

## Inflammation and the Osteogenic Regulation of Vascular Calcification A Review and Perspective

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Arterial biomineralization processes have been afflicting humans for  $\geq 5$  millennia, as realized in 2003 via the computed tomographic imaging of Ötzi, the intriguing “ice mummy” discovered in the Tyrolean Alps.<sup>1</sup> Patchy abdominal atherosclerotic calcification was readily detected in the postmortem of this  $\approx 40$ -year-old hunter of the early Copper Age, by 2000 years a predecessor of King Tutankhamen.<sup>1</sup> Today, an epidemic of vascular calcification is emerging within our aging and dysmetabolic populace.<sup>2,3</sup> 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.<sup>4,5</sup> Moreover, as in skeletal development – where unique biology controls matrix mineralization in membranous bone, endochondral bone, dentin, and enamel,<sup>6,7</sup> mechanistic diversity exists in the pathobiology of vascular calcium deposition.<sup>2,4,5,8</sup> 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<sup>4,5,9</sup> (Table 1). Although we touch on the subject, the reader is referred to other contemporary reviews for in-depth consideration of pathogenic differences.<sup>2,4,5</sup>

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.<sup>10</sup> 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 mellitus, and uremia to arterial calcium deposition.<sup>2,4,11</sup> 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.<sup>5,12</sup>

### Inflammatory Cytokines in the Initiation and Progression of Arterial Calcification: Lessons Learned From LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> Mice

Some degree of vascular inflammation is a frequent concomitant of most forms of arterial calcification.<sup>13,14</sup> Sites of

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

### RANKL and Atherosclerotic Calcification

*Receptor Activator of Nuclear Factor  $\kappa$ B Ligand/Osteoprotegerin 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)<sup>-/-</sup> mouse.<sup>34</sup> OPG-deficient mice develop severe medial and intimal arterial calcification in conjunction with high-turnover osteoporosis driven by excessive osteoclast formation.<sup>34</sup> OPG was first shown to function as an antagonistic “faux receptor” of receptor activator of nuclear factor  $\kappa$ B ligand (RANKL), the TNF superfamily member that signals via its receptor activator of nuclear factor  $\kappa$ B on monocyte/macrophage progenitors to promote the formation of bone-resorbing osteoclasts.<sup>7,35</sup> 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.<sup>35</sup> RANKL expression is readily detected in T cells and macrophages near atherosclerotic lesions and within cytokine-stimulated endothelium.<sup>35</sup> Intriguingly, RANKL has been shown recently to promote osteochondrogenic mineral-

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**Table 1. Common Histoanatomic Forms of Vascular Calcification and Clinical Settings<sup>2,4,5,11,99</sup>**

Type	Characteristics	Histopathology	Disease Biology	Risk Factors
Calcific aortic valve disease, also known as calcific aortic stenosis or aortic valve calcification	Calcification of aortic valve leaflets	Intracellular lipid and extracellular lipoprotein accumulation in valves	Mixed picture of membranous, endochondral, and dystrophic calcification	Hypercholesterolemia <ul style="list-style-type: none"> <li>● Low-density lipoprotein cholesterol</li> <li>● Advanced age</li> </ul>
	Stenosis with variable regurgitation	Subendothelial thickening	Runx2/Cbfa1, Msx2, and Sox9 osteogenic transcription factors expressed in VICs <ul style="list-style-type: none"> <li>● <math>\beta</math>-Catenin osteogenic programs (Wnt3a)<sup>135</sup></li> <li>● Promoted by oxidative stress</li> <li>● Promoted by matrix stiffness<sup>144</sup></li> </ul>	Bicuspid aortic valve
	Increased myocardial workload	Displaced, split elastic lamina	Notch and NO synthase 3 genetics contribute in bicuspid disease <sup>170</sup>	
	Left ventricular hypertrophy, heart failure, syncope, and sudden death	Fibrofatty expansion between fibrosa and ventricularis <p>Multiple early calcification foci at base of lesions (regions of highest mechanical stress) with later coalescence</p> <p>Woven bone with marrow in <math>\approx</math>13% of specimens<sup>169</sup></p> <p>Amorphous nodules of calcium phosphate accrue via epitaxial mineral deposition</p>	Cell-mediated inflammatory cells observed in bicuspid and senile calcific disease	T2DM <sup>46</sup> Metabolic syndrome <sup>45</sup> Hypertension <sup>154</sup> Tobacco use Male sex
AMC, also known as medial artery calcification, Mönckeberg sclerosis, or medial calcific sclerosis	Calcification of the arterial tunica media	Circumferential, contiguous, and confluent calcification of tunica media	Arterial activation of "membranous" ossification	T2DM <sup>63-65</sup>
	Reduced vascular compliance and impaired Windkessel physiology	Circumferential adventitial inflammation with fibrofatty expansion	Msx2 and Osx early	Advanced age
	Increased lower extremity amputation risk in T2DM	AKP2-positive matrix vesicles mediate calcification, associated with elastin lamellae	Runx2 late	CKD (see below)
	Increased pulse pressure	Usually spares coronary arteries except in setting of CKD and calcium-based phosphate binders <sup>24</sup>	Adventitial-medial Wnt signaling direct osteogenic differentiation of CVCs in tunica media	Autonomic neuropathy <sup>171,172</sup>
	Increased myocardial workload		Paracrine Wnt signals from adventitial myofibroblasts	
	Myocardial infarction and stroke		$\beta$ -Catenin osteogenic programs activated in media	
	Elevated lipidaceous markers of oxidative stress		TNF and BMP2 dependent, osteogenic signals propagated by hydrogen peroxide <sup>10</sup>	
Autonomic neuropathy <sup>171,172</sup>		Low-grade systemic inflammation		
Atherosclerotic intimal calcification	Calcification of atherosclerotic plaques	Eccentric, lumen-deforming, type Vb intimal plaque <sup>173</sup>	Mostly endochondral ossification picture in addition to lipid core mineralization and fibrous microcalcification	Hypercholesterolemia <sup>4</sup>

(Continued)

Table 1. Continued

Type	Characteristics	Histopathology	Disease Biology	Risk Factors
	Angina	Some calcification present in most atherosclerotic plaques	Focal inflammation	Low-density lipoprotein cholesterol
	Acute coronary syndrome	Patchy distribution	Oxidized low-density lipoprotein in intima and macrophage-derived signal promote osteogenic differentiation of mural CVCs	Hypertension <sup>154</sup>
	Myocardial infarction, stroke, and sudden death	Several mechanisms	Runx2/Cbfa1, Sox9 >> Msx2 expression	Type 1 diabetes mellitus
	Reduced vascular compliance	Lipid core calcification	RANKL, TNF, and IL6 dependent	T2DM
	Peripheral arterial disease and claudication	Fibrous calcification/apoptotic bodies	Macrophage and T-cell sources of RANKL <sup>28,127</sup>	Tobacco use
	Mitral and aortic annulus calcification are both associated with atherosclerotic intimal calcification <sup>172</sup>	Endochondral ossification with matrix vesicles, AKP2 positive Elastinolysis		Rheumatoid arthritis <sup>174</sup> Systemic lupus erythematosus <sup>175,176</sup>
Vascular calcification of end-stage kidney disease CKD5	CKD5 (GFR <15 cc/min/1.73 m <sup>2</sup> ) in concert with any of the above	All the above <sup>11,97</sup>	Impaired serum calcium phosphate homeostasis	Any of the above <sup>11</sup>
	Impaired serum calcium phosphate homeostasis	VSMC apoptosis <sup>158</sup>	Vascular smooth muscle cells elaborate mineralizing matrix vesicles and apoptotic bodies, stimulated by elevated serum calcium and phosphate	Hyperphosphatemia
	Often represents acceleration of antecedent calcific vasculopathy	Elastinolysis (cathepsin S)	Low-grade systemic inflammation, reduced serum fetuin impairs matrix vesicle clearance by VSMCs	Hypercalcemia
	40% diabetic At any level of CKD, patients with diabetes mellitus have greater vascular calcium loads Can and does occur without antecedent disease (eg, pediatric populations)	AMC predominates with T2DM as cause of CKD Coronary artery medial calcification can be seen in this setting of CKD <sup>24</sup> Frequent mitral annular calcification observed	Dialysis induces VSMC apoptosis Phosphate upregulates Runx2/Cbfa1 and Msx2 expression in VSMCs via Ptt1 <sup>116,177</sup> Vascular TNF, BMP2, and Msx2 increased, coregulated in CKD <sup>90</sup>	Renal osteodystrophy, low turnover bone disease <sup>178</sup> <sup>174</sup> Excessive use of calcium-based phosphate binders and excessive PTH suppression <sup>178,179</sup>
Calcific uremic arteriopathy, also known as calciphylaxis	GFR <30 cc/min/1.73 m <sup>2</sup> , chronic >> acute	Arteriolar (<0.6 mm) medial calcification of dermal, pulmonary, and mesenteric vascular beds <sup>180,181</sup>	Few detailed mechanistic studies	CKD4 or CKD5 <sup>181</sup>
	Warfarin treatment, diabetes	Fibroproliferative arteriole occlusion and fatty tissue necrosis	BMP4 expressed in periarteriolar dermal tissue <sup>182</sup>	Warfarin treatment
	Painful violaceous dermal nodules that progress to dermal necrosis	Periarteriolar inflammation	Reduced MGP- and fetuin-dependent matrix vesicle internalization by VSMCs	T2DM

(Continued)

Table 1. Continued

Type	Characteristics	Histopathology	Disease Biology	Risk Factors
	Lower extremities, buttocks, and pannus frequently affected		Reduced MGP- dependent inhibition of osteogenic BMP2/4 signaling	Obesity
	Greatly elevated markers of systemic inflammation (ESR and CRP)			Severe secondary hyperparathyroidism
	High mortality			? Vitamin K deficiency

CRP indicates C reactive protein; CVC, calcifying vascular cell of Demer; ESR, erythrocyte sedimentation rate; GFR, glomerular filtration rate; Pit1, phosphate transporter SLC20A1; PTH, parathyroid hormone; VIC, valve interstitial cell.

ization of vascular smooth muscle cells (VSMCs)<sup>36</sup> and aortic valve interstitial cells<sup>37</sup> in vitro. Via the receptor activator of nuclear factor  $\kappa$ B expressed in VSMCs, RANKL upregulates bone morphogenetic protein 4 (BMP4) expression, thus providing an autocrine stimulus for osteogenic differentiation (see also the “Of BMPs and Wnts” section below).<sup>36</sup> These dual and disparate actions of RANKL on the skeletal monocyte/macrophage lineage versus VSMCs likely explain the

Table 2. Features of ApoE<sup>-/-</sup> and LDLR<sup>-/-</sup> Murine Models of Arterial Calcification

Parameter	Murine Disease Model	
	ApoE <sup>-/-</sup> Mouse	LDLR <sup>-/-</sup> Mouse
Diet-induced hypercholesterolemia	Yes <sup>33,183</sup>	Yes <sup>33,184</sup>
Diet-induced atherosclerosis	Yes <sup>183</sup>	Yes <sup>185</sup>
Diet-induced diabetes mellitus with hypertriglyceridemia	No <sup>33</sup>	Yes <sup>33,44</sup>
Diet-induced obesity	Less so <sup>33</sup>	Yes <sup>33</sup>
“Spontaneous” arterial chondroid metaplasia	Yes (accelerated by drug-induced diabetes mellitus) <sup>54,98</sup>	No <sup>29,44</sup>
Early diet-induced nonendochondral <sup>7</sup> medial artery calcification	No <sup>54</sup>	Yes <sup>29,167</sup>
Late diet-induced endochondral <sup>7</sup> atherosclerotic calcification	Yes <sup>54</sup>	Yes <sup>28,29,167</sup>
Hemodynamically significant calcific aortic valve disease occurs with progression	Not known (valve thickening seen with CRI) <sup>186</sup>	Yes (concomitant ApoB100/100 genotype) <sup>125,187</sup>
Exaggerated inflammatory response and susceptibility to mortality with gram-negative sepsis	Yes <sup>53,188</sup>	Less so <sup>53,189*</sup>
Arterial calcification accelerated by chronic renal insufficiency	Yes <sup>140</sup>	Yes <sup>118</sup>

For C57Bl/6 background, arterial calcification is greater in male animals in both apoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice.<sup>39,190</sup>

\*Sepsis susceptibility with exaggerated inflammation includes: apoE<sup>-/-</sup> > LDLR<sup>-/-</sup> > wild-type C57Bl/6 mice.<sup>53,188,189</sup>

intriguing phenotype of OPG-null mice.<sup>34</sup> Of note, although the vascular calcification of OPG deficiency occurs in the complete absence of atheroma formation,<sup>34</sup> calcified lesions begin to form in arteries only in the postpartum period with copious CD3<sup>+</sup> T-cell infiltrates, a few F4/80<sup>+</sup> macrophages, and cathepsin K<sup>+</sup> osteoclast-like cells.<sup>34,38</sup> This suggests that, in vivo, inflammatory signals absent in utero are necessary for vascular disease initiation and progression in OPG<sup>-/-</sup> animals. In addition, as first observed in the diabetic LDLR<sup>-/-</sup> mouse,<sup>39</sup> serum levels of OPG are higher in patients with diabetes mellitus.<sup>40,41</sup> Because OPG is expressed in VSMCs,<sup>42</sup> such increases in the setting of type 2 diabetes mellitus (T2DM) presumably reflect a vascular defense that helps prevent excessive RANKL signaling via negative feedback regulation.<sup>28</sup>

#### *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<sup>34</sup>; however, this caveat has been addressed recently.<sup>28</sup> Inhibition of RANKL via administration of recombinant OPG has been evaluated in 2 very different murine models of vascular disease<sup>33</sup>: the LDLR<sup>-/-</sup> mouse<sup>28</sup> and the apoE<sup>-/-</sup> mouse.<sup>43</sup> It is important to highlight that, whereas both models encompass impaired cholesterol metabolism and atherosclerosis on the C57Bl/6 background, the arteriosclerotic disease processes exhibited by these 2 pre-clinical models are very distinct (Table 2).<sup>33</sup> As Schreyer et al<sup>33</sup> first showed, although 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 mellitus and obesity with hypertriglyceridemia. However, the male LDLR<sup>-/-</sup> mouse develops both of these relevant characteristics alongside arterial calcification in response to challenge with fatty diet—possessing compositions typical of westernized societies,<sup>33,44</sup> a clinically important stimulus for vascular disease.<sup>45,46</sup> Early medial artery calcification is followed by progressively severe atherosclerotic disease in this model (see below).<sup>29</sup> Furthermore, the diet-induced systemic low-grade inflammation, characterized by low but measurable levels of circulating TNF in obese LDLR<sup>-/-</sup> mice<sup>29,47</sup> and diabetic humans,<sup>48–50</sup> is not seen and apparently does not contribute to vascular inflammation in the apoE<sup>-/-</sup> model<sup>43,51</sup> even when streptozotocin is admin-

istered to induce diabetes mellitus.<sup>52</sup> However, in response to other stimuli, such as lipopolysaccharide administration or *Klebsiella* infection, apoE<sup>-/-</sup> mice exhibit exaggerated TNF induction and increased mortality.<sup>53</sup> Finally, in the apoE-null mouse, vascular calcification quickly evolves on the backdrop of VSMC chondroid metaplasia<sup>8</sup> that is observed over time even on mouse chow, that is, in the absence of cholesterol-rich dietary challenge.<sup>54</sup> By comparison, evolution of arterial calcification in the LDLR<sup>-/-</sup> mouse is more protracted and elicited by the clinically relevant Western diet (42.00% of calories from fat and 0.15% cholesterol), accruing vascular mineral deposition via sequentially distinct mechanisms.<sup>28,29</sup> 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<sup>-/-</sup> mice, biochemically quantifiable after acid extraction.<sup>29</sup> 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,<sup>55,56</sup> because every little inorganic phosphate staining is evident by von Kossa at this stage.<sup>29</sup> Similar observations have been described in human specimens.<sup>57</sup> With progression, however, massive aortic sinus and subintimal cholesterol deposits accrue, with atherosclerotic calcification visualized within the cholesterol clefts and degenerating atheromata.<sup>29</sup> During this second phase, chondroid metaplasia clearly contributes to vascular calcium accrual in male LDLR<sup>-/-</sup> mice,<sup>28</sup> as observed in apoE<sup>-/-</sup> mice.<sup>8</sup> The extent of medial calcium is, thus, increased on Alizarin red staining,<sup>29,57</sup> and the von Kossa method for visualizing inorganic phosphate now reveals massive medial and atherosclerotic calcium phosphate deposition in male LDLR<sup>-/-</sup> mice fed fatty diets.<sup>28,29</sup> Thus, when placed on high-fat westernized diets, the male LDLR<sup>-/-</sup> 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).

#### *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 pharmacological 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<sup>-/-</sup> and apoE<sup>-/-</sup> mice.<sup>28,43</sup> Morony et al<sup>28</sup> first evaluated the male LDLR<sup>-/-</sup> mouse, the dynamics of endogenous RANKL/OPG signaling during disease initiation and progression, and the impact of exogenous OPG administration. Serum RANKL measurements demonstrated that progression of vascular disease over 5 months of dietary cholesterol challenge closely tracks the progressive recovery of circulating RANKL after an early phase of diet-induced suppression.<sup>28</sup> Early diet-induced increases in OPG, a presumed adaptive mechanism to protect against untoward RANKL signaling,<sup>36</sup> exhibited no dynamic change with progression.<sup>28</sup> As predicted from studies of

OPG<sup>-/-</sup> mice,<sup>34</sup> male LDLR<sup>-/-</sup> mice treated with exogenous OPG exhibit reduced arterial calcification and diminished aortic osteochondrogenic differentiation.<sup>28</sup> However, no change in atherosclerosis (ie, the size of arterial atheroma) was observed.<sup>28</sup> Intriguingly, 3 sources of vascular RANKL production were identified in this LDLR<sup>-/-</sup> model: (1) the F4/80<sup>+</sup> monocyte-macrophage population in closest proximity to lesions undergoing chondroid metaplasia; (2) the endothelial cells overlying atheroma; and (3) the CD3<sup>+</sup> T cells at the adventitial-medial junction.<sup>28</sup> 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<sup>-/-</sup> mice, as in LDLR<sup>-/-</sup> mice, OPG administration apparently does not affect atheroma lesion size.<sup>43</sup> However, OPG significantly increases fibrous cap size and thickness and reduces matrix metalloproteinase (MMP) 12 levels, potentially stabilizing the lesion but not directly assessed<sup>43</sup> (see below). Nonsignificant, tantalizing trends for reductions in numbers of macrophages and T cells were also observed in response to OPG administration. Unlike male LDLR<sup>-/-</sup> mice, where diet-induced obesity increases circulating TNF levels,<sup>29</sup> basal TNF levels are below the limits of detection in apoE<sup>-/-</sup> animals and, thus, not measurably changed by OPG administration.<sup>43</sup> Unfortunately, calcification was not scored in this recent study.<sup>43</sup> However, Bennett et al<sup>31</sup> have applied murine genetics to carefully detail the important role for endogenous OPG in the calcification of advanced atherosclerotic lesions of apoE<sup>-/-</sup> mice by generating and evaluating OPG<sup>-/-</sup>;apoE<sup>-/-</sup> mice. In this model, congenitally deficient OPG<sup>-/-</sup>;apoE<sup>-/-</sup> 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.<sup>31</sup> Plaque stability was not assessed in OPG<sup>-/-</sup>;apoE<sup>-/-</sup> mice, but OPG was shown to increase MMP9 activity *in vitro*,<sup>31</sup> and MMP9 promotes intraplaque hemorrhage *in vivo* in advanced atherosclerotic lesions of apoE-null animals.<sup>58,59</sup> However, congenitally deficient MMP9<sup>-/-</sup>;apoE<sup>-/-</sup> mice exhibit increased lesion size after disease initiation versus MMP9-replete siblings,<sup>60</sup> suggesting that stage-specific roles of MMP9 exist in atherosclerosis and sclerosis.<sup>58</sup> As a modulator of MMP9, OPG could potentially exert adverse, as well as beneficial, arteriosclerotic actions during pharmacological manipulation of RANKL signaling.<sup>31</sup> Thus, as in the LDLR<sup>-/-</sup> mouse, OPG limits arterial calcium accumulation in the apoE-null mouse. OPG may regulate plaque stability, but the differential responses of pharmacological versus genetic manipulation of OPG on vascular histopathology in apoE<sup>-/-</sup> mice highlight the need for a more detailed assessment of impact on plaque formation, stability, and regression.

In summary, antagonism of RANKL signaling cascades holds much promise for modulation of atherosclerotic calcification.<sup>61</sup> Of note, a humanized antibody that antagonizes human RANKL has been developed for prevention of fractures in osteoporosis<sup>62</sup>; based on preclinical studies of Helas et al<sup>32</sup> using a "humanized RANKL" murine model, 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.

### Medial Artery Calcification and Diabetic Arteriosclerosis

#### 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.<sup>63,64</sup> The earliest studies were reported for Pima Indians, a native American population with increased risk for T2DM.<sup>63,64</sup> 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.<sup>65</sup> Why, then, does increased arterial stiffness (arteriosclerosis) in T2DM, arising from AMC without peripheral atherosclerosis, contribute to the increased risk for lower extremity amputation?<sup>66</sup> Conduit vessel stiffening from any cause<sup>67</sup> compromises normal arterial Windkessel physiology,<sup>67,68</sup> thus impairing uniform distal tissue perfusion throughout the cardiac cycle.<sup>69,70</sup>

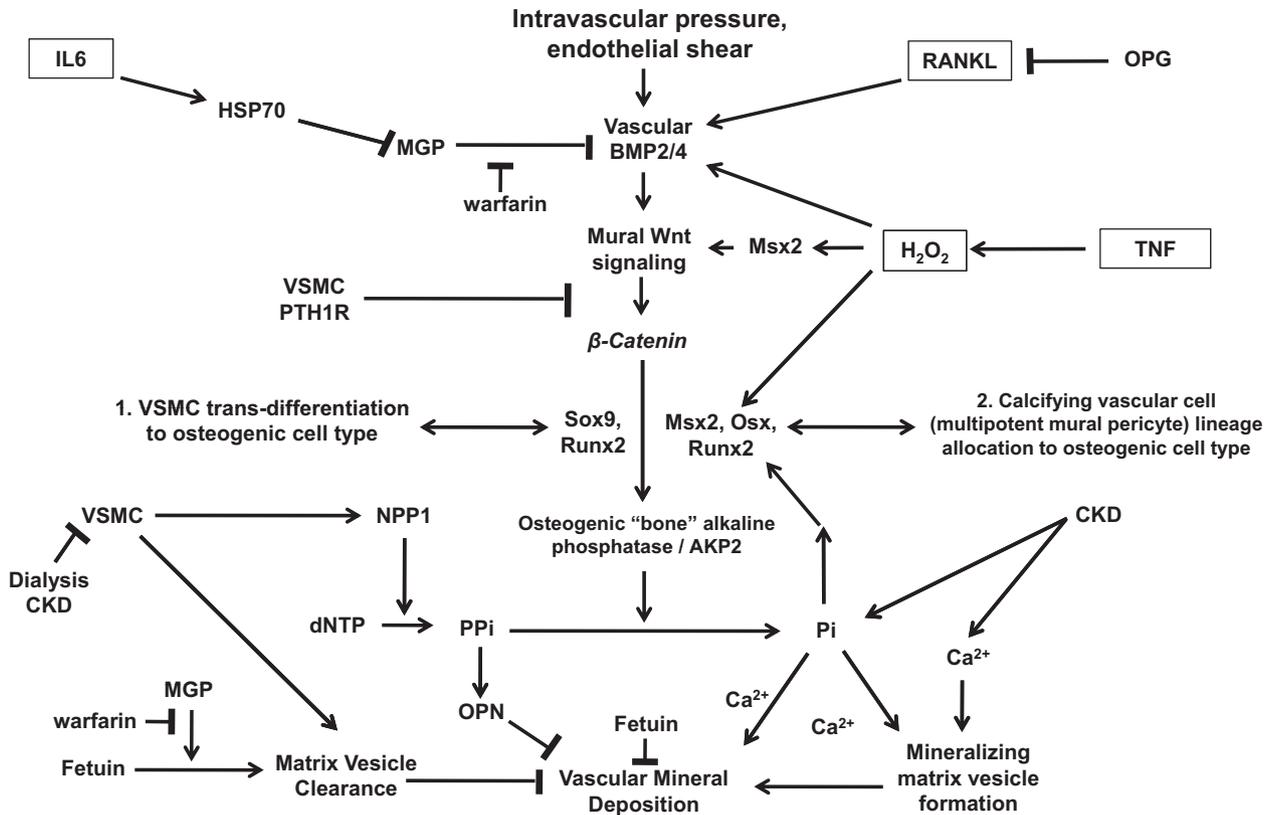
At this point, however, it should be re-emphasized that critical limb ischemia arising from atherosclerotic plaque formation and arterial stenosis in the femoropopliteal bed is a well-recognized contributor to lower extremity amputation risk; moreover, atherosclerotic calcification also contributes to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

#### Mechanisms of Medial Artery Calcification in T2DM: Clues From the Field of Bone Biology and the LDLR<sup>-/-</sup> Mouse

During skeletal mineralization, bone formation can occur via either endochondral (preceding cartilage template required) or membranous (nonendochondral; no cartilage required) processes.<sup>7</sup> Osteo/chondrocytic transcription factors, such as Sox9, osteoblast transcription factor runt related transcription factor (Runx) 2, osteoblast transcription factor muscle seg-

ment homeobox homolog (Msx) 2, Msx1, and osteoblast transcription factor osterix (Osx), play critical roles in promoting either endochondral (Sox9, Runx2, and Osx) or membranous (Msx2, Msx1, Runx2, and Osx) bone formation.<sup>7</sup> In bone, polypeptide morphogens, such as bone morphogenetic proteins (BMPs) and wingless/mouse mammary tumor virus integration site family (Wnts) induce these osteoblast DNA binding proteins along with  $\beta$ -catenin, a transcription coadapter indispensable for bone formation.<sup>7,85</sup> A common feature of active osteogenic mineralization is induction of alkaline phosphatase (AKP) 2, the “bone” alkaline phosphatase that degrades the plentiful and endogenous mineralization inhibitor inorganic pyrophosphate (PPi; Figure).<sup>7</sup> Of note, Sox9, Runx2, Msx2, and AKP2 have all been described as being expressed in calcifying human arterial segments<sup>86</sup> and are upregulated by stimuli that promote arterial calcification (Figure).

The molecular mechanisms controlling initiation and progression of medial artery calcification in T2DM have been studied recently in detail in the male LDLR<sup>-/-</sup> mouse (Table 2), a model in which obesity, diabetes mellitus, and osteogenic arterial calcification programs are induced in response to high-fat diets possessing compositions characteristic of westernized societies.<sup>2,29,39,44,87</sup> Importantly, diet-induced disease in male LDLR<sup>-/-</sup> mice<sup>2,29,44,87</sup> closely tracks molecular and physiological characteristics of T2DM patients afflicted with valve<sup>88,89</sup> and arterial<sup>86,90</sup> 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 (ie, fat-derived cytokines).<sup>48–50,91,92</sup> TNF is the prototypic inflammatory cytokine, elaborated not only by adipocytes but also by adipose tissue macrophages that infiltrate fat with “diabesity.”<sup>48,93,94</sup> Tintut et al<sup>95</sup> first identified that TNF and a macrophage-derived signal stimulated the mineralization of aortic calcifying vascular cells (CVCs) in vitro. Subsequently, we demonstrated that infliximab-mediated inhibition of TNF signaling in vivo in the LDLR<sup>-/-</sup> mouse downregulated the osteogenic Msx2-Wnt gene regulatory program in aortas of diabetic LDLR<sup>-/-</sup> mice.<sup>29</sup> Concomitant reductions in early vascular calcium load was also observed with infliximab.<sup>29</sup> Although 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.<sup>29</sup> 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.<sup>29</sup> Others have now also confirmed the important role for Msx2 in TNF-dependent induction of AKP2 and mineralization in VSMCs (Figure).<sup>96</sup> Koleganova highlighted the significance of these preclinical studies to human disease biology in those afflicted with arterial calcification of renal failure<sup>90</sup>; TNF, Msx2, and BMP2 expressions were correlated with osteogenic differentiation in both calcified and noncalcified vessel segments of patients with chronic kidney disease (CKD) 5.<sup>90</sup>



**Figure.** Inflammation and osteogenic regulation of vascular calcification: a review and working model. Osteochondrocytic cells that promote vascular matrix mineralization can arise from at least 2 sources: transdifferentiation of VSMCs (ie, a type of phenotypic modulation in which the mature VSMC phenotype is replaced, and reprogrammed to that of an osteochondrocytic cell) or osteogenic lineage allocation from a multipotent mesenchymal progenitor (ie, 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 VSMCs not only provides substrate for nucleation but also loss of cellular defenses. Multiple paracrine inhibitors control pro-osteogenic signals provided by BMP/Wnt signaling, RANKL, and TNF actions and nucleation/aggregation/epitaxial propagation of apatitic calcium phosphate deposition. Via heat shock protein 70 (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 downregulates 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.<sup>10,88,168</sup> Although clearly an important stimulus for vascular BMP2 expression,<sup>131</sup> 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 posited recently but has yet to be established.<sup>152</sup> See text for details and additional references.

Thus, in summary, these data<sup>90</sup> and others<sup>86,88,89</sup> confirm the clinical relevance of the osteogenic relationships established in the LDLR<sup>-/-</sup> murine model of calcific vasculopathy (Figure). Obligatory diet-induced diabetes in the LDLR<sup>-/-</sup> 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, and Sox9) are ectopically induced in the arteries and valves of diabetic LDLR<sup>-/-</sup> mice. Mechanistic insights possible via preclinical studies point to both transdifferentiation of VSMCs and osteochondrogenic lineage allocation of multipotent mesenchymal progenitors by these osteogenic transcription factors.<sup>4,97</sup> Because streptozotocin drug-induced diabetes mellitus also accelerates osteochondral metaplasia in the apoE<sup>-/-</sup> mouse,<sup>98</sup> further evaluation of this model may help elucidate the pathobiological mechanisms whereby hyperglycemia promotes arterial mineralization.

**Inflammation, Fetuin, and Matrix Vesicle Metabolism: Novel Insights Into the Calcific Vasculopathy of CKD**

CKD, particularly CKD5, represents a “perfect storm” of calcific vasculopathy (Table 1).<sup>11</sup> Antecedent diabetes mellitus, hypertension, and dyslipidemia intersect with phosphate retention, low turnover bone disease, and dialysis-induced systemic inflammation, and VSMC apoptosis synergizes to drive ferocious arterial calcium accrual (Figure).<sup>11</sup> 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 articles.<sup>11,99,100</sup> 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, also known as fetuin A, α-2-Heremans-Schmid glycoprotein, is a serum protein synthesized by the liver.<sup>99</sup> As first demonstrated by Rochette et al,<sup>101</sup> fetuin avidly binds

amorphous calcium phosphate and maintains the solubility of supersaturated serum calcium phosphate<sup>102</sup> via the formation of calciprotein particles that inhibit insoluble calcium phosphate crystal aggregate formation (Figure).<sup>103–106</sup> Consistent with these observations, Schafer et al<sup>106</sup> demonstrated widespread soft tissue calcification in fetuin-deficient mice. Reynolds and colleagues<sup>107,108</sup> recently identified that fetuin also plays a critical role in VSMC-mediated removal of procalcific matrix vesicles. In response to hypercalcemia and hyperphosphatemia, common stimuli in dialysis patients,<sup>102</sup> 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.<sup>107,108</sup> Serum-derived fetuin and matrix vesicle-associated matrix Gla protein (MGP) are required for VSMC-mediated uptake and clearance of vesicles (Figure).<sup>107,108</sup> 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.<sup>109,110</sup> In end-stage renal disease, fetuin levels are inversely related to the extent of coronary calcification, providing yet another link among inflammation, oxidative stress, and arterial calcium accumulation.<sup>111,112</sup> Similar results have been noted in patients with calcific aortic stenosis.<sup>113</sup>

In summary, the cumulative evidence overwhelmingly points to an important role of fetuin in limiting arterial calcium deposition. It remains to be determined whether 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<sup>114</sup>) is associated with reductions in fetuin but is significant only in the setting of inflammation.<sup>115</sup> 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,<sup>11,116</sup> VSMC BMP2-dependent phosphate transport,<sup>117</sup> and BMP7-corrected hyperphosphatemia<sup>118,119</sup> to the pathobiology of vascular calcification in the setting of CKD.

### **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 end-stage renal disease, and calcific uremic arteriopathy (Table 1).<sup>5</sup> However, over the past 2 years, multiple groups have identified the important role of oxidative stress signaling in vascular activation of osteogenic gene regulatory programs.<sup>88,120</sup> Byon et al<sup>120</sup> first demonstrated that the osteochondrocytic transcription factor Runx2 is activated by hydrogen peroxide and supports bone AKP2 expression and matrix mineralization in cultured vascular smooth muscle cells (Figure). Similarly, membranous ossification programs<sup>121,122</sup> elaborated by Msx2-Wnt signaling cascades are

also dependent on peroxide signals elaborated from mitochondrial activity and downstream of TNF-stimulated NADPH oxidases.<sup>123,124</sup> Very recently, Miller et al<sup>88</sup> demonstrated colocalization of oxidative stress signaling and the osteogenic transcription factors Msx2 and Runx2 in calcifying human aortic valves. However, the sources of oxidative stress were shown to arise from uncoupling of NO synthase and failures in the enzymatic defenses (eg, catalase) that restrain peroxide accumulation.<sup>88</sup> 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.<sup>125</sup> This suggests that an oxylipid,<sup>126</sup> in addition to oxylipid-responsive cytokines, such as TNF<sup>29,95</sup> and RANKL,<sup>28,127</sup> is required for vascular calcification, as first proposed by Demer and colleagues.<sup>95,126,127</sup>

In summary, although sources of oxidative stress may differ with vascular venue and disease state<sup>88</sup> (and the signaling cascades have yet to be fully elucidated<sup>123</sup>) oxidative stress signals provide important stimuli. With inflammatory cytokine signals, this helps provide a unifying theme for arterial elaboration of osteogenic mineralization processes.

### **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 postnatal life.<sup>128</sup> Via an autocrine Wnt signaling loop, BMP2 promotes osteoblast commitment and the induction of the bone AKP2,<sup>129</sup> the latter an important enzymatic mediator of osteogenic matrix mineralization (Figure).<sup>129</sup> Almost 2 decades ago, Bostrom et al<sup>130</sup> identified the presence of BMP2 in calcified atherosclerotic plaques and demonstrated the important role for BMP2 in CVC mineralization. With studies by Tanimura et al,<sup>55</sup> 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.<sup>123</sup> Csiszar et al<sup>131</sup> demonstrated recently that TNF, hydrogen peroxide, and high intravascular pressure, stimuli commonly encountered with diabetes mellitus, hypertension, and the metabolic syndrome, all upregulate the expression of BMP2 in endothelial cells. 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<sup>130,132,133</sup> (Figure). 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<sup>36</sup> or MGP.<sup>30</sup> Importantly, in addition to being entrained to TNF,<sup>29</sup> the vascular osteogenic Wnt signaling cascades discussed previously<sup>87,123,124</sup> (Figure) are also activated downstream of BMP2 *in vivo*.<sup>2</sup> Of note, these canonical Wnt signals drive osteochondrocytic differentiation of multipotent vascular pericytes *in vitro*<sup>134</sup> and promote the arterial calcification of T2DM *in vivo*<sup>87</sup>; via multiprotein cell surface receptor complexes containing LRP5 or LRP6, the Wnt polypeptide family contributes to

bone morphogenesis and skeletal integrity<sup>135</sup> in conjunction with the BMPs.<sup>136</sup> Intriguingly, along with many Wnt ligands, LRP5 and LRP6 are expressed in endothelial cells and VSMCs.<sup>137</sup> The precise reasons why vascular expression of these osteogenic morphogens does not always lead to arterial mineralization are still unclear. This presumably reflects the local balance between agonists and antagonists of BMP/Wnt signaling<sup>123</sup> (eg, MGP and Dkk1, respectively; Figure); the important roles of PPI,<sup>138</sup> fetuin,<sup>107</sup> and osteopontin<sup>139</sup> as osteogenic mineralization inhibitors; elastin metabolism<sup>140–143</sup>; and the impact of matrix stiffness<sup>144</sup> on the osteogenic potential of vascular mesenchymal progenitors. Nevertheless, strategies that can selectively inhibit the activation or the actions of vascular osteogenic BMP/Wnt signaling or augment vascular defenses that prevent mineralization hold promise for limiting arterial calcium accumulation.

### **PPI and MGP: Overlapping Consequences of Genetic and Inflammation-Induced Deficiency in 2 Key Vascular Defenses**

Elegant genetic studies in mice and humans have highlighted the important roles for PPI and MGP as noninflammatory inhibitors of vascular mineralization. Karsenty et al<sup>145</sup> first identified that murine deficiency in the BMP2/4 antagonist MGP<sup>132</sup> results in chondroid metaplasia of the arterial tunica media, panarterial calcification, and vascular rupture. Similarly, Johnson et al<sup>138</sup> showed that murine deficiency in the mineralization inhibitor PPI, arising from genetic disruption of the ectoenzyme ectonucleotide pyrophosphatase/phosphodiesterase 1 also results in arterial calcification with chondroid metaplasia. The relevance of the PPI/ectonucleotide pyrophosphatase/phosphodiesterase 1 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 because of ectonucleotide pyrophosphatase/phosphodiesterase 1 loss-of-function mutations.<sup>146</sup> How, then, is inflammation connected to these critically important inhibitors of mineralization? Overtly, the induction of AKP2 by TNF<sup>95</sup> and downstream osteogenic BMP-Wnt pathways<sup>29,87,135</sup> (described above) hydrolyzes PPI to destroy this inhibitor during vascular mineralization (Figure).<sup>147</sup> Moreover, depletion of PPI markedly downregulates osteopontin,<sup>148</sup> the even more potent inducible inhibitor of vascular mineralization.<sup>139</sup> However, until very recently, the relationship between inflammation and MGP insufficiency was less clear. In a series of very insightful studies, Yao et al<sup>149</sup> identified that MGP is a Gla-dependent inhibitor of BMP2 and BMP4, osteogenic morphogens that upregulate AKP2 expression.<sup>30,132,149</sup> Yao et al<sup>30</sup> went on to show that interleukin (IL) 6, an inflammatory cytokine important in diabetic vascular disease, increases the expression and secretion of heat shock protein (HSP) 70, an endogenous MGP-binding protein and antagonist of MGP function that is highly expressed in calcifying atherosclerotic plaques. Thus, by inducing heat shock protein 70, inflammatory signals provided by IL6 potentiate vascular BMP2/4 actions by nullifying MGP<sup>30</sup> (Figure). Whether other inflammatory cytokines participate in this hierarchy of regulated mineralization is unknown. Nevertheless, these newer data point to how 2 axes, genetic

defenses against vascular mineralization and inflammation-induced arterial osteogenic programs, functionally intersect to regulate arterial calcification.<sup>4</sup>

### **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 transdifferentiation, VSMC apoptosis, and osteochondrocytic lineage allocation of multipotent mesenchymal cells all contribute but to varying extents dependent on pathophysiologic setting and disease stage (Figure). It has been posited that marrow-derived circulating osteoprogenitors may also contribute to vascular mineralizing cell types,<sup>150–152</sup> but this has yet to be unambiguously established.

To date, approaches to prevent and/or reverse macrovascular calcification have largely been unsuccessful,<sup>153</sup> attributed in part to this mechanistic heterogeneity and the intrinsic proinflammatory actions of vascular calcium phosphate that provide a “feed-forward” stimulus for disease.<sup>25,26</sup> 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.<sup>154</sup> 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.<sup>24</sup> 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 low-density lipoprotein cholesterol reduction and anti-inflammatory actions.<sup>12,155,156</sup> Unfortunately, simple strategies that seek to “scavenge” redox signals elaborated by inflammation do not offer significant clinical benefits for most individuals at risk.<sup>80</sup> Approaches that target the OPG/RANKL pathway,<sup>61</sup> the calcium sensing receptor,<sup>157</sup> and other calcitropic signals<sup>39,116,119</sup> or key vascular proteases<sup>140,143</sup> offer hope, but individually may be insufficient in some clinical settings, particularly dialysis-dependent renal failure.<sup>100,158</sup> Except for a few prescient reports on the relationships between arterial pressure and vascular BMP-Wnt signaling,<sup>131,159–163</sup> remarkably few studies have examined the mechanistic links between hypertension and signals that regulate arterial calcification.<sup>164</sup> Given that endothelin-dependent signals control both vascular calcium homeostasis and blood pressure,<sup>165,166</sup> the paucity of such studies represents an unmet scientific need. Moving forward with mech-

anistic insights, pharmacological strategies can be crafted that newly acknowledge disease complexity<sup>4,5</sup> and, thus, antagonize with sophistication the combination of pathobiological processes that promote vascular mineralization during disease initiation and progression.<sup>167</sup> The future holds great promise for the development of these successful therapeutics and the medical management necessary to address a burgeoning clinical need.<sup>5</sup>

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