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Inflammation and the Osteogenic Regulation of Vascular Calcification: A Review & Perspective

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Pathologic calcification; arteriosclerosis; atherosclerosis; inflammation; oxidative stress; Wnt proteins; bone morphogenetic proteins

I. Introduction

Arterial biomineralization processes have been afflicting humans for at least 5 millenia, as realized in 2003 via the computed tomographic imaging of Ötzi, the intriguing “Ice Mummy” discovered in the Tyrolean Alps[1]. Patchy abdominal atherosclerotic calcification was readily detected in the post mortem of this 40-ish year old hunter of the early Copper Age – by 2000 years a predecessor of King Tutankhamen[1]. Today, an epidemic of vascular calcification is emerging within our aging and dysmetabolic populace[2, 3]. Although vascular calcification was once considered only a passive process of dead and dying cells, work from laboratories worldwide has now highlighted that arterial biomineralization is an actively regulated form of calcified tissue metabolism[4, 5]. Moreover, as in skeletal development – where unique biology controls matrix mineralization in membranous bone, endochondral bone, dentin, and enamel[6, 7] -- mechanistic diversity exists in the pathobiology of vascular calcium deposition [2, 4, 5, 8]. Five common forms of vascular calcification -- each possessing unique histoanatomic characteristics and clinical settings with overlapping yet distinct molecular mechanisms -- have been described to date[4, 5, 9] (Table 1). Although we touch upon the subject, the reader is referred to other contemporary reviews for in-depth consideration of pathogenic differences[2, 4, 5].

In this brief review and perspective, we recount recent data that emphasize inflammation and oxidative stress signaling as key contributors to the pathogenesis of vascular mineral deposition[10]. Furthermore, we highlight differences between the low density lipoprotein receptor (LDLR)-deficient and apolipoprotein E (apoE)-deficient murine models (Table 2) that help articulate the multifaceted contributions of dyslipidemia, diabetes, and uremia to arterial calcium deposition[2, 4, 11]. We end by summarizing the importance of considering these disease stage- and context-specific contributions arterial mineralization when crafting therapeutic strategies to address the disease burden of vascular calcification that increasingly afflicts our patients[5, 12].

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II. Inflammatory cytokines in the initiation and progression of arterial calcification: Lessons learned from LDLR-/- and apoE-/- mice

Some degree of vascular inflammation is a frequent concomitant of most forms of arterial calcification[13, 14] Sites of inflammation relevant to disease biology may not only include the atherosclerotic intima and media, but also the tunica adventitia[15-18]. Of note, calcification of the elastic lamina with elastinolysis in the absence of overt histologic inflammation has been reported,[19-23] and intimal CD68+ macrophage accumulation is more commonly associated with atherosclerotic vs. medial calcification[24]. However, because calcium phosphate mineral deposition itself elicits inflammatory responses[25] -- including tumor necrosis factor (TNF) production by macrophages[26, 27] -- a primary role for inflammation in the pathogenesis of clinically relevant vascular calcification was unproven until very recently[28-32]. In this section, we review this new data -- and also highlight distinctions between the LDLR-/- and apoE-/- murine disease models[33] (Table 2) that provide insights into the mechanistic complexities of inflammation-dependent arterial calcium accumulation.

II.A.1 RANKL/OPG signaling and atherosclerotic calcification

The first robust evidence for the primary contributions of inflammatory cytokine signaling to pathogenesis of vascular calcification arose from the generation and evaluation of the osteoprotegerin (OPG)-/- mouse[34]. OPG-deficient mice develop severe medial and intimal arterial calcification in conjunction with high-turnover osteoporosis driven by excessive osteoclast formation[34]. OPG was first shown to function as an antagonistic “faux receptor” of RANKL (receptor activator of NFκB ligand), the TNF superfamily member that signals via its receptor RANK on monocyte/macrophage progenitors to promote the formation of bone-resorbing osteoclasts[7, 35]. In bone, the antagonist OPG is expressed alongside RANKL in the osteoblast lineage, However, OPG is also expressed in vascular smooth muscle cells and endothelial cells of large arteries – a venue where RANKL is normally absent but induced with inflammation[35]. RANKL expression is readily detected in T-cells and macrophages near atherosclerotic lesions, and within cytokine-stimulated endothelium[35]. Intriguingly, RANKL has been recently shown to promote osteochondrogenic mineralization of VSMCs (vascular smooth muscle cells) [36] and aortic VICs (valve interstitial cells)[37] in vitro. Via the RANK expressed in VSMCs, RANKL upregulates BMP4 (bone morphogenetic protein 4) expression, thus providing an autocrine stimulus for osteogenic differentiation (see also section IV below) [36]. These dual and disparate actions of RANKL upon the skeletal monocyte/macrophage lineage vs. VSMCs likely explain the intriguing phenotype of OPG-null mice[34]. Of note, although the vascular calcification of OPG deficiency occurs in the complete absence of atheroma formation[34], calcified lesions begin to form in arteries only in the post-partum period with copious CD3+ T-cell infiltrates, a few F4/80+ macrophages, and cathepsin K+ osteoclast-like cells [34, 38]. This suggests that, in vivo, inflammatory signals absent in utero are necessary for vascular disease initiation and progression in OPG-/- animals. Additionally, as first observed in the diabetic LDLR-/- mouse[39], serum levels of OPG are higher in patients with diabetes[40, 41]. Since OPG is expressed in VSMCs[42], such increases in the setting of type II diabetes presumably reflect a vascular defense that helps prevents excessive RANKL signaling via negative feedback regulation[28].

II.A.2 Perturbations in RANKL/OPG signaling and the pathobiology of arteriosclerosis

Although compelling, the “spontaneous” vascular calcification observed in response to the genetic lesioning in OPG deficient mice did not ensure contributions to the pathobiology of arteriosclerosis[34]; however, this caveat has been recently addressed.[28] Inhibition of RANKL via administration of recombinant OPG has been evaluated in two very different murine models of vascular disease[33] -- the LDLR-/-mouse[28] and the apoE-/- mouse[43]. It is important to

highlight that, while both models encompass impaired cholesterol metabolism and atherosis on the C56Bl/6 background, the arteriosclerotic disease processes exhibited by these two preclinical models are very distinct (Table 2)[33]. As LeBoeuf first showed, while both models develop atheroma in response to cholesterol-containing fatty diets, the apoE-null mouse never develops the clinically relevant contributions of insulin-resistant diabetes and obesity[33]. However, the male LDLR-/- mouse develops both of these relevant characteristics alongside arterial calcification in response to challenge with fatty diet possessing compositions typical of Westernized societies[33, 44] – a clinically important stimulus for vascular disease[45, 46]. Early medial artery calcification is followed by progressively severe atherosclerotic disease in this model (see below)[29]. Furthermore, the diet- induced systemic low-grade inflammation -- characterized by low but measurable levels of circulating TNF in obese LDLR-/- mice[29, 47] and diabetic humans[48-50] -- is not seen and apparently does not contribute to vascular inflammation in the apoE-/- model[43, 51] even when streptozotocin is administered to induce diabetes[52]. However, in response to other stimuli such as lipopolysaccharide administration or Klebsiella infection, apoE-/-mice exhibit exaggerated TNF induction and increased mortality[53]. Finally, in the apoE-null mouse, vascular calcification quickly evolves upon the backdrop of VSMC chondroid metaplasia[8] that is observed over time even on mouse chow – i.e., in the absence of cholesterol-rich dietary challenge[54]. By comparison, evolution of arterial calcification in the LDLR-/- mouse is more protracted and elicited by the clinically relevant Western diet (42% of calories from fat, 0.15% cholesterol), accruing vascular mineral deposition via sequentially distinct mechanisms[28, 29]. At early stages, vascular calcification can be histologically detected by Alizarin red staining within the tunica media of major conduit arteries of diabetic, male LDLR-/- mice -- biochemically quantifiable following acid extraction.[29] Atheromata are not uniformly present at this early stage, and if present do not stain for calcium. As with atherosclerosis, the initial calcium deposition within the tunica media may be elastin organized phospholipid vesicles[55, 56], since very little *inorganic* phosphate staining is evident by von Kossa at this stage[29]. Similar observations have been described in human specimens[57]. With progression, however, massive aortic sinus and subintimal cholesterol deposits accrue, with atherosclerotic calcification visualized within the cholesterol clefts and degenerating atheromata[29]. During this second phase, chondroid metaplasia clearly contributes to vascular calcium accrual in male LDLR-/- mice[28] as observed in apoE-/- mice[8]. The extent of medial calcium is thus increased upon Alizarin red staining[29, 57], and the von Kossa method for visualizing inorganic phosphate now reveals massive medial and atherosclerotic calcium phosphate deposition in male LDLR-/- mice fed fatty diets[28, 29]. Thus, when place on high fat westernized diets, the male LDLR-/- mouse sequentially elaborates an early arterial medial calcification program (Table 1) that with disease progression is augmented by processes of atherosclerotic intimal calcification (Table 1; see also Table 2).

II.A.3 Inhibition of RANKL signaling as a therapeutic approach to arteriosclerotic calcification

As noted above, OPG is an endogenous inhibitor of RANKL signaling that limits arterial calcium accumulation during development. Recently, the impact of pharmacologic inhibition of RANKL by OPG has been evaluated in the above preclinical models of atherosclerosis and arterial calcification. Interestingly, very distinct responses are observed with OPG administration in LDLR-/- and apoE-/- mice[28, 43]. Demer and colleagues first evaluated the male LDLR-/- mouse, the dynamics of endogenous RANKL/OPG signaling during disease initiation and progression, and the impact of exogenous OPG administration[28]. Serum RANKL measurements demonstrated that progression of vascular disease over 5 months of dietary cholesterol challenge closely tracks the progressive recovery of circulating RANKL following an early phase of diet-induced suppression [28]. Early diet-induced increases in OPG – a presumed adaptive mechanism to protect against untoward RANKL signaling[36] – exhibited no dynamic change with progression[28]. As predicted from studies of OPG-/-mice[34], male LDLR-/- mice treated with exogenous OPG exhibit reduced arterial calcification and

diminished aortic osteochondrogenic differentiation[28]. However, no change in atherosis – i.e. the size of arterial atheroma – was observed[28]. Intriguingly, three sources of vascular RANKL production were identified in this LDLR-/- model: (i) the F4/80+ monocyte-macrophage population in closest proximity to lesions undergoing chondroid metaplasia; (ii) the endothelial cells overlying atheroma; and (iii) the CD3+ T-cells at the adventitial-medial junction[28]. Whether any one source of RANKL production represents the lynchpin for the OPG-dependent inhibition of progressive vascular mineral accrual in this model remains to be determined.

In apoE-/- mice, as in LDLR-/- mice, OPG administration apparently does not affect atheroma lesion size[43]. However, OPG significantly increases fibrous cap size and thickness and reduces MMP12 levels, potentially stabilizing the lesion but not directly assessed[43] (see below). Non-significant, tantalizing trends for reductions in numbers of macrophages and T-cells were also observed in response to OPG administration. Unlike male LDLR-/- mice -- where diet-induced obesity increases circulating TNF levels[29] -- basal TNF levels are below the limits of detection in apoE-/- animals and thus not measurably changed by OPG administration [43]. Calcification was, unfortunately, not scored in this recent study[43]. However, Bennett and colleagues have applied murine genetics to carefully detail the important role for endogenous OPG in the calcification of advanced atherosclerotic lesions of apoE-/- mice by generating and evaluating OPG-/-;apoE-/- mice[31]. In this model, congenitally deficient OPG-/-;apoE-/- mice exhibit atherosclerotic lesions of increased size in the innominate artery, with significantly increased areas of calcification and aortic calcium accumulation measured during disease progression[31]. Plaque stability was not assessed in OPG-/-;apoE-/- mice, but OPG was shown to increase MMP9 (matrix metalloproteinase 9) activity in vitro[31], and MMP9 promotes intraplaque hemorrhage in vivo in advanced atherosclerotic lesions of apoE-null animals[58, 59]. However, congenitally deficient MMP9-/-;apoE-/- mice exhibit increased lesion size following disease initiation vs. MMP9-replete siblings[60], suggesting that stage-specific roles of MMP9 exist in atherosis and sclerosis[58]. As a modulator of MMP9, OPG could potentially exert adverse as well as beneficial arteriosclerotic actions during pharmacological manipulation of RANKL signaling [31]. Thus, as in the LDLR-/- mouse, OPG limits arterial calcium accumulation in the apoE – null mouse. OPG may regulate plaque stability -- but the differential responses of pharmacologic vs. genetic manipulation of OPG on vascular histopathology in apoE-/- mice highlight the need for more a detailed assessment of impact upon plaque formation, stability, and regression.

In summary, antagonism of RANKL signaling cascades holds much promise for modulation of atherosclerotic calcification[61]. Of note, a humanized antibody that antagonizes human RANKL has been developed for prevention of fractures in osteoporosis[62]; based upon preclinical studies of Hofbauer et al using a “humanized RANKL” murine model[32], this same reagent might be useful in treatment of cardiovascular calcification. However, the net impact on vascular physiology – vascular compliance, Windkessel-dependent conduit function, distal tissue perfusion, arterial remodeling and plaque stability – has yet to be determined.

II.B.1. Medial artery calcification, arteriosclerosis, and lower extremity amputation risk in T2DM

The relationship between arteriosclerotic medial artery calcification (AMC; Table 1) and the risk of lower extremity amputation in T2DM has been appreciated for 2 decades[63, 64]. The earliest studies were reported for Pima Indians, a native American population with increased risk for T2DM[63, 64]. Subsequent studies from Finland identified that radiographic femoral medial artery calcification – not atherosclerotic calcification – was the single best predictor of lower extremity amputation in T2DM[65]. Why, then, does increased arterial stiffness (arteriosclerosis) in T2DM -- arising from AMC without peripheral atherosclerosis -- contribute to the increased risk for lower extremity amputation?[66] Conduit vessel stiffening

from any cause[67] compromises normal arterial Windkessel physiology[67, 68], thus impairing uniform distal tissue perfusion throughout the cardiac cycle[69, 70].

At this point, however, it should be re-emphasized that critical limb ischemia (CLI) arising from atherosclerotic plaque formation and arterial stenosis in the femoropopliteal bed is a well-recognized contributor to lower extremity amputation risk; moreover, atherosclerotic calcification also contributes to conduit vessel stiffness[71-74]. Medical strategies such as statins that reduce atherosclerotic disease burden also improve outcomes in patients with peripheral arterial disease (PAD)[71, 75]. Reductions in ankle-brachial indices (ABIs) provide a clinically useful tool for identifying symptomatic individuals at risk[72, 73]. Increased mobility, reduced claudication, limb salvage, and improved ABIs can often be achieved by surgical or percutaneous vascular interventions[71] -- more successfully so in stenosed distal femoropopliteal segments[76, 77] than proximal segments[78], and less successfully so in patients with diabetes[79-81]. However, in the setting of T2DM, PAD arises with contributions from both medial artery calcification and atherosclerosis[74]. Furthermore, in T2DM, ABIs are frequently elevated[82] – *not reduced* – due to medial calcific sclerosis[74, 82]. While elevated ABIs do not necessarily convey increased risk for atherosclerotic disease[83], an ABI $\geq$ 1.3 does indicate the presence of arteriosclerosis – i.e., arterial stiffening -- and concomitantly portends lower extremity amputation[84]. In summary, the clinical evaluation of PAD in patients with T2DM requires special consideration, including assessment of toe-brachial indices in lieu of ABIs[82].

II.B.2. Mechanisms of medial artery calcification in T2DM: Clues from the field of bone biology and the LDLR-/- mouse

During skeletal mineralization, bone formation can occur via either endochondral (preceding cartilage template required) or membranous (non-endochondral; no cartilage required) processes[7]. Osteo/chondrocytic transcription factors such as Sox9, Runx2/Cbfa1, Msx2, Msx1, and Osx play critical roles in promoting either endochondral (Sox9, Runx2, Osx) or membranous (Msx2, Msx1, Runx2 and Osx) bone formation[7]. In bone, polypeptide morphogens such at BMPs (bone morphogenetic proteins) and Wnts (wingless/mouse mammary tumor virus integration site family) induce these osteoblast DNA binding proteins along with β-catenin, a transcription co-adapter indispensible for bone formation[7, 85]. A common feature of active osteogenic mineralization is induction of AKP2, the "bone" alkaline phosphatase that degrades the plentiful and endogenous mineralization inhibitor, inorganic pyrophosphate (PPi) (Figure 1)[7]. Of note, Sox9, Runx2, Msx2, and AKP2 have all been described as being expressed in calcifying human arterial segments[86], and are upregulated by stimuli that promote arterial calcification (Figure 1).

The molecular mechanisms controlling initiation and progression of medial artery calcification in T2DM have recently been studied in detail in the male LDLR-/- mouse (Table 2) – a model in which obesity, diabetes, and osteogenic arterial calcification programs are induced in response to high fat diets possessing compositions characteristic of westernized societies [2, 29, 39, 44, 87]. Importantly, diet-induced disease in male LDLR-/- mice[2, 29, 44, 87] closely tracks molecular and physiological characteristics of T2DM patients afflicted with valve[88, 89] and arterial[86, 90] calcification. A critical clue to the pathogenesis of AMC in this setting arose from recognition that T2DM induces a low grade systemic inflammatory state, programmed in part by adipokines – i.e., fat-derived cytokines[48-50 91, 92]. TNF is the prototypic inflammatory cytokine, elaborated not only by adipocytes but also by adipose tissue macrophages (ATM) that infiltrate fat with "diabesity."[48, 93, 94]. Demer et al first identified that TNF and a macrophage-derived signal stimulated the mineralization of aortic calcifying vascular cells (CVCs) in vitro[95]. Subsequently, we demonstrated that infliximab-mediated inhibition of TNF signaling in vivo in the LDLR-/- mouse down-regulated osteogenic Msx2-Wnt gene regulatory program in aortas of diabetic LDLR-/- mice[29]. Concomitant reductions in early vascular

calcium load was also observed with infliximab[29]. While diet-induced abnormalities in fasting glucose and lipid profiles were not improved, dosing with infliximab did decrease serum 8-F-isoprostane levels, an oxylipid and marker of oxidative stress in T2DM[29]. Conversely, local augmentation of TNF tone in the aortic wall with a SM22-TNF transgene activated aortic Msx2-Wnt signaling in the absence of diet-induced disease, demonstrating the important role of TNF in the initiation of macrovascular disease in T2DM[29]. Others have now also confirmed the important role for Msx2 in TNF-dependent induction of AKP2 and mineralization in VSMCs (Figure 1)[96]. Koleganova highlighted the significance of these preclinical studies to human disease biology in those afflicted with arterial calcification of renal failure[90]; TNF, Msx2, and BMP2 expression were correlated with osteogenic differentiation in both calcified and non-calcified vessel segments of patients with CKD5 (chronic kidney disease)[90].

Thus, in summary, these data[90] and others[86, 88, 89] confirm the clinical relevance of the osteogenic relationships established in the LDLR-/- murine model of calcific vasculopathy (Figure 1). Obligatory diet-induced "diabesity" in the LDLR-/- model is an important feature of this model that is highly relevant to the burgeoning disease burden of westernized societies. As in diseased humans vessels, osteogenic transcription factors (Msx2, Runx2, Osx, Sox9) are ectopically induced in the arteries and valves of diabetic LDLR-/- mice. Mechanistic insights possible via preclinical studies point to both (a) trans-differentiation of VSMCs; and (b) osteochondrogenic lineage allocation of multipotent mesenchymal progenitors by these osteogenic transcription factors[4, 97]. Since streptozotocin drug-induced diabetes also accelerates osteochondral metaplasia in the apoE-/- mouse[98], further evaluation of this model may help elucidate the pathobiological mechanisms whereby hyperglycemia promotes arterial mineralization.

II.C. Inflammation, fetuin, and matrix vesicle metabolism: Novel insights into the calcific vasculopathy of chronic kidney disease (CKD)

CKD, particularly CKD5, represents a "perfect storm" of calcific vasculopathy (Table 1)[11]. Antecedent diabetes, hypertension, and dyslipidemia intersect with phosphate retention, low turnover bone disease, and dialysis-induced systemic inflammation and VSMC apoptosis synergize to drive ferocious arterial calcium accrual (Figure 1)[11]. Arterial calcification of CKD5 and in calcific uremic arteriolopathy (also called calciphylaxis; Table 1) have been reviewed in detail, and the reader is referred to these excellent manuscripts[11, 99, 100]. However, fetuin biology as relevant to the arterial calcification in CKD5 is worthy of special consideration, particularly within the context of inflammation-mediated vascular disease.

Fetuin - a.k.a. fetuin A, alpha-2-Heremans-Schmid glycoprotein, AHSG -- is a serum protein synthesized by the liver[99]. As first demonstrated by Jahnen-Dechent, fetuin avidly binds amorphous calcium phosphate, and maintains the solubility of supersaturated serum calcium phosphate[101] via the formation of calciprotein particles that inhibit insoluble calcium phosphate crystal aggregate formation (Figure 1) [102-106]. Consistent with these observations, Jahnen-Dechent, Ketteler, and colleagues demonstrated widespread soft tissue calcification in fetuin-deficient mice[106]. Shanahan recently identified that fetuin also plays a critical role in VSMC-mediated removal of pro-calcific matrix vesicles[107, 108]. In response to hypercalcemia and hyperphosphatemia – common stimuli in dialysis patients[101] – VSMCs elaborate matrix vesicle and apoptotic bodies that not only can nucleate extracellular matrix deposition but might also help facilitate clearance of vascular calciprotein particles[107, 108]. Serum-derived fetuin and matrix vesicle-associated matrix Gla protein (MGP) are required for VSMC-mediated uptake and clearance of vesicles (Figure 1)[107, 108]. Importantly, fetuin is an "inverse" acute phase reactant, decreased by inflammation via inhibition of the CCAAT/enhancer binding protein-DNA interactions that support fetuin gene transcription in hepatocytes[109, 110]. In ESRD, fetuin levels are inversely related to extent of coronary calcification, providing yet

another link between inflammation, oxidative stress, and arterial calcium accumulation[111, 112]. Similar results have been noted in patients with calcific aortic stenosis[113].

In summary, the cumulative evidence overwhelmingly points to an important role of fetuin in limiting arterial calcium deposition. It remains to be determined if normalization or augmentation of serum fetuin reduces vascular calcification in a model of inflammation-induced vascular disease. Of note, increased patient mortality in CKD5 – an outcome related to the extent of vascular calcification[114] – is associated with reductions in fetuin but is significant only in the setting of inflammation[115]. This suggests that other signals elaborated by inflammation independent of fetuin suppression – such as reactive oxygen species – must play an important pathophysiological role. Finally, the reader is referred to outstanding recent reviews highlighting the critical contributions of hyperphosphatemia[11, 116], VSMC BMP2-dependent phosphate transport[117], and BMP7-corrected hyperphosphatemia[118, 119] to the pathobiology of vascular calcification in the setting of CKD.

III. Oxidative stress signaling and vascular calcification: Peroxide paves an osteogenic path

As noted above, when coupled with the clinical setting, histoanatomic and molecular characteristics distinguish aortic valve calcification, atherosclerotic calcification, diabetic medial artery calcification, vascular calcification of ESRD, and calcific uremic arteriolopathy (Table 1)[120]. However, over the past two years multiple groups have newly identified the important role of oxidative stress signaling in vascular activation of osteogenic gene regulatory programs[88, 121]. Chen first demonstrated that the osteochondrocytic transcription factor Runx2/Cbfa1 is activated by hydrogen peroxide (H_20_2) and supports bone alkaline phosphatase (AKP2) expression and matrix mineralization in cultured vascular smooth muscle cells (Figure 1)[121]. Similarly, membranous ossification programs[122, 123] elaborated by Msx2-Wnt signaling cascades are also dependent upon peroxide signals elaborated from mitochondrial activity and downstream of TNF stimulated NADPH oxidases[124, 125]. Very recently, Miller, Heistad, and colleagues demonstrated co-localization of oxidative stress signaling and the osteogenic transcription factors Msx2 and Runx2/Cbfa1 in calcifying human aortic valves[88]. However, the sources of oxidative stress were shown to arise from uncoupling of nitric oxide synthase (NOS) and failures in the enzymatic defenses (e.g., catalase) that restrain peroxide accumulation[88]. Using the “Reversa” mouse model, they subsequently demonstrated that elevated cholesterol levels are required for calcification and sustained vascular induction of the osteogenic transcription factors Msx2 and Runx2/Cbfa1[126]. This suggests that an oxylipid[127] -- in addition to oxylipid-responsive cytokines such as TNF[29, 95] and RANKL[28, 128] -- is required for vascular calcification, as first proposed by Demer[95, 127, 128].

In summary, although sources of oxidative stress may differ with vascular venue and disease state[88] -- and the signaling cascades have yet to be fully elucidated[124] -- oxidative stress signals provide important stimuli. With inflammatory cytokine signals, this helps provide a unifying theme for arterial elaboration of osteogenic mineralization processes.

IV. Of BMPs and Wnts: Osteogenic morphogens as proximal mediators of vascular calcification

BMP2 is a powerful osteogenic morphogen that promotes bone formation during skeletal development and also maintains skeletal integrity and supports fracture repair during post-natal life[129]. Via an autocrine Wnt signaling loop, BMP2 promotes osteoblast commitment and the induction of the bone alkaline phosphatase (AKP2) [130], the latter an important enzymatic mediator of osteogenic matrix mineralization (Figure 1)[130]. Almost two decades ago, Bostrom, Demer and colleagues identified the presence of BMP2 in calcified atherosclerotic plaques and

demonstrated the important role for BMP2 in CVC mineralization[131]. With Anderson's studies[55], this provided the first molecular insights into the biology of vascular calcium deposition and vascular BMP2 expression -- now mechanistically integrated with pathophysiological states that initiate calcific vasculopathy[124]. Ungvari and colleagues recently demonstrated that TNF, H_20_2, and high intravascular pressure –stimuli commonly encountered with diabetes, hypertension, and the metabolic syndrome -- all upregulate the expression of BMP2 in endothelial cells[132]. This provides a morphogenetic cue that reinforces osteogenic differentiation of multipotent vascular mesenchymal cells such as pericytes and CVCs that reside within the vascular wall [131, 133, 134](Figure 1). Moreover, as mentioned above, RANKL stimulates BMP4 production by VSMCs, providing an autocrine stimulus for osteochondrogenic transdifferentiation -- if not held in check by BMP4 antagonists such as noggin[36] or MGP[30]. Importantly, in addition to being entrained to TNF[29], the vascular osteogenic Wnt signaling cascades previously discussed[87, 124, 125] (Figure 1) are also activated downstream of BMP2 in vivo[2]. Of note, these canonical Wnt signals drive osteochondrocytic differentiation of multipotent vascular pericytes in vitro[135] as well as promote the arterial calcification of type II diabetes in vivo[87]; via multiprotein cell surface receptor complexes containing LRP5 or LRP6, the Wnt polypeptide family contributes to bone morphogenesis and skeletal integrity[136] in conjunction with the BMPs[137]. Intriguingly, along with many Wnt ligands, LRP5 and LRP6 are expressed in endothelial cells and VSMCs[138]. The precise reasons why vascular expression of these osteogenic morphogens does not always lead to arterial mineralization is still unclear. This presumably reflects the local balance between agonists and antagonists of BMP/Wnt signaling[124] (e.g., MGP and Dkk1, respectively; Figure 1); the important roles of inorganic pyrophosphate[139], fetuin[107], and osteopontin[140] as osteogeonic mineralization inhibitors; elastin metabolism[141-144]; and the impact of matrix stiffness[145] upon the osteogenic potential of vascular mesenchymal progenitors. Nevertheless, strategies that can selectively (a) inhibit the activation or the actions of vascular osteogenic BMP/Wnt signaling; or (b) augment vascular defenses that prevent mineralization hold promise for limiting arterial calcium accumulation.

V. Inorganic pyrophosphate (PPi) and Matrix Gla protein (MGP): Overlapping consequences of genetic and inflammation-induced deficiency in two key vascular defenses

Elegant genetic studies in mice and humans have highlighted the important roles for inorganic pyrophosphate and MGP as non-inflammatory inhibitors of vascular mineralization. Karsenty and colleagues first identified that murine deficiency in the BMP2/4 antagonist MGP[133] results in chondroid metaplasia of the arterial tunica media, pan-arterial calcification, and vascular rupture[146]. Similarly, Terkeltaub showed that murine deficiency in the mineralization inhibitor PPi, arising from genetic disruption of the ectoezyme NPP1 (ectonucleotide pyrophosphatase/phosphodiesterase I) also results in arterial calcification with chondroid metaplasia[139]. The relevance of the PPi/NPP1 axis to human genetic disease is established via identification that generalized arterial calcification of infancy (OMIM #208000), a rare congenital disorder, arises from PPi deficiency due to NPP1 loss-of-function mutations[147]. How, then, is inflammation connected to these critically important inhibitors of mineralization? Overtly, the induction of AKP2 by TNF[95] and down-stream osteogenic BMP-Wnt pathways[29, 87, 136] (described above) hydrolyzes PPi to destroy this inhibitor during vascular mineralization (Figure 1)[148]. Moreover, depletion of PPi markedly down-regulates osteopontin[149], the even more potent inducible inhibitor of vascular mineralization[140]. However, until very recently, the relationship between inflammation and MGP insufficiency was less clear. In a series of very insightful studies, Bostrom identified that MGP is a Gla-dependent inhibitor of BMP2 and BMP4[150], osteogenic morphogens that upregulate AKP2 expression[30, 133, 150]. Bostrom went on to show that IL6, an inflammatory cytokine important in diabetic vascular disease, increases the expression and

secretion of HSP70, an endogenous MGP binding protein and antagonist of MGP function that is highly expressed in calcifying atherosclerotic plaques[30]. Thus, by inducing HSP70, inflammatory signals provided by IL6 potentiate vascular BMP2/4 actions by nullifying MGP[30] (Figure 1). Whether other inflammatory cytokines participate in this hierarchy of regulated mineralization is unknown. Nevertheless, these newer data point to how two axes – genetic defenses against vascular mineralization and inflammation –induced arterial osteogenic programs – functionally intersect to regulate arterial calcification[4].

VI. Summary and Future Directions

The fund of knowledge available to the field of arterial calcification and vascular mineral metabolism has dramatically grown in recent years. Our understanding of this disease biology has been enabled by incredible advancements in bone and mineral research that occurred alongside innovative investigation in cardiovascular medicine and insightful human studies from astute clinician-scientists. As in bone, mechanistic heterogeneity exists in the different forms of vascular mineral deposition, and also during stages of disease initiation and disease progression. Moreover, there is heterogeneity in the sources and mechanisms of mineralizing vascular cell types; osteochondrocytic VSMC trans-differentiation, VSMC apoptosis, and osteochondrocytic lineage allocation of multipotent mesenchymal cells all contribute, but to varying extents dependent upon pathophysiologic setting and disease stage (Figure 1). It has been posited that marrow derived circulating osteoprogenitors may also contribute to vascular mineralizing cell types[151-153], but this has yet to be unambiguously established.

To date, approaches to prevent and/or reverse macrovascular calcification have largely been unsuccessful[154], due in part to this mechanistic heterogeneity and the intrinsic pro-inflammatory actions of vascular calcium phosphate that provide a “feed-forward” stimulus for disease[25, 26]. In addition, clinical setting dramatically alters the metabolic milieu and rate-limiting pathophysiology of calcific vasculopathy (Table 1) – risk factors that differentially impact disease initiation and progression[155]. Moreover, not all human medial calcific sclerosis may be associated with overt inflammation; indeed, use of oral calcium-based phosphate binders alone increases coronary artery medial calcification in CKD – a vascular bed not usually afflicted by medial sclerosis[24]. However, recent data highlight the fundamental contributions of inflammation, oxidative stress, and osteogenic morphogen signaling in this vascular disease. With careful patient selection and consideration of the diseased vascular segment, intervention with a potent statin may yet play a clinically important role via LDL cholesterol reduction and anti-inflammatory actions[12, 156, 157]. Unfortunately, simple strategies that seek to “scavenge” redox signals elaborated by inflammation do not offer significant clinical benefits for most individuals at risk[80]. Approaches that target the OPG/RANKL pathway[61], the calcium sensing receptor[158] and other calciotropic signals[39,116, 119], or key vascular proteases[141, 144] offer hope --- but individually may be insufficient in some clinical settings, particularly dialysis-dependent renal failure[100,159]. Except for a few prescient reports on the relationships between arterial pressure and vascular BMP-Wnt signaling[132, 160-164], remarkably few studies have examined the mechanistic links between hypertension and signals that regulate arterial calcification[165]. Given that endothelin-dependent signals control both vascular calcium homeostasis and blood pressure[166, 167], the paucity of such studies represents an unmet scientific need. Moving forward with mechanistic insights, pharmacological strategies can be crafted that newly acknowledge disease complexity[4, 5], and thus antagonize with sophistication the combination of pathobiological processes that promote vascular mineralization during disease initiation and progression[168]. The future holds great promise for the development of these successful therapeutics and the medical management necessary to address a burgeoning clinical need[5].

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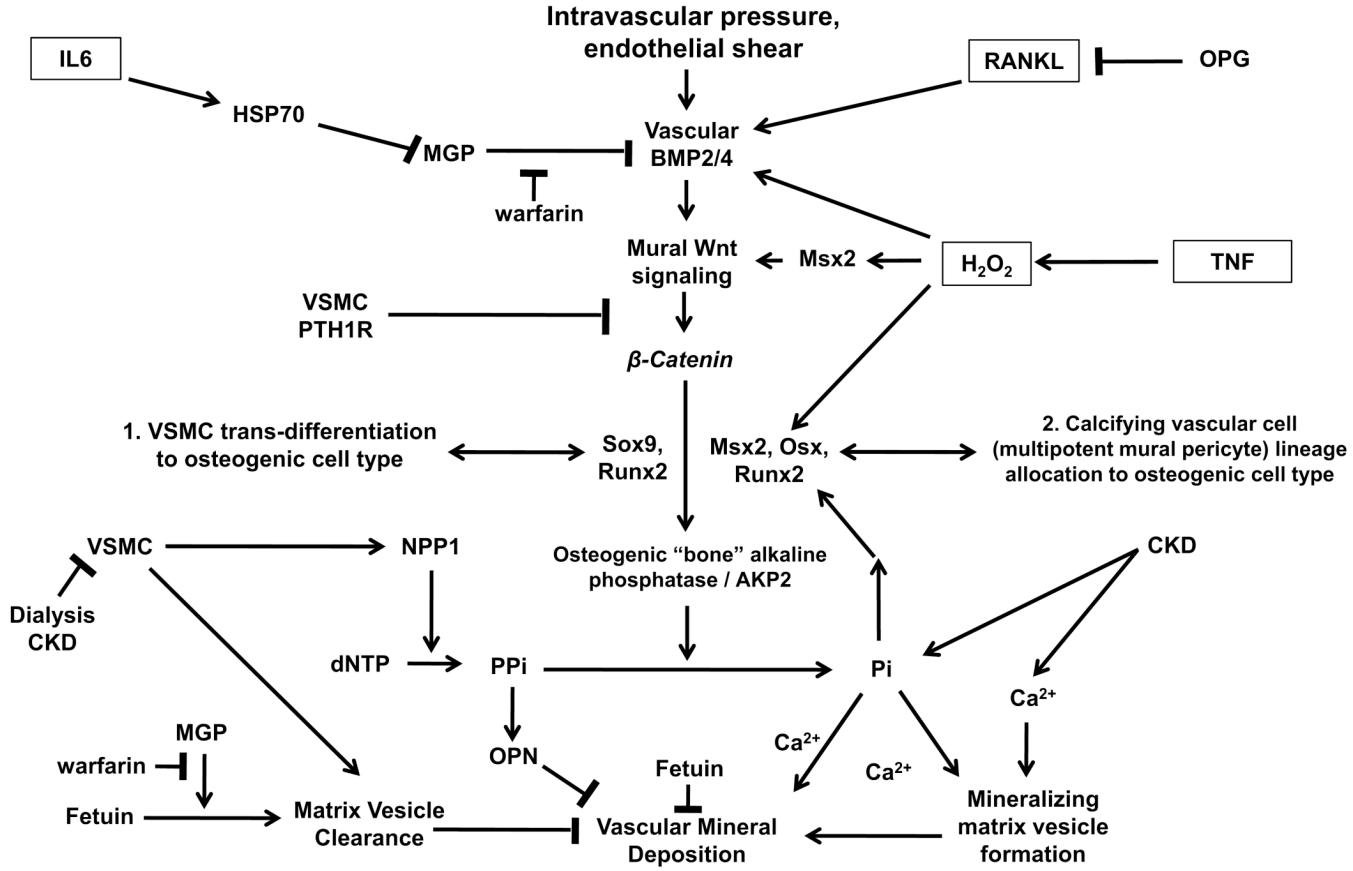

Figure 1. Inflammation and Osteogenic Regulation of Vascular Calcification: A Review and Working Model

Osteochondrocytic cells that promote vascular matrix mineralization can arise from at least two sources: (1) trans-differentiation of VSMCs – i.e., a type of phenotypic modulation in which the mature VSMC phenotype is replaced, and reprogrammed to that of an osteochondrocytic cell; or (2) osteogenic lineage allocation from a multipotent mesenchymal progenitor – i.e., a cell that has the potential to become an osteoblast, chondrocyte, VSMC, or adipocyte. Both processes are triggered by key inflammatory cytokines and oxidative stress signaling (boxed). VSMCs also elaborate apoptotic bodies and matrix vesicles that can nucleate mineral deposition – but also may play a role in removing vascular calciprotein particles via fetuin and MGP-dependent cellular uptake. Thus, apoptosis of VSMC not only provides substrate for nucleation, but also loss of cellular defenses. Multiple paracrine inhibitors control (a) pro-osteogenic signals provided by BMP/Wnt signaling, RANKL and TNF actions; and (b) nucleation/aggregation/epitaxial propagation of apatitic calcium phosphate deposition. Via HSP70-mediated inhibition of MGP and AKP2-mediated PPi degradation, inflammatory cytokines such as IL6 and TNF impair MGP and PPi defense mechanisms, respectively. Inflammation also down-regulates expression of serum fetuin, an import hepatocyte-derived inhibitor of soft tissue mineral deposition. Not shown are the enzymatic defense mechanisms such as catalase and glutathione peroxidase that reduce vascular oxidative stress[10, 88, 169]. Although clearly an important stimulus for vascular BMP2 expression[132], remarkably few studies have examined the molecular mechanisms whereby hypertension activates vascular osteogenic signaling cascades. Of note, contribution of marrow-derived osteogenic endothelial progenitor cells as an additional source of mineralizing vascular mesenchymal progenitors has been recently posited, but has yet to be established[153]. See text for details and additional references.

Table 1

Common Histoanatomic Forms of Vascular Calcification & Clinical Settings [2, 4, 5, 11, 99]

Type	Characteristics	Histopathology	Disease Biology	Risk Factors
Calcific Aortic Valve Disease (CAVD) a.k.a. calcific aortic stenosis or aortic valve calcification	• Calcification of aortic valve leaflets • Stenosis with variable regurgitation • Increased myocardial workload • Left ventricular hypertrophy,heart failure, syncope, and sudden death	• Intracellular lipid and extracellular lipoprotein accumulation in valves • Subendothelial thickening • Displaced, split elastic lamina • Fibro-fatty expansion between fibrosa and ventricularis • Multiple early calcification foci at base of lesions (regions of highest mechanical stress) with later coalescence • Woven bone with marrow in ~ 13% of specimens[170] • Amorphous nodules of calcium phosphate accrue via epitaxial mineral deposition	• Mixed picture of membranous, endochondral, and dystrophic calcification. • Runx2/Cbfa1, Msx2, Sox9 osteogenic transcription factors expressed in VICs – β-catenin osteogenic programs (Wnt3a)[136] – promoted by oxidative stress – promoted by matrix stiffness[145] • Notch, NOS3 genetics contribute in biscuspid disease[171] • Cell mediated inflammatory cells observed in bicuspid as well as senile calcific disease.	• Hypercholesterolemia – LDL cholesterol • Advanced age • Bicuspid aortic valve • Type II diabetes[46] • Metabolic syndrome[45] • Hypertension[155] • Tobacco use • Male gender
Arterial Medial Calcification (AMC) a.k.a. medial artery calcification, Mönckeberg sclerosis, or medial calcific sclerosis	• Calcification of the arterial tunica media • Reduced vascular compliance, impaired Windkessel physiology • Increased lower extremity amputation risk in type II diabetes • Increased pulse pressure • Increased myocardial workload • MI, stroke	• Circumferential, contiguous & confluent calcification of tunica media • Circumferential adventitial inflammation with fibro-fatty expansion • AKP2-positive matrix vesicles mediate calcification, associated with elastin lamellae • Usually spares coronary arteries except in setting of chronic kidney disease & calcium-based phosphate binders[24]	• Arterial activation of “membranous” ossification – Msx2, Osx early – Runx2 late • Adventitial-medial Wnt signaling direct osteogenic differentiation of CVCs in tunica media – paracrine Wnt signals from adventitial myofibroblasts – β-catenin osteogenic programs activated in media	• Type II diabetes[63-65] • Advanced age • CKD (see below) • Autonomic neuropathy[172]

Type	Characteristics	Histopathology	Disease Biology	Risk Factors
	• Elevated lipidaceous markers of oxidative stress • Autonomic neuropathy		• TNF and BMP2 dependent, osteogenic signals propagated by H202[10]. • Low grade systemic inflammation	
Atherosclerotic Intimal Calcification (AIC)	• Calcification of atherosclerotic plaques • Angina • Acute coronary syndrome • MI, stroke, sudden death • Reduced vascular compliance • Peripheral arterial disease, claudication • Mitral and aortic annulus calcification are both associated with AIC[173].	• Eccentric, lumen deforming, type Vb intimal plaque[174] • Some calcification present in most atherosclerotic plaques • Patchy distribution • Several mechanisms – Lipid core calcification – Fibrous calcification/ apoptotic bodies – Endochondral ossification with matrix vesicles, AKP2-positive • Elastinolysis	• Mostly endochondral ossification picture in addition to lipid core mineralization • Focal inflammation • Oxidized LDL in intima and macrophage-derived signal promote osteogenic differentiation of mural CVCs • Runx2/Cbfa1, Sox9≫Msx2 expression • RANKL, TNF, and IL6 dependent • Macrophages and T cells sources of RANKL[28, 175]	• Hypercholesterolemia[4] – LDL cholesterol • Hypertension[155] • Type I diabetes • Type II diabetes • Tobacco use • Rheumatoid arthritis[176] • Systemic lupus erythematosis[177, 178]
Vascular calcification of end-stage kidney disease CKD5	• CKD5(GFR <15 cc/ min/1.73 m^2) in concert with any of the above • Impaired serum calcium phosphate homeostasis • Often represents acceleration of antecedent calcific vasculopathy – 40% diabetic – At any level of CKD, patients with diabetes	• All the above[11, 97] • VSMC apoptosis[15] • Elastinolysis (cathepsin S) • AMC predominates with type II diabetes as cause of CKD • Coronary artery medial calcification can be seen in this setting setting of chronic kidney disease[24] • Frequent mitral annular calcification observed	• Impaired serum calcium phosphate homeostasis • Vascular smooth muscle cells elaborate mineralizing matrix vesicles and apoptotic bodies, stimulated by elevated serum calcium and phosphate • Low grade systemic inflammation, reduced serum fetuin impairs matrix vesicle clearance by VSMC • Dialysis induces VSMC apoptosis • Phosphate upregulates Runx2/Cbfa1 and Msx2	• Any of the above[11] • Hyperphosphatemia • Hypercalcemia • Renal osteodystrophy, low turnover bone disease[180] • Excessive use of calcium based phosphate binders, excessive PTH suppression[180, 181]

Type	Characteristics	Histopathology	Disease Biology	Risk Factors
	have greater vascular calcium loads • Can and does occur without antecedent disease e.g. pediatric populations.		expression in VSMCs via Pit1[116, 179] • Vascular TNF, BMP2, Msx2 increased, co-regulated in CKD[90]	
Calcific Uremic Arteriolopathy (CUA) a.k.a. calciphylaxis	• GFR < 30 cc/min/ 1.73 m^2, chronic ≫ acute • Warfarin treatment, diabetes • Painful violaceous dermal nodules that progress to dermal necrosis • Lower extremities, buttocks, pannus frequently affected • Greatly elevated markers of systemic inflammation (ESRand CRP) • High mortality	• Arteriolar (<0.6 mm) medial calcification of dermal, pulmonary & mesenteric vascular beds[182, 183] • Fibroproliferative arteriole occlusion and fatty tissue necrosis • Periarteriolar inflammation	• Few detailed mechanistic studies • BMP4 expressed in periarteriolar dermal tissue[184] • Reduced MGP- and fetuin-dependent matrix vesicle internalization by VSMCs • Reduced MGP-dependent inhibition of osteogenic BMP2/4 signaling.	• CKD4 or CKD5[183] • Warfarin treatment • Type II diabetes • Obesity • Severe secondary hyperparathyroidism • ? Vitamin K deficiency

AKP2, bone alkaline phosphatase; BMP, bone morphogenetic protein; CKD, chronic kidney disease; CRP, C reactive protein; CUA, calcific uremic arteriolopathy; CVC, calcifying vascular cell of Demer; ESR, erthyrocyte sedimentation rate; GFR, glomerular filtration rate; MGP, matrix gla protein; MI, myocardial infarction; Msx2, osteoblast transcription factor muscle segment homeobox homolog 2; NOS, nitric oxide synthase; Osx, osteoblast transcription factor osterix; Pit1, phosphate transporter SLC20A1; PTH, parathyroid hormone; RANKL, receptor activator of NF-kappaB ligand; Runx2, osteoblast transcription factor runt related transcription factor 2; TNF, tumor necrosis factor; VIC, valve interstitial cell; VSMC, vascular smooth muscle cells.

Table 2
Features of ApoE-/- and LDLR-/- Murine Models of Arterial Calcification*

	Murine Disease Model	
Parameter	**ApoE-/- Mouse**	**LDLR-/- mouse**
Diet-induced hypercholesterolemia	Yes[33, 185]	Yes[33, 186]
Diet-induced atherosclerosis	Yes[185]	Yes[187]
Diet–induced diabetes	No[33]	Yes[33, 44]
Diet-induce obesity	No[33]	Yes[33]
"Spontaneous" arterial chondroid metaplasia	Yes[54, 98] (accelerated by drug-induced diabetes)	No[29, 44]
Early diet-induced non-endochondral[7] medial artery calcification	No[54]	Yes[29, 168]
Late diet-induced endochondral[7] atherosclerotic calcification	Yes[54]	Yes[28, 29, 168]
Hemodynamically -significant calcific aortic valve disease occurs with progression	Not known (valve thickening seen with CRI)[188]	Yes[126, 189] (concomitant ApoB100/100 genotype)
Exaggerated inflammatory response and susceptibility to mortality with gram-negative sepsis	Yes[53, 190]	Less so**[53, 191]
Arterial calcification accelerated by chronic renal insufficiency (CRI)	Yes[141]	Yes[118]

* C57Bl/6 background. Arterial calcification is greater in male animals in both apoE -/- and LDLR-/- mice[39, 192]

** Sepsis susceptibility with exaggerated inflammation: apoE -/- > LDLR-/- > wild-type C57Bl/6 mice[53, 190, 191]