Calcification and Cardiovascular Health
New Insights Into an Old Phenomenon

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Uremic cardiovascular disease is characterized by accelerated calcifying atherosclerosis and valvular heart disease. Vascular calcification develops at 2 different sites within the vessel wall. Although intimal plaque calcification is a feature of genuine atherosclerosis, medial calcification is restricted to the smooth muscle cell layer and especially to the elastic laminae of arterial vessels (Figure 1). Both entities can be frequently observed in chronic kidney disease (CKD) patients. Dialysis patients with intimal calcifications are elderly and characterized by a history of “traditional” risk factors (eg, smoking and dyslipidemia) before the start of dialysis, whereas those with medial calcifications are, on average, 20 years younger and characterized by a longer time on dialysis treatment and a higher incidence of derangements in their calcium (Ca) balance. Another recent study in incident dialysis patients showed that those with rapid arterial calcification progress already had calcified coronary arteries before reaching the dialysis stage. This emphasizes that diagnostic, preventive, and therapeutic measures need to be initiated in early CKD stages. The clinical importance of this notion is stressed by a number of reports demonstrating that coronary artery and valvular calcifications occur prematurely and are very prevalent in dialysis patients and that they are independent risk factors of cardiovascular death in this patient group. Such calcifications can, therefore, serve to at least partially explain why cardiovascular mortality is dramatically increased in the uremic as compared with the nonuremic patient population, and why it is not appropriately explained by the traditional Framingham risk factors. One of the mechanisms by which medial vascular calcification feeds into cardiovascular mortality may be via the associated increase in aortic pulse wave velocity. Calcified arteries become stiffer, causing quicker return of the systolic pulse wave from the periphery, thereby increasing left ventricular afterload. Through this mechanism, a high aortic pulse wave velocity is associated with increased left ventricular mass.

Based on registry data and cross-sectional analyses, an increased Ca×P product because of hyperphosphatemia and/or hypercalcemia (derived from a positive Ca balance, eg, Ca-containing P binders) is thought to be a key determinant of cardiovascular mortality and progression factors of unwanted calcifications in uremia. In 2 studies focusing on young dialysis patients with childhood-onset renal disease, coronary artery calcifications (quantified by electron-beam computed tomography) were highly prevalent in the age group of 20- to 40-year-old patients and associated with an increased Ca×P product, high parathyroid levels, an increased Ca intake, and inflammation. However, a few reports could not demonstrate associations between the extent of calcifications and hyperphosphatemia or an increased Ca×P product. This may relate to the difficulty of detecting an association between a long-term event, such as extrasosseous calcification, on the one hand, and rapidly changing serum parameters, such as P, Ca, or intact parathyroid hormone levels, on the other hand. In addition, as discussed below, in the “inflamed patient,” serum may not be the right location to assess the risk for calcification, because Ca and P may rapidly deposit in tissues resulting in a “pseudonormal” Ca×P product.

For a long time, the view was held that extrasosseous calcification in dialysis patients results from supersaturation of serum with Ca and P ions, that is, passive precipitation. In serum, as compared with an aqueous solution, the chemical solubility of Ca and P ions is much better, and solubility of these ions is partly achieved by body temperature, pH, and the ionic strength (NaCl concentration) of serum. However, serum still is a “metastable” Ca and P solution, and if the numerical chemical solubility product of Ca×P dramatically exceeds the ion concentrations in biological solutions in mammals, then precipitation of Ca and P must be actively prevented in physiological conditions. Based on these brief considerations, 3 pathways, which are not mutually exclusive, may contribute to unwanted extrasosseous calcification in CKD (Table): (1) true passive precipitation of Ca and P in the presence of excessively high extracellular concentrations, (2) the presence or upregulation of inducers of cellular osteogenic transformation and hydroxyapatite formation, and (3) deficiency of calcification inhibitors.

The present review focuses on the discussion of those factors for which current data point to a potentially significant clinical role. However, it needs to be stressed that additional factors modifying calcification processes were identified, including fibrillin-1, carbonic anhydrase, bone morphogenic...
proteins-2 and -7, and others, but their role in clinical disease states currently remains less well defined.

Calcification Induction: Vascular Smooth Muscle Cell Osteogenic Differentiation by P and Ca

Hyperphosphatemia is one of the most potent independent predictors of cardiovascular mortality in dialysis patients. It was believed that this observation relates to progressive cardiovascular calcification in hyperphosphatemic patients with a raised Ca×P product leading to passive hydroxyapatite formation. Although such passive precipitation might indeed contribute to tumor-like soft tissue calcifications in dialysis patients (an example of severe periarticular calcifications in the hip region of a dialysis patient imaged by skeletal scintigraphy is shown in Figure 2), recent in vitro studies suggest that the key process in vascular, in particular, medial calcification seems to be an active, cell-mediated event. By raising P concentrations in cell culture media of vascular smooth muscle cells (VSMCs), deposition of hydroxyapatite and a phenotypic switch of VSMCs into osteoblast-like cells was induced. However, blocking cellular P entry by inhibitors of the sodium-P cotransporter, PTT-1, was able to prevent osteogenic differentiation and, more importantly, hydroxyapatite formation despite the continued presence of a high P extracellular milieu. Thus, high intracellular P in VSMCs induces osteogenic differentiation, that is, the formation of matrix vesicles and de novo expression of bone-related proteins, such as alkaline phosphatase, osteopontin, osteocalcin, and collagen I. Of note, high Ca conditions in vitro act synergistically with hyperphosphatemia in inducing osteoblast-like transformation of VSMCs. The combination of supraphysiological Ca and P concentrations in the cell culture medium potently stimulated matrix vesicle and apoptotic body formation, which both serve as the imminent calcification matrices.

Calcification Inhibition: Fetuin-A Is the Key Systemic Inhibitor

Fetuin-A (α2-Schmid Heremans glycoprotein) is a hepatocyte-derived serum protein (molecular weight, ~60 kDa). Serum concentrations are high, with levels between 0.5 and 1.0 g/L in average populations (note that currently available assays differ to some degree regarding their normal ranges). Fetuin-A has been known for a long time to represent the most prominent component of the α2-band of the serum electrophoresis. In the late seventies, Lebreton et al showed that fetuin-A levels were inversely related to episodes of severe acute infections and, thus, negatively predict acute-phase reactions. Fetuin-A may act as a soluble transforming growth factor-β antagonist (by possessing a transforming growth factor-β–receptor II–like domain) and may interfere with insulin receptor autophosphorylation and tyrosine kinase activity leading to insulin resistance.

The dominant biological function of fetuin-A is its calcification inhibitory property that potently limits hydroxyapatite crystal formation. It is estimated that fetuin-A is responsible for about half of the precipitation inhibitory properties within the extracellular space. Fetuin-A molecules have been
shown to form stable colloidal spheres with Ca and P, so-called calciprotein particles, in ex vivo settings.\(^{20}\) Related to this finding in a cell-free environment, experimental data from studies of etidronate-treated rats demonstrated fetuin-A as the main part (80%) of a high molecular mass complex further consisting of Ca×P mineral (18%) and matrix Gla protein ([MGP] 2%).\(^{21}\) Calciprotein particles possibly fulfill clearance functions for small calcification nuclei. In addition to the function of fetuin-A in calciprotein particles, in vitro studies in VSMCs undergoing osteogenic differentiation have shown that fetuin-A is taken up by these cells and inhibits both formation, as well as intracellular calcification, of matrix vesicles before their extrusion.\(^{22}\)

The above biochemical properties of fetuin-A were confirmed by genetic deletion of the fetuin-A gene in mice (fetuin-A\(^{-/-}\) mice). Such mice developed osteosclerotic calcifications, but the severity and distribution of calcifications depended on the genetic background of the animals.\(^{23}\) Fetuin-A\(^{-/-}\) mice on a C57Bl/6 background showed a relative resistance against calcification requiring additional stimuli, such as active vitamin D treatment, to develop soft-tissue hydroxyapatite deposition.\(^{23}\) In contrast, fetuin-A\(^{-/-}\) mice on a DBA/2 background spontaneously developed severe and progressive soft-tissue and organ calcifications.\(^{23}\) The reasons underlying this genetic disparity are presently unknown but may be related to the presence of hypomagnesemia in the latter strain. One of the prominent and functionally relevant features of the fetuin-A\(^{-/-}\) DBA/2 strain is myocardial calcification leading to a phenotype of "myocardial stiffness" characterized by cardiac fibrosis, diastolic dysfunction, impaired tolerance to ischemia, and catecholamine resistance, as measured in isolated hearts (Langendorff apparatus) by ex vivo echocardiography.\(^{24}\) Whether dystrophic calcification in uremic patients causes similar functional disturbances has not yet been examined.

**Fetuin-A Deficiency: Studies in CKD Patients**

Because cardiovascular calcifications are so highly prevalent in dialysis patients and associated with poor survival, it seemed tempting to hypothesize that a lack of calcification inhibitors may predict mortality. Indeed, in a cohort of \(>300\) hemodialysis patients, the lowest tertile of serum fetuin-A levels was associated with a significantly increased all-cause and cardiovascular mortality.\(^{25}\) As expected, fetuin-A was also inversely correlated with C-reactive protein levels underlining its nature as a negative acute-phase reactant.\(^{25}\) To demonstrate the functional calcification-inhibitory capacity of fetuin-A deficiency, we used sera from selected dialysis patients of this cohort and from a small group of calciphylaxis (see below) patients in an ex vivo \(^{45}\)CaCl\(_2\)-radioisotope assay, which enables quantification of serum-induced inhibition of Ca×P precipitation. Sera from these patients were significantly less effective at inhibiting Ca×P crystal formation than normal serum with appropriate fetuin-A concentrations, and this lack of efficacy could be reversed by the addition of purified fetuin-A in quantities that restore serum levels to normal.\(^{23,25}\)

Stenvinkel et al\(^{26}\) recently confirmed the mortality risk prediction by fetuin-A deficiency in \(\sim300\) incident dialysis patients. In addition, a specific fetuin-A point mutation (Thr256Ser) was described in this cohort and was shown to predict particularly low fetuin-A levels and to be associated with an adverse prognosis compared with patients carrying alternative polymorphisms.\(^{26}\) Hypoalbuminemia was strongly correlated with fetuin-A deficiency, suggesting an involvement of both factors in the so-called malnutrition–inflammation–atherosclerosis syndrome.\(^{26}\) In prevalent peritoneal dialysis patients, fetuin-A deficiency was also linked to features of the malnutrition–inflammation–atherosclerosis syndrome and to both cardiovascular events and mortality.\(^{27}\) Moreover, this study demonstrated an association between low fetuin-A levels and the magnitude of valvular calcification.\(^{27}\) In a smaller cohort of hemodialysis patients, Moe et al\(^{28}\) showed a significant association between coronary calcification and fetuin-A deficiency, and the same relationship was recently published in a study investigating fetuin-A levels and aortic calcification.\(^{29}\) The statistical significance of these associations between low fetuin-A levels and calcifications was...
relatively weak, but still these results should be regarded as quite remarkable, because calcification is a slow, progressive process with an unknown starting point (probably in early CKD phases, see Reference 2), and fetuin-A levels may fluctuate on a short-term basis. Taken together, these observations indicate that fetuin-A deficiency may, indeed, be an important inflammation-related link between cardiovascular calcification and mortality in dialysis patients.

In patients with normal renal function, as well as in predialysis CKD patients, the available information on the relationship among fetuin-A levels, the degree of calcification, and mortality is currently limited. In ~1000 patients from the Heart and Soul study focusing on cardiovascular risk patients, mostly without renal dysfunction, high fetuin-A levels were found to be strongly associated with hyperlipidemia and features of the metabolic syndrome but not with outcome parameters.30 Mehrotra et al31 studied patients with diabetes mellitus spanning CKD stages 1 to 4. Surprisingly, the magnitude of coronary artery calcification correlated with increased rather than with decreased fetuin-A levels in this cohort. On the one hand, this finding may be specific for a diabetic cohort, where fetuin-A levels may also be linked to insulin resistance.19 On the other hand, these data may imply that fetuin-A upregulation initially acts as a systemic defense mechanism ("early warning system") trying to protect from or counteract against vascular calcifications in their early stages. Such an interpretation is indirectly supported by immunohistochemical findings showing strong fetuin-A deposition, but not synthesis, in areas of vascular calcification.28 When in chronic long-term uremia the calcification burden finally increases beyond a certain point, compensatory systems, such as fetuin-A release, may become exhausted, and fetuin-A deficiency may start a vicious cycle of even more progressive extraosseous calcification and fetuin-A consumption. If this hypothesis of initial adaptive fetuin-A overproduction and later exhaustion of the system is correct, future research should attempt to identify factors influencing fetuin-A expression and secretion. Currently, very few of such factors are known, but, based on preliminary observational and in vitro work, may include insulin, lipids, or steroid hormones as inducers and some uremic toxins or cytokines as suppressors.

Calcification Inhibition: MGP and Protection of the Medial Layer
MGP belongs to a family of N-terminal γ-carboxylated (Gla) proteins which require a vitamin K–dependent γ-carboxylation for their biological activation.16,32,33 MGP is a pivotal inhibitor of cartilage and arterial calcification.16,32–34 Major progress in the understanding of MGP actions was gained by the investigation of mice deficient for MGP (MGP−/−). The phenotype of these mice is characterized by severe medial calcification of the aorta leading to lethal ruptures of the bone-like aorta within weeks after birth.34

High local MGP expression is usually found in the vicinity of atherosclerotic plaques, especially in the lipid-rich regions surrounding calcified areas.35 As in the case of fetuin-A, this pattern is interpreted as a local attempt to counteract and limit vascular calcification. Recently, immunohistochemical localization studies of MGP using selective antibodies distinguishing among total, active γ-carboxylated, and inactive undercarboxylated MGP were performed.38 Severely calcified arteries from diabetic patients exhibited a very high proportion of undercarboxylated MGP in close spatial association with calcifications pointing to local or systemic vitamin K deficiency. Blood levels of total MGP were recently measured and found to be inversely correlated with the magnitude of coronary artery calcifications in 115 subjects with suspected coronary artery disease.37 Such measurements may, however, be meaningless, because it is unclear to which degree serum levels reflect activation, tissue production, and deposition. Furthermore, a recent experimental study clearly demonstrated no effect of systemic transgenic MGP overexpression (ApoE promoter) on the vascular calcification phenotype in MGP−/− mice, whereas only VSMC-specific MGP overexpression (SM22α promoter) rescued the MGP−/− mice from calcification.38

In the normal population, 2 pieces of evidence, meanwhile, connect vitamin K deficiency to the risk of vascular calcification. First, the Rotterdam study group identified that a low vitamin K2 (menaquinone) intake was strongly associated with coronary artery disease–related and all-cause mortality and with the severity of aortic calcifications.39 Second, the long-term use of vitamin K–antagonist–based oral anticoagulation in patients with aortic valve disease was associated with higher coronary and valvular Ca scores as compared with a cohort without anticoagulation.40 There is only a small amount of data available concerning the vitamin K and MGP status in patients with CKD. Vitamin K plasma levels are difficult to measure, and it is unclear whether these levels represent the vitamin K pools, for example, in the liver, and, as pointed out above, the value of measuring total MGP plasma concentrations is currently not defined.

However, in a calcification-prone population, such as in patients with CKD, arterial MGP activation may represent a particularly important issue, and the question must be raised as to whether the use of vitamin K–antagonist–based anticoagulation could have potentially harmful consequences. Indeed, warfarin use was already identified as a risk factor for the development of calciphylaxis.41,42 Calciphylaxis (calcific uremic arteriolopathy) is a rare, disastrous, and often lethal manifestation of vascular calcification in CKD patients and is characterized by calcifications of cutaneous arterioles with subsequent exulcerations and superinfection. These considerations caused the Vascular Calcification Work Group of the National Kidney Foundation to put forward efforts to better understand the role of vitamin K antagonists in the vascular disease of CKD patients, including the implementation of a calciphylaxis registry and investigating warfarin use in relation to outcomes based on available registry data.43

Osteoprotegerin: Calcification Inhibitor or Inducer?
Osteoprotegerin (OPG) is a key regulator of osteoclast activation by acting as a soluble decoy receptor scavenging the osteoclast activator receptor activator of nuclear factor κB ligand (RANKL) Figure 3). Increased OPG availability prevents binding of RANKL to its receptor, receptor activator of nuclear factor κB. In genetic knockout animal
models, OPG deficiency not only leads to pronounced osteoporosis but is associated with calcifications of the aorta and of renal arteries.\textsuperscript{44} So far it is unclear whether extraosseous calcifications are induced by the release of excess amounts of Ca and P from the bone by osteoclast activation or whether OPG possesses local calcification-inhibitory properties within the vessel wall similar to MGP.\textsuperscript{44–46} The recently published clinical results concerning OPG in dialysis patients are somewhat puzzling; Nitta et al\textsuperscript{47} demonstrated that OPG levels were elevated when compared with the normal population and that increased rather than low OPG levels were independently associated with the severity of aortic calcification in their study cohort. Furthermore, Morena et al\textsuperscript{48} published a study identifying high OPG levels as a strong and independent predictor of mortality in dialysis patients, whereas low soluble RANKL levels predicted a positive outcome. This study, however, is difficult to interpret, because the sample size was not large, and the observed associations of OPG levels with survival turned out to show a U-shaped curve in patients with signs of inflammation (C-reactive protein cutoff, >12 mg/L) in a multivariate analysis, so both high and low levels predicted impaired outcomes under this condition. However, comparable observations were already made in non-CKD populations in which higher OPG levels also predicted an increased cardiovascular risk.\textsuperscript{49} Thus, the integrated biological influence of OPG on cardiovascular calcifications currently remains somewhat obscure.

**Calcification Inhibition: Pyrophosphates Act as Local Tissue Protectors**

Local pyrophosphate release is regulated by 3 factors: (1) the rate-limiting enzyme ecto-nucleotide pyrophosphatase phosphodiesterase-1 (ENPP-1), (2) the transmembrane transporter progressive ankylosis locus (ANK) encoded by the progressive ankylosis locus, and (3) a membrane-bound tissue nonspecific alkaline phosphatase (TNAP).\textsuperscript{50} Activity of ENPP-1 and intact ANK control and prevent tissue calcification, whereas increased TNAP activity causes local hyperphosphatemia and a concomitant decrease in the local pyrophosphate defense triggering and enhancing calcification (Figure 4).

Genetic ANK deficiency in mice causes periarticular calcifications with secondary progressive inflammatory arthritis, and ENPP-1 deficiency is associated with soft-tissue calcifications, especially in the vicinity of tendons and ligaments.\textsuperscript{51,52} Both models do not spontaneously develop vascular calcifications. Of note, idiopathic infantile arterial calcification, a disastrous and lethal disorder, which leads to ubiquitous vascular calcifications in small children, was identified recently as a pyrophosphate-related calcification disorder caused by a loss-of-function mutation of ENPP-1.\textsuperscript{53} Such patients might benefit from bisphosphonates, thus repleting a pyrophosphate-like molecule.

Lomashvili et al\textsuperscript{54} documented that a relative systemic pyrophosphate deficiency may develop in end-stage CKD patients because of removal of these small molecules during hemodialysis. As in the case of MGP measurements, the
interpretation of this finding is hampered by the fact that we do not know whether circulating pyrophosphate concentrations reflect local production or concentrations in tissues. However, recent reports about benefits of bisphosphonate therapy in cases of calciphylaxis and of bisphosphonate-induced regression of coronary calcification scores in dialysis patients underscore the therapeutic potential of bisphosphonates. However, the bisphosphonate effects on the bone remain a matter of concern in this context, because bisphosphonates block bone turnover and, at least in renal transplant patients, induce adynamic bone disease to a high percentage. Adynamic bone disease, in turn, is characterized by an inability to take up and buffer excess Ca and P concentrations and is a known risk factor for cardiovascular calcifications in dialysis patients.58

Calciﬁcation Inhibition and Induction: Integrated Models of Central Players

We now know that, in addition to supersaturation of Ca and P in biological tissues, factors such as osteogenic differentiation and calcification inhibitors are determinants of protection from the progression of extraosseous calcifications. But is there a hierarchy among procalcifying pathomechanisms? Murshed et al recently attempted to give a complex experimental answer to this question. By using an array of in vitro and in vivo models, the authors created different settings of hyperphosphatemia and hypophosphatemia, collagen matrix availability, and calcification inhibitor deﬁciencies. Their key results can be summarized as described here. First, extracellular P availability, rather than Ca, regulates bone mineralization (in vitro). Second, extracellular P is a key modiﬁer of extraosseous calcifications, in particular if pyrophosphate availability is limited. Extraosseous calcifications could be prevented by hypophosphatemia in MGP- and ANK-deﬁcient mice, and vascular calcifications could be induced de novo by hyperphosphatemia in ENPP1- and ANK-deﬁcient mice, respectively. Third, the localization of calcifications depends on the joint presence of sufﬁcient amounts of P, low amounts of pyrophosphate (eg, because of high local TNAP activity), and the correct matrix consisting of type I collagen. Thus, cutaneous coexpression of TNAP (causing local hyperphosphatemia and pyrophosphate deﬁciency) and collagen I led to skin calcifications, whereas a lack of collagen I coexpression did not permit local Ca and P crystal formation despite high TNAP availability.

It seems likely that deﬁciencies of other calcification inhibitors, such as fetuin-A and OPG, may also modify calcification in such complex experimental settings. Furthermore, the role of Ca in extraosseous calcifications was also not addressed and will probably remain unclear for awhile, because achieving controlled and isolated hypercalcemia or hypocalcemia is technically very difﬁcult in vivo. Nevertheless, these seminal observations explained a lot about the interactions between Ca-regulatory factors and identiﬁed therapeutically modifiable determinants (ie, P and pyrophosphates).

In a similar attempt to combine various procalcifying and anticalcifying factors in vivo, we investigated the cooperative role of high dietary P intake and renal failure induced by 5/6-nephrectomy in fetuin-A−/− mice on the relatively calcification-resistant C57Bl/6 background. Wild-type and fetuin-A−/− mice with normal renal function or low P feeding served as controls. The latter groups all failed to develop either increases in their serum Ca×P product or extraosseous calcifications. Only the combination of renal insufﬁciency plus a high-P diet led to an increased serum Ca×P product of >10 mmol²/L² but not to tissue calcifications in wild-type mice. In contrast, fetuin-A−/− mice exposed to the same interventions developed signiﬁcant calcifications of the myocardium, lungs, and heart valves despite a much lower serum Ca×P product (<7 mmol²/L²). This ﬁnding demonstrates another problem in the complexity of calcification processes, namely that proposed risk indicators, such as an elevated Ca×P product, do not give proper information on the calcification risk if other factors, such as the calcification inhibition potential, is severely deranged. We have, therefore, proposed that in the “inﬂamed” patient with low serum fetuin-A, current guidelines on target Ca×P products in dialysis patients may not apply.13

Conclusions

Vascular and soft-tissue calcification is not a random process of passive Ca and P precipitation, but usually actively regulated by several inhibitors and inducers. Because unwanted extraosseous calcification is a major predictor of impaired cardiovascular health in both the normal and the uremic population, treatment strategies to prevent or regress calcifications are in need. In this respect, vitamin K status, fetuin-A release, pyrophosphate availability, and P balance may become prime targets for future therapeutic intervention in calcification-prone individuals. Improved diagnostic tools clearly identifying the burden and functional relevance of calcification and, thus, high-risk individuals, in relation to the responsible dysregulations of their calcification-protection, are warranted. Another challenge lying ahead is to dissect which factors govern cardiovascular calcification in patients with normal renal function, those with early CKD, and those with advanced or end-stage CKD. Based on the studies
covered in this review, it cannot be assumed that therapeutic attempts can follow a “one-for-all” approach, and stage-specific interventions may ultimately evolve.

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References


