reduced FACE1 expression concurrent with prelamin A expression.\textsuperscript{34} Ectopic expression of prelamin A in VSMCs in vitro resulted in accumulation of DNA damage, indicated by formation of γH2AX and 53BP1 foci.\textsuperscript{33} Prelamin A interferes with DNA repair by delaying recruitment of 53BP1 to sites of DNA damage,\textsuperscript{34} thus setting into motion a domino effect, leading to irreparable cellular damage, and the cell eventually undergoes premature cellular senescence via p16-dependent pathways, finally resulting in osteogenic differentiation of VSMCs.\textsuperscript{33} Whether prelamin A expression is a cause or consequence of premature VSMC senescence, and the precise mechanism(s) of how prelamin A accumulates and induces DNA damage have yet to be elucidated. Importantly, prelamin A–positive VSMCs are also found in advanced atherosclerotic plaques, further supporting the notion that premature VSMC aging is a process that might concurrently promote MAC and atherosclerosis in PAD.\textsuperscript{55}

### Role for Neuropathy in VSMC Calcification/ Dysfunction

Although the accumulation of DNA damage and premature aging may provide new insights into treatment targets for PAD, it does not account for the specific peripheral artery disease seen in patients with diabetes mellitus. In diabetic patients, neuropathy is a common complication, and the risk of PAD increases with the presence of peripheral neuropathy of the lower extremities.\textsuperscript{56,57} In diabetes mellitus, multiple cellular stressors, including inflammation, enhanced oxidative stress, and dysregulation of the AGE/RAGE system, can potentially result in nerve and vessel damage.\textsuperscript{58}

Importantly, sympathetic denervation of the smooth muscle cell layer of the tunica media has been speculated to be of importance in the etiology of MAC.\textsuperscript{58} MAC in the feet arteries was detected in 92% of 60 diabetic and nondiabetic patients after undergoing uni- or bilateral lumbar sympathectomy.\textsuperscript{59} The vascular tree receives sympathetic innervation that controls blood vessel resistance. These efferent fibers are found along the outer border of the tunica media and secrete their neuroregulators into the extracellular fluid surrounding the VSMCs. Thus, it is plausible that crosstalk exists between the vasculature and the nerves that supply them, although there is scant evidence as to how this interaction is orchestrated and what the consequence of this regulation might be.

Substance P has previously been suggested to be involved in vascular remodeling in pulmonary hypertension\textsuperscript{60}; in a rat model of monocrotaline-induced inflammation of pulmonary vessels, reactive oxygen species were thought to lead to increased substance P, via inhibiting substance P degradation.\textsuperscript{61} Secretoneurin and neuropeptide Y are also thought to regulate VSMC migration and proliferation, but further work is required to determine how these pathways might be deregulated in PAD.\textsuperscript{62-64}

MAC is frequently seen in the pedal arteries of patients with neuropathy-related complications, including foot ulceration and Charcot osteoarthropathy (Charcot foot) in diabetes mellitus patients.\textsuperscript{65,66} Indeed, an invariant feature of Charcot foot is the presence of MAC in the arteries,\textsuperscript{56} and Charcot foot greatly predisposes to amputation and mortality. The significance of the RANKL/OPG system in Charcot foot has been demonstrated in vivo as well as in vitro over the past few years.\textsuperscript{58} RANKL binds to its receptor RANK on osteoclastic precursors and induces NF-κB signaling, further driving downstream osteoclastic activity and bone resorption, which may further affect vascular calcification.\textsuperscript{57} Furthermore, RANKL/RANK have been shown to be a potent promoter of osteo/chondrogenic differentiation of VSMCs, suggesting a possible feedback between bone and vascular calcification in this condition.\textsuperscript{68,69}

The upstream activators of the RANKL/OPG have not been clarified, but there is evidence that the usual suspects of oxidative stress and a proinflammatory microenvironment play crucial roles.\textsuperscript{58} The exact type of nerve damage in Charcot foot patients is also not clear. However, the loss of nerve-derived peptides has been linked to an overactive RANKL/OPG axis to drive disease progression. Immunohistological analysis in Charcot foot patients has noted reduced expression of calcitonin gene–related peptide,\textsuperscript{70} a neurotransmitter that modulates bone growth, repair, and remodeling in nociceptive C fibers.\textsuperscript{71} Interestingly, calcitonin gene–related peptide is also known to have vasodilatory properties and the lack thereof is thought to contribute to age-related hypertrophy in rats.\textsuperscript{72,73} It is not known whether calcitonin gene–related peptide modulates the RANKL-mediated bone resorption in Charcot foot. More work is needed to elucidate the role of calcitonin gene–related peptide and other nerve-derived factors to find missing links connecting diabetic neuropathy and vascular calcification, particularly in defining whether neuropathy affects VSMC phenotypes.

### Conclusions

Vascular calcification has been a subject of intense study for centuries, owing much to its prevalence in aging, atherosclerosis, and cardiovascular disease. Abundant evidence has linked MAC and PAD; yet, little is known about the mechanisms driving MAC in this complicated, multifactorial disease. To date, a transgenic animal model for PAD is lacking, and current existing disease models for PAD rely on surgical intervention to induce lower extremity arterial occlusion. Nonetheless, insights from studies of human aging and diabetes mellitus and CKD (which frequently coexist with and aggravate symptoms associated with PAD) converge toward an essential role played by VSMCs in orchestrating the events leading to vascular calcification, and these calcified/aged VSMCs may impact directly and indirectly to promote plaque rupture (summarized in Figure 3). However, several open questions remain. Specifically, to what extent do individuals with PAD exhibit sustained DNA damage? Is this DNA damage related to oxidative stress or do other factors play a role? On a similar note, can oxidative stress explain the pathogenesis of nerve damage in neuropathy? It would also be intriguing to know whether prelamin A accumulation is a hallmark of PAD? Finally, how do neurotrophins regulate VSMC phenotype? Undoubtedly, there is a pressing need for a better understanding of the interplay of various risk factors associated with these disorders and modulation of VSMC phenotypes, as well as improvements in the sensitivity of PAD and MAC detection, for instance, via...
new imaging modalities, such as 18F-sodium fluoride, F18-FDG PET/computed tomography, to achieve better patient management and improve the overall quality of life of patients with PAD.

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None.

References


Peripheral arterial disease is an increasingly prevalent health problem often considered secondary to atherosclerosis. However, recent evidence points toward medial arterial calcification as a potential driver in peripheral arterial disease.

This review examines the relationship between medial arterial calcification and atherosclerosis in peripheral arterial disease.

Potential novel risk factors in peripheral arterial disease progression include oxidative stress, DNA damage, vascular aging, and neuropathy.
Medial Arterial Calcification: An Overlooked Player in Peripheral Arterial Disease

Chin Yee Ho and Catherine M. Shanahan

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