A current understanding of vascular calcification in CKD

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VASCULAR CALCIFICATION is defined as the inappropriate and pathological deposition of mineral in the form of calcium phosphate salts into the vascular tissues. While this can happen with normal aging, it is accelerated in certain disease states, including diabetes mellitus, cardiovascular disease, and specific genetic diseases (57, 114). One of the most common causes of vascular calcification is chronic kidney disease (CKD) (14). Originally thought to occur primarily in end-stage renal disease (ESRD) and in patients on dialysis, new animal models are demonstrating that vascular calcification likely begins much earlier, possibly as early as CKD stage 2 (23).

Once hypothesized to be a benign finding, vascular calcification in the CKD and ESRD populations is associated with significant morbidity and mortality (64, 96). This inappropriate calcification in the vessel wall causes increased wall stiffness, elevated pulse pressure, and left ventricular hypertrophy. Together, these cause left ventricular stress and decreased coronary perfusion. Cardiovascular disease is the leading cause of mortality in patients with CKD and ESRD, and vascular calcification has been shown to be an independent predictor of vascular morbidity and mortality (65, 72). Vascular calcification has even been shown to affect pediatric ESRD patients on dialysis (33).

Vascular calcification can occur in two areas of the vessel wall, the intima and the media. The intimal layer of the artery is comprised of endothelial cells surrounded by a thick outer layer of elastic fibers. Calcification at this site is associated with dyslipidemia and, along with inflammation and thickening of the intimal layer, causes atherosclerosis. This process forms plaques on the inner vessel wall which are unstable and capable of rupturing. They are also prone to cause thrombus formation and resulting occlusive disease (28, 94). The media is made up of smooth muscle cells in a framework of loose connective tissue (mainly elastin), and calcification here is often secondary to diabetes mellitus or CKD. This can also be referred to as Monckeberg’s arteriosclerosis, specifically in the setting of medial calcification without any luminal narrowing (32). While patients with CKD can develop both types of vascular calcification, calcification of the media is more specific to CKD and is the exclusive form of vascular calcification observed in pediatric CKD patients (106). A better understanding of the pathology of vascular calcification is critical to developing better therapies for this population at high risk for cardiovascular disease. This review will focus primarily on established and novel mechanisms that contribute to the medial calcification observed in CKD.

The Pathology of Vascular Calcification

Phenotypic changes in vascular smooth muscle cells. Before deposition of calcium in the vessel wall (Fig. 1), vascular smooth muscle cells (VSMC) undergo transdifferentiation into cells that resemble bone-formative cells. These phenotypically distinct cells downregulate production of smooth muscle-specific genes such as smooth muscle (SM) α-actin and SM22α. Simultaneously, these cells upregulate markers of osteochondrogenesis, including Runx2 (Cbfa1), osterix, osteopontin (OPN), osteocalcin, and alkaline phosphatase. These osteoblast/chondrocyte-like cells lose their contractile properties but are able to produce a collagen matrix and form calcium- and phosphorus-rich matrix vesicles that are capable of initiating mineralization of the vascular wall. Phenotypic changes in VSMC appear to be an essential step in the pathological calcification pathway (56). Runx2, a key transcription factor involved in normal osteoblast differentiation, is a critical component of this phenotypic change. The absence of VSMC-derived Runx2 prevents the morphological change of these cells and prevents mineralization (115, 117).
Elastin degradation. Elastin is the most abundant protein in the aortic wall, and its degradation has been shown to occur in the setting of uremia and CKD. In vitro, the addition of \( \text{elastin} \), an elastin-derived peptide, increases calcification of VSMC grown in a high-phosphate medium (42). Mice with elastin degradation secondary to uremia do not develop arterial medial calcification (AMC) unless placed on a high-phosphate diet, and it is believed that this breakdown of elastin serves as a site of calcium deposition in the setting of mineral dysregulation (78). Elastin degradation is thought to be caused by a certain class of proteases known as matrix metalloproteases (MMP), specifically, MMP-2 and MMP-9, which are upregulated in CKD and bind and degrade elastin. Cathepsin-S, a third protease known to be elevated in CKD mouse models, is also thought to be responsible for mediating the elastin degradation seen in these uremic states (1). The degradation of elastin causes overexpression of transforming growth factor (TGF)-\( \beta \), which is involved in osteoblast differentiation and directly hastens VSMC calcification (79). Elastin disruption also contributes to the increased arterial stiffness seen in CKD due to loss of its role in normal arterial hemodynamics (26).

Risk Factors for Vascular Calcification in CKD

Vascular calcification associated with CKD was initially believed to be a passive process where the dysregulation of calcium and phosphorous resulted in a precipitation of mineral that deposited in the vessel wall. While abnormal calcium and phosphorous levels do play a critical role in calcification in CKD, it is now understood that this is a highly regulated, active, and complex process that closely resembles skeletal bone formation.

Abnormal calcium and phosphorous balance. Sporadic hypercalcemia and an increased calcium load due to use of activated vitamin D and vitamin D receptor agonists along with calcium-based phosphorus binders are observed in the CKD/ESRD population. When VSMC are exposed to a medium rich in calcium, they undergo osteogenic differentiation with subsequent matrix mineralization, possibly due to secondary increased phosphate transport intracellularly (126). L-type calcium channels are also involved in vascular calcification, as in vitro blockade of these channels with verapamil decreases calcification of VSMC (10). Blockade of the annexins, a family of multifunctional calcium-binding proteins expressed on the surface of most cell types, also decreases calcification in cultured VSMC (11). Elevated extracellular calcium levels are implicated in matrix vesicle release and have been shown to promote cell death, which in turn releases apoptotic bodies, both of which are capable of further potentiating the calcification process and will be discussed in more detail below (102).

While an increased calcium-phosphorus product (Ca \( \times \) P) is also important for the mineralization of vascular extracellular matrix, it has been shown that at a given Ca \( \times \) P product,
calcium was a stronger inducer of calcification than phosphorus in vascular organ culture. Aortic rings of both pre-dialysis CKD and dialysis patients were exposed in vitro to media with various concentrations of calcium and phosphorus with the Ca × P held constant. Those aortic rings exposed to media with higher calcium concentrations showed more significant calcification (107). While is it unknown whether elevated calcium is a stronger vascular calcification inducer than phosphorus in vivo, additive/synergistic effects are likely as the use of calcium-based phosphorus binders in dialysis patients is associated with progressive arterial calcification while non-calcium-based binders do not have this deleterious effect (5).

Hyperphosphatemia due to reduced renal phosphate clearance is commonly seen in patients with CKD and ESRD. Phosphate is not only an integral component of hydroxyapatite, the calcium mineral seen in bone and in vascular calcification, but is also a part of the signaling cascade that triggers vascular calcification. Multiple experiments have demonstrated that culturing VSMC in a high-phosphate medium will increase calcification in a dose-dependent manner. This is also associated with the upregulation of proteins involved in bone formation and the phenotypic differentiation seen in the VSMC (29, 104). Calcification of the coronary arteries is independently associated with hyperphosphatemia in ESRD patients, and elevated serum phosphate levels, even those in the high-normal range, are correlated with a higher risk of myocardial infarction and mortality in CKD patients (53, 123).

The role of hyperphosphatemia as a significant risk factor in the development of vascular calcification may be related to the role of sodium-dependent phosphate transporters (59). Of the three types of sodium-dependent phosphate transporters, type I and II are expressed primarily in the kidney and the intestinal epithelium and play a large role in phosphate homeostasis in the body. In contrast, the type III sodium-dependent phosphate transporters, Pit-1 and Pit-2, are expressed ubiquitously in cells, including in the kidney, brain, heart, lung, and liver as well as osteoblasts and VSMC. They are thought to be involved in local and intracellular phosphate homeostasis (118, 125). Pit-1 and Pit-2 both likely play a role in pathological calcification.

VSMC cultured in high-phosphate media have been shown to undergo osteochondrogenic differentiation and calcify in vitro. This is dependent on the actions of Pit-1 and was demonstrated when the transporter was knocked down in VSMC using small interfering (si) RNA. These VSMC, cultured in high-phosphate media, demonstrated significantly less sodium-dependent phosphate uptake and calcium deposition. The same study confirmed that Pit-1 was necessary for the phosphate-induced phenotypic change in the VSMC, as the cultured cells treated with Pit-1 siRNA did not have increased levels of Cbfal (Runx2) or OPN mRNA when grown in a high-phosphate environment (60). In the presence of elevated phosphate, Pit-1 has been shown to cause phosphorylation of Erk 1/2, which in turn leads to the osteogenic phenotype conversion seen before matrix mineralization (35).

VSMC-specific Pit-1 knockdown mice underwent partial renal ablation to produce CKD and were then placed on a high-phosphate diet. Further investigation revealed that Pit-2 had been upregulated in these VSMC in compensation for the loss of Pit-1. Phosphate uptake and phosphate-induced calcification were restored with overexpression of Pit-2 in Pit-1 knockdown cultured VSMC, and similarly knockdown of Pit-2 in the VSMC of Pit-1-deficient mice decreased phosphate transport and phosphate-induced calcification (15). A better understanding of the functions of Pit-1 and Pit-2 will undoubtedly lead to a new understanding of the mechanism of vascular calcification in CKD.

Even before the development of hyperphosphatemia, abnormal levels of the counterregulatory hormones parathyroid hormone (PTH) and FGF-23 are involved in phosphate homeostasis and contribute to CKD-mineral bone disease (MBD) (105). FGF-23 has recently emerged as a major phosphaturic hormone, and clinical trials demonstrate an association between FGF-23 levels and vascular calcification in both healthy and CKD populations (18, 99). However, the addition of FGF-23 to cultured human VSMC did not increase the calcium content of the cells in either control or high-phosphate media (101). It is not yet clear whether FGF-23 is a causative agent of calcification or simply a biomarker of disease. PTH functions to maintain normal serum calcium and phosphorus levels, and it too may play a role in vascular calcification. Elevated PTH levels are one of the main factors associated with progression of vascular calcification in hemodialysis patients, and treatment of secondary hyperparathyroidism with calcimimetics prevents progression of calcification and may actually attenuate vascular calcification in this population (47, 90, 121). In vitro, rats treated with PTH infusions develop significant vascular calcification independently of diet or renal function, which suggests that PTH is a direct or indirect cause of vascular calcification (74).

**Failed anticalcific mechanisms.** Under normal physiological conditions, there exist a multitude of regulatory molecules that act as inhibitors of mineral formation to prevent widespread tissue calcification. In patients with CKD, levels or function of these inhibitors may be abnormal, which in turn can predispose to or accentuate ectopic calcification (73). One of these inhibitors is matrix Gla protein (MGP). Expressed in bone, cartilage, kidney, lung, heart, and smooth muscle cells, MGP acts to bind calcium ions and clear excess calcium, as well as bind calcium crystals and inhibit crystal growth (86). Calcium binding to MGP is weakened by excess phosphate ions, and MGP expression is downregulated with vitamin D deficiency, both of which are seen in CKD (24, 95). Vitamin K acts as a cofactor for this process. Vitamin K antagonists, such as warfarin, are used commonly in CKD patients and interfere with the generation of active MGP (88). Adenine-induced CKD mice treated with therapeutic doses of warfarin develop significant vascular calcification, and warfarin use is associated with vascular calcification in the hemodialysis population (48, 69). Circulating levels of dephosphorylated uncarboxylated (inactive) MGP have been found to increase with worsening stages of CKD, and these elevated levels are associated with the severity of aortic calcification. Measuring this inactive form of MGP could prove to be a useful marker of cardiovascular disease in patients with CKD (100).

Another important inhibitor of calcification is fetuin-A. This glycoprotein is synthesized in the liver and is normally present in high levels in the circulation, where it binds calcium ions...
and hydroxyapatite. It is found in skeletal bone and teeth, where it plays a key role in bone formation and resorption (98). Fetuin A has been shown to decrease apoptosis of VSMC and is also loaded into matrix vesicles, decreasing the formation of intravesicular basic calcium phosphate crystals (93). Mice deficient in fetuin A show increased susceptibility to widespread calcification, and fetuin A added to bovine VSMC cultured in known calcification media inhibited calcification in a dose-dependent fashion (21). It was originally hypothesized that since fetuin A is a negative acute-phase reactant, the inflammatory state seen in dialysis patients causes the serum levels of fetuin A to decrease, and this further precipitates soft tissue calcification (22, 54). However, other studies have not demonstrated a clear indirect relationship between fetuin A levels and the degree of calcification in the CKD or hemodialysis populations (40, 50, 70). This discrepancy may be due to the presence of calciprotein particles, which will be discussed later in this review.

OPN, a phosphoprotein, is normally found in bones and teeth and acts as a regulator of mineralization by inhibiting apatite crystals and promoting osteoclast function (97). Not normally found within the vasculature, it is seen at high levels in calcified arteries and potentially is upregulated to counteract the advancement of vascular calcification (51). OPN null mice do not spontaneously calcify, but calcification is synergistically enhanced when these mice are bred with MGP null mice. OPN is present in high levels in the arteries of calcifying MGP knockout mice, and its absence in OPN MGP double knockout mice contributes to significantly increased vascular calcification because of the loss of two inhibitors of mineralization (27). While it has been shown that plasma levels of OPN can be significantly elevated in CKD patients compared with controls, plasma levels do not seem to directly correlate with vascular calcification in the CKD population (7). This could be due to the fact that OPN is an important regulatory molecule in many other physiological processes, including inflammation, chemotaxis, immune cell activation, and apoptosis as well as in smooth muscle cell remodeling. How this multifunctional molecule contributes to calcification in various stages of CKD is intriguing and is still being investigated.

Pyrophosphate (PPi), a natural inhibitor of hydroxyapatite formation, is normally produced by VSMC and is typically found in plasma at sufficient levels to prevent vascular calcification. Circulating PPi’s role as an inhibitor of vascular calcification was demonstrated very clearly when aortas were transplanted between wild-type (WT) mice and those lacking ectonucleotide pyrophosphate phosphodiesterase (ENPP1), the enzyme responsible for the generation of PPi. Aortas from ENPP1 knockouts transplanted into WT mice showed no further calcification, and WT aortas placed into ENPP1 knockout mice developed significant calcification (63). Humans lacking pyrophosphate have extensive arterial calcification early in life. This is seen in the genetic disease idiopathic infantile arterial calcification, an autosomal recessive condition that leads to a loss of function of ENPP1. Children with this disease often die within the first 6 mo of life due to heart failure (30). Pseudoxanthoma elasticum (PXE), another heritable mineralizing disease in humans, is associated with calcifications of the eyes, skin, and arteries. It has been known that PXE is due to mutations in ATP-binding cassette subfamily C member 6 (ABCC6), an ATP-dependent efflux transporter present in the liver. New evidence in mouse models has revealed that ABCC6 is responsible for transporting PPi into the circulation, and the lack of plasma PPi causes the extensive mineralization in PXE (45). Even though PPi is normally cleared by the kidneys, ESRD patients on hemodialysis have been found to have low circulating levels of PPi. This is explained in part by the fact that PPi is removed by dialysis, but there is likely decreased production or increased extrarenal metabolism of PPi in CKD (62).

Why patients with CKD develop demineralizing bone disease and vascular calcification (dubbed the “calcification paradox”) may not only be related to abnormal systemic mineral balance but may also be caused by dysfunction of local mineral homeostasis. In normal bone, the skeletal mass is regulated by a dynamic balance of continual bone production (performed by osteoblasts) and bone resorption (performed by osteoclasts) under the control of the RANK/RANKL/OPG signaling pathway. RANK is a type I membrane protein receptor found on the surface of osteoclast precursors that, when activated, stimulates osteoclastogenesis and osteoclast activity (43). Its activating ligand is RANKL, a type II membrane protein and member of the TNF superfamily, which is expressed by osteoblasts (25). RANKL production is upregulated by PTH and calcitriol (44, 120). Osteoprotegerin (OPG), another member of the TNF family, is a potent inhibitor of osteoclast differentiation. OPG binds to RANKL and prevents this ligand from binding to its receptor, RANK (127). OPG is produced by osteoblasts and other cell types, including those of the arterial cell wall, and is a key protein involved in regulation of skeletal mass and turnover (82). It is also involved in vascular calcification, as OPG knockout mice develop spontaneous vascular calcification along with osteoporosis. Several studies demonstrate that RANKL added to cultured VSMC stimulated calcification. These studies suggest that RANKL stimulates vascular calcification while OPG plays a protective role (19, 80). In humans, calcified arteries show decreased levels of OPG by RT-PCR (122). However, in CKD and hemodialysis patients plasma levels of OPG are increased 1.4–1.9 times above normal, and levels directly correlate with degree of coronary artery calcification (71). While RANKL and OPG likely play a role in vascular calcification, the cause and effect relationship of these molecules in the setting of CKD is still unclear.

**Emerging Concepts in Vascular Calcification**

A new look at extracellular vesicles. The participation of extracellular, membrane-bound vesicles in biomineralization has long been recognized. Matrix vesicles (MV), originally discovered in 1967, are now considered an integral component of normal skeletal bone formation (4). While the role of MV is well established in bone osteogenesis, new evidence is demonstrating that smooth muscle cells as well as leukocyte-derived MV may also play a role in vascular calcification. In addition, other types of extracellular vesicles have been proposed to participate in vascular calcification including apoptotic bodies and ectosomes. Evidence supporting a potential role for these particles in vascular calcification is summarized below.

MV have been identified in both atherosclerotic plaques associated with intimal calcification and in nonatherosclerotic vessels associated with arterial medial calcification (119).
These submicroscopic particles with a size of 30–300 nm are released from VSMC and, like their bone counterparts, contain the proteins, lipids, and microRNAs necessary to actively propagate the calcification cascade. They contain the same enzymes such as tissue nonspecific alkaline phosphatase which are critical in altering the local microenvironment to precipitate ectopic calcification (75). These pathological MV are also dependent on annexins II, V, and VI for mineral deposition identical to MV from normal osteoblasts and chondrocytes (12). The MV ultimately form hydroxyapatite crystals, which are deposited on a collagen matrix.

MV are released under different circumstances in the patient with CKD; elevated calcium and phosphate levels have been shown to induce release of MV from cultured VSMC. Elevated calcium levels also enhance the mineral formation from these MV (52). In addition, recent evidence suggests that autophagy may regulate MV cargo and formation (103). When autophagy was inhibited, phosphate-treated VSMC released more MV, and these particles had increased alkaline phosphatase activity, suggesting that they may have enhanced calcific potential. While this needs to be validated in vivo, it is hypothesized that CKD patients, who may have a disturbance of induction or maintenance of autophagy, may have exacerbated calcification due to the increased quantity of MV released along with increased calcific potential of those MV (17).

Finally, new evidence is demonstrating that macrophages, integral components of atherosclerotic plaques, may also play a role in the biogenesis of procalcific microparticles with properties similar to MV. Cultured macrophages that have been stimulated with calcium and phosphorus produce extracellular vesicles that are fully capable of mineralizing matrix. While it is unclear whether macrophages are directly associated with arterial medial calcification, this is hypothesized to be a new mechanism which could contribute to early intimal microcalcifications in the CKD population (76).

A second type of extracellular vesicle, the apoptotic body, is known to contribute to vascular calcification in CKD. Elevated phosphorus is believed to be responsible for VSMC apoptosis via inactivation of Bcl-2 and activation of the Bcl-2-associated death promoter (BAD). This proapoptotic protein causes activation of caspase-3 and results in cell death (112). In vitro, elevated calcium levels have also been shown to cause VSMC apoptosis, and there is synergy of elevated phosphorus and calcium in inducing apoptosis (92). The apoptotic bodies released from these dying cells can then propagate vascular calcification in one of several ways. The apoptotic bodies have been shown to accumulate very high levels of intracellular calcium and can act as a direct nidus for calcification, an effect which is increased in the presence of calcium and phosphorus. Also, the calcium released at the vessel wall of apoptotic cells markedly increases the calcium concentration of the local microenvironment, which can be taken up by local VSMC, raising the intracellular calcium level of these surrounding cells. In fact, when VSMC are cultured in vitro, inhibiting apoptosis significantly reduces the degree of calcification (89).

A third type of extracellular vesicle, the ectosome (or microparticle), is plasma membrane derived and 50–1,000 nm in size, with significant procoagulant activity due to exposed phosphatidylserine and tissue factor (8, 67, 77). Microparticles are found in atherosclerotic plaques, and circulating levels increase early in the development of atherosclerosis (13, 58). It has also been shown that elevated levels of microparticles are seen in patients with ESRD and may be predictive of cardiovascular mortality (3). The reason for this is not yet entirely clear, but in the ESRD patient population circulating endothelial microparticles directly correlated with aortic pulse wave velocity and the carotid augmentation index (2). As far as microparticles’ role in vascular calcification, one study has demonstrated that a high number of circulating microparticles are seen in menopausal women with coronary calcification and the level of microparticles is directly correlated with the calcium score (46). Further mechanistic properties of these microparticles need to be determined to understand whether they are involved in the medial vascular calcification seen in CKD.

**MicroRNAs and vascular calcification.** Discovered about 20 years ago, microRNAs (miRNAs; miR) are now known to be an essential constituent of gene expression and protein synthesis. The main role of these small (~22-nucleotide) noncoding RNAs in gene regulation is their posttranscriptional repression of target mRNAs, which can occur via translational repression, mRNA degradation, or mRNA alteration. As more is learned about these small epigenetic regulatory molecules, it is no surprise that miRNAs have been discovered to play an essential role in vascular calcification.

Many miRNAs are involved in vascular calcification, and they have been implicated in all stages of the calcification process. miRNA-135a, miRNA-762, miRNA-714, and miRNA-712 were all found to be upregulated in klotho homozygous mutant mice that demonstrated significant vascular calcification. The calcium efflux proteins NCX1, PMCA1, and NCKX 4 have been identified as potential targets of these miRNAs, and thus would be transcriptionally downregulated when these miRNAs were active, potentially enhancing intracellular calcium loading in the cells and increasing calcification potential. Indeed, when inhibitors of these identified miRNA were added to VSMC cultured in a high-phosphate, high-calcium medium the degree of intracellular calcium and calcification decreased (34).

miR-133a is known to suppress Runx2 and impede the osteoblastic differentiation of mesenchymal cells and has now been shown to play a role in the regulation of the osteogenetic differentiation of cultured VSMC. When VSMC overexpress miR-133a in the presence of β-glycerophosphate (which induces osteogenic differentiation), these cells had decreased alkaline phosphatase activity, osteocalcin secretion, and Runx2 expression along with decreased evidence of mineralization. Similarly, inhibition of miR-133a was found to increase alkaline phosphatase activity, osteocalcin secretion, and Runx2 expression (61). Similarly, miR-204 has been shown to suppress the osteoblastic differentiation of mesenchymal progenitor cells by downregulating Runx2. A separate study using cultured VSMC demonstrated that VSMC overexpression of miR-204 with β-glycerophosphate caused decreased levels of alkaline phosphatase, osteocalcin, and Runx2 protein. Inhibition of miR-204 caused opposite effects. To test the effect of miR-204 in vivo, mice were treated with vitamin D3 to induce calcification and simultaneously with a miR-204 agonir (miRNA mimic). In these mice, Runx2 was not upregulated and aortic calcification was similar to controls (16). Similarly, miRNA-30b and miRNA-30c (which target and downregulate Runx2) were shown to be downregulated by bone morphoge...
nomic protein-2 (BMP-2), and the loss or knockdown of these miRNAs in vivo increased Runx2 expression and calcification (6). miR-21 hasn’t been directly related to vascular calcification, but several studies have shown that it does seem to play an important role in maintenance of VSMC phenotype and injury response (49).

Independent of Runx2 expression, miR-221 and miR-222 synergistically act as promoters of VSMC transdifferentiation and calcification. This was demonstrated on examination of cultured mouse aortic smooth muscle cells. Levels of miR-221 and miR-222 were significantly downregulated when smooth muscle cells were treated with a procalcific medium. It was further shown that these two miRNAs may promote calcification via their actions on ENPP1 and Pit-1, causing alterations in phosphorus regulation (66).

miR-223 is key component in osteoclast differentiation, and increased levels of this miRNA have been found in damaged skeletal muscle cells and cardiomyocytes. VSMC cultured in high-phosphate media show significant upregulation of miR-223, and similar elevations are seen in ApoE−/− mice. The reported targets of miR-223 (Mef2c and RhoB) are known to play a role in VSMC contractility and differentiation. In this same study, levels of miR-143 and miR-145 are downregulated in the ApoE−/− mice (91). These have both been shown to be involved in the regulation of the VSMC contractile phenotype via their targets PHACTR4, CFL1, and SSH2 (39).

miR-29a/b regulates the vascular calcification cascade by downregulating ADAMTS-7. This metalloproteinase degrades cartilage oligomeric matrix protein (COMP), and COMP deficiency leads to vascular calcification by loss of its inhibitory effect on BMP-2 osteogenic signaling. miR-29a/b levels were found to be depressed in nephrectomized rats treated with calcitriol to induce vascular calcification, and the overexpression of miR-29a/b in high phosphate-stimulated VSMC in vivo decreased calcification (20).

The target mRNA for miR-125b is not entirely clear, but it has been demonstrated that human coronary artery smooth muscle cells grown in osteogenic media and calcified aortas from ApoE−/− mice have decreased levels of miR-125b. The addition of anti-miR-125b to the cultured human cells increased alkaline phosphatase activity, Runx2 expression, and matrix mineralization (31). Calcification of cultured rat aortic smooth muscle cells grown in a high-phosphate medium was decreased when these cells were treated with miR-125b mimics. This in vitro finding may be due to a significant reduction in protein expression of the multifunctional transcription factor Ets1 by miR-125b (124).

Human studies in this area remain very limited. Patients with CKD stage 3 and 4 have decreased circulating levels of several miRNAs involved in the VSMC phenotype including miR-125b, miR-145, and miR-155 (9). While this study did not investigate vascular calcification, the abnormalities in miRNA may play a role in initialization and propagation of the calcification pathway in the CKD population. As more is discovered about the role of miRNAs in the pathophysiology of vascular calcification, this may prove to be both an effective biomarker and therapeutic target for patients with CKD. The microRNAs known to play a role in vascular calcification are summarized in Table 1.

### Table 1. Abnormalities of microRNA in vascular calcification

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<th>miRNA</th>
<th>Levels in Vascular Calcification</th>
<th>Potential Target(s)</th>
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<tr>
<td>miR-29a</td>
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<td>ADAMTS-7</td>
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<tr>
<td>miR-29b</td>
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<td>ADAMTS-7</td>
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<td>miR-30b</td>
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<td>Runx2</td>
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<tr>
<td>miR-30c</td>
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<td>Runx2</td>
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<tr>
<td>miR-125b</td>
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<td>Ets1</td>
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<tr>
<td>miR-133a</td>
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<td>Runx2</td>
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<td>miR-135a</td>
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<td>NCX1, PMCA1, NCKX4</td>
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<td>↑</td>
<td>Mef2c, RhoB</td>
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<td>miR-712</td>
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<td>miR-762</td>
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<td>NCX1, PMCA1, NCKX4</td>
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More to fetuin than we thought: the discovery of calciprotein particles. Fetuin was isolated over 50 years ago and has long been known to bind calcium with strong affinity and act as an inhibitor of mineralization (98, 116). Only over the past decade has it become clear that fetuin’s role in ectopic calcification involves the formation of fetuin mineral complexes, otherwise known as calciprotein particles. These complexes were first discovered in mice treated with high-dose etidronate. Shortly after administration of the bisphosphonate, serum calcium and phosphorous levels became markedly elevated, but ionized calcium levels did not increase. Further investigation revealed that the elevated calcium and phosphorous levels were due to the formation of a serum mineral-protein complex that contained calcium, phosphorous, and large amounts of fetuin (85). A large fraction of circulating fetuin becomes associated with these complexes and clearance of these complexes from the bloodstream is seen over time, which coincides with decreasing serum fetuin levels (36, 84). Follow-up studies demonstrated that fetuin’s capability to inhibit mineralization is due to its ability to form these colloidal complexes, dubbed calciprotein particles (CPP), and not simply to its binding to ionized calcium (36, 83).

Further characterization has determined that CPPs are formed in a two-stage process. The first stage consists of a spontaneous development of an amorphous particle with a diameter of ~500 Å. This primary CPP slowly transforms into a secondary CPP, which is elongated and spherical in shape with a diameter of ~1,000 Å. The structure of the secondary CPP is more complex, consisting of an octacalcium phosphate mineral core surrounded by a high-density fetuin-A monolayer (38). A nanoparticle-based assay to measure calcification propensity has been developed based on timing the transformation (APWV), and on longitudinal follow-up was independently associated with increased risk of all-cause mortality (110). CPPs act to remove excess mineral from circulation and may be a defense mechanism against ectopic calcification. Similar to lipoprotein particles, the CPPs are taken up by the reticu-
loendothelial system for removal from circulation. Mice injected with CPP demonstrated that systemic CPPs are taken up by Kupffer cells in the liver and marginal zone macrophages in the spleen. This process is quite rapid, with over half of CPPs cleared by 10 min (41). However, they are also likely involved in potentiating vascular calcification, especially in pathological states such as CKD. Naked calcium phosphate crystals at levels of 40 μg/ml strongly stimulate secretion of TNF-α and IL-1β from macrophages, and uptake of calcium phosphate crystals at similar levels by macrophages induces oxidative stress and apoptosis. Compared with basic calcium phosphate in vitro, CPPs markedly decrease the inflammatory cytokine expression and oxidative stress markers in the macrophage. However, high concentrations of CPP in a dose-dependent manner stimulate inflammatory cytokines and cause apoptosis in macrophages, especially when the levels of CPPs are greater than 80 μg/ml (111). While this has yet to be linked to vascular calcification in vivo, it is conceivable that circumstances such as CKD with increased CPP production or decreased clearance could predispose to a proinflammatory and proapoptotic condition ideal for exacerbation of ectopic mineralization.

Animal and human studies are demonstrating a likely clinical association between CPP and vascular calcification, particularly in the CKD population. Rats with vitamin D-induced arterial calcification demonstrated a significant correlation between the degree of calcification and serum CPP levels along with an absence of CPPs in control rats (87). Rats with renal failure secondary to adenine administration develop vascular calcification, which coincides with the appearance of CPPs. Again, these mineral complexes are not detected in control rats (68). These animal studies have been further supported by human clinical studies in multiple scenarios. The first was the detection of CPPs in the effluent of a peritoneal dialysis patient who was diagnosed with calcifying peritonitis (37). In a cohort of 200 predialysis CKD patients, serum CPP levels were strongly associated with APWV and although direct calcification was not examined, increased APWV is often due to arterial medial calcification in the CKD population (108, 110). In a study of 12 hemodialysis patients and 73 nondialysis CKD patients, the fetuin-A reduction ratio (RR; the ratio of CPP to total fetuin) correlated with coronary artery calcification scores (CACS) while total fetuin levels did not (35). An analysis of CKD stages 3 and 4, hemodialysis, and peritoneal dialysis patients demonstrated higher levels of fetuin-A RR in dialysis patients compared with CKD patients. The highest levels were seen in patients clinically diagnosed with calciphylaxis (calcific uremic arteriolopathy), a severe form of vascular calcification that is accompanied by thrombosis and skin necrosis (109). Although more information still has to be gathered regarding the role of CPPs in CKD-associated vascular calcification, it is likely that they will be a useful tool in the future both as a marker and therapeutic adjunct in the treatment of vascular calcification.

**Conclusion**

High cardiovascular morbidity and mortality rates continue to be a very large burden in the CKD and ESRD patient population. Vascular calcification contributes to this cardiovascular disease, and currently there are very limited treatment options for either the prevention or treatment of vascular calcification in CKD. At present, prevention and management of hyperphosphatemia is the cornerstone of treatment. Even with dietary phosphorus restriction and the emergence of well-tolerated, safe non-calcium-based binders, vascular calcification and cardiovascular disease are still prominent in patients with CKD (113). Despite the fact that much insight has recently been gained into the mechanisms of ectopic calcification, more investigation and comprehension of this complicated process are needed. Only with better understanding of the pathophysiology of vascular calcification will effective therapeutic options be possible for the prevention and treatment of this complex disease.

**REFERENCES**


Review

NEW INSIGHTS INTO VASCULAR CALCIFICATION IN CKD


