Chapter 7
The Role of Chronic Kidney Disease in Ectopic Calcification

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What Is Chronic Kidney Disease?

Chronic kidney disease (CKD) is a progressive disorder, characterised by a gradual decline in functional nephrons and a reduction in glomerular filtration rate (GFR). CKD is defined by a GFR below 60 ml/min/1.73m^2 [1]. As the GFR deteriorates further, there is a graded increase in the risk of cardiovascular morbidity [2]. CKD culminates in end-stage kidney disease (ESKD); by this stage, patients require dialysis or renal transplantation.

Cardiovascular disease is the most common cause of death in CKD patients receiving dialysis. The rate of cardiovascular mortality in dialysis patients in their 20s is comparable to octogenarians [3]. The high risk of cardiovascular mortality in CKD patients is strongly correlated with vascular calcification. In ESKD there are a number of risk factors including disturbances in mineral metabolism, secondary hyperparathyroidism (SHPT) and a build-up of uraemic toxins that predispose patients to CKD bone mineral disorder (BMD) and to ectopic calcification [4].

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The Risk Factors of CKD and Their Association with Ectopic Calcification

Dysregulated Mineral Metabolism: CKD Leads to Hyperphosphataemia, which is Exacerbated by Klotho Deficiency and Ineffective Fibroblast Growth Factor 23 (FGF23)

The kidney is a major regulator of serum phosphorous (P); it is important for both P excretion and regulating the resorption of P and calcium (Ca) to accommodate bone turnover. In CKD there is a decline in renal function, and P excretion by the kidney is impaired; therefore, raised serum P is associated with CKD.

The kidney relies on autocrine signalling from FGF23 to maintain P homeostasis [5–7]. When serum P levels are high, FGF23 is synthesised by bone osteocytes and osteoblasts to raise circulating levels of FGF23. FGF23 binds to the fibroblast growth factor receptor (FGFR) on the basolateral membrane of the kidney tubules, and this has two downstream effects to reduce serum P towards homeostatic levels [8]:

1. Increased P excretion. FGF23 blocks the synthesis and increases endocytosis of the Na/P cotransporter on the apical membrane of the kidney tubule [6]. The reduced number of P transporters decreases P reabsorption from the filtrate, therefore increasing P excretion.
2. Reduced vitamin D levels to reduce Ca and P resorption. FGF23 blocks the synthesis of 1α-hydroxylase (required for activation of vitamin D) and upregulates the synthesis of 24 hydroxylase (an enzyme that deactivates vitamin D). The combined effect is reduced levels of active vitamin D [6]. This prevents further increase of serum P as vitamin D promotes bone turnover and resorption of Ca and P.

In patients with CKD, the initial rise in serum P is compensated for by increased FGF23 production, and P homeostasis is maintained. As CKD progresses and the GFR declines further, the kidneys are unable to react to sufficiently lower P despite high serum FGF23 levels.

This is exacerbated by a klotho deficiency linked to CKD. Klotho is a protein required to confer the FGFR specific to FGF23, and in the absence of klotho, FGF23 is unable to bind to the FGFR and act on the kidney tubule to reduce serum P towards normal levels [9]. This P retention leads to persistent hyperphosphataemia and stimulates a further increase in FGF23 levels; however homeostatic mechanisms can no longer restore the P balance.

A chronic increase in serum P prevails [10] and increases parathyroid hormone (PTH) secretion. In a state of klotho deficiency, FGFRs in the parathyroid gland are unable to respond to high circulating levels of FGF23, and this negative feedback mechanism to prevent excessive PTH secretion is lost.

Dysregulated Mineral Metabolism: Vitamin D Deficiency in CKD and Reduced Ca Intake

Vitamin D is a term used to describe several related compounds, it is obtained as previtamin D and exists in the circulation in the inactive form of 25-hydroxy vitamin D. 25-hydroxy vitamin D is hydrolysed by the 1α-hydroxylase enzyme to the active form 1α,25-dihydroxy vitamin D. The majority of 1α-hydroxylase is expressed in the kidney; therefore the kidney plays a key role in regulating the activation of vitamin D.

Patients with CKD are often deficient in vitamin D. The detrimental effects of vitamin D deficiency on bone mineral disorders such as rickets, led to the discovery of vitamin D in the early 1900s and are now widely known as reviewed by [11].

Vitamin D in its active form, 1α,25-dihydroxy vitamin D (referred to as vitamin D here in) is important for Ca intake and homeostasis by three key mechanisms:

1. Increase Ca absorption from the small intestine. Vitamin D increases transcription of the Ca channel TRPV6 and calbindin in the small intestine to increase the efficiency of Ca absorption from 10% to 40% [12].
2. Increase resorption of Ca and P from bone. Vitamin D upregulates RANKL expression in osteoblasts and drives the maturation of pre-osteoclasts to osteoclasts which resorb Ca and P from bone and release it into the circulation [13].
3. Reduce PTH secretion. Vitamin D increases expression of the vitamin D receptor (VDR) and the calcium-sensing receptor (CaSR) in the parathyroid glands to increase their sensitivity to both Ca and vitamin D. The effect of vitamin D on the parathyroid gland is to downregulate PTH expression in order to prevent excessive Ca and P resorption and extensive bone turnover [14].

Vitamin D deficiency leads to an initial reduction in serum Ca. Low levels of circulating Ca stimulate the parathyroid gland to secrete PTH. The state of vitamin D deficiency affects two negative feedback mechanisms of PTH secretion. Vitamin D cannot downregulate PTH expression or upregulate the expression of the CaSR in the parathyroid gland to increase its sensitivity to circulating Ca.

Dysregulated Mineral Metabolism: CKD Is a State of SHPT Resulting in Hypercalcaemia and Hyperphosphataemia

PTH plays a key role in maintaining mineral homeostasis, it acts on multiple regulatory pathways, and PTH itself is regulated by multiple negative feedback mechanisms.

The parathyroid gland is well known to contain CaSRs, and PTH is the primary regulator of serum Ca; it is secreted when serum Ca levels are low [15]. For

example, the low serum Ca levels observed in early CKD stimulate PTH secretion; PTH has three mechanisms of action to increase serum Ca:

1. PTH increases Ca reabsorption from the kidney tubule.
2. PTH upregulates transcription of CYP27B1 in the kidney to increase 1α-hydroxylase activation of vitamin D and leads to increased serum vitamin D levels.
3. PTH increases resorption of Ca and P from bone. PTH increases bone turnover in a similar manner to vitamin D by upregulating the expression of RANKL in osteoblasts. RANKL drives the maturation of pre-osteoclasts to mature osteoclasts, which resorb Ca and P from the bone matrix and release it into the circulation.

Hyperphosphataemia also upregulates PTH secretion. PTH promotes P and Ca resorption from bone raising serum P levels further; however PTH also increases P excretion by the kidney; therefore its overall effect is to decrease serum P [16].

In a state of health, PTH increases serum Ca and reduces serum P levels to restore homeostasis; the stimuli to upregulate PTH secretion are removed, therefore acting as a negative feedback mechanism. Excessive PTH secretion is also prevented by FGF23 and vitamin D, which act as a negative feedback mechanism and bind to the FGFRs and vitamin D receptors (VDRs) in the parathyroid gland to downregulate PTH secretion.

CKD is a state of disrupted mineral metabolism; multiple mechanisms lead to high serum P and low serum Ca levels, both of which stimulate PTH secretion (Fig. 7.1). The kidney is unable to excrete P despite the high levels of PTH; serum Ca levels are restored by increased Ca and P resorption from bone; however this further increases the serum P levels. Chronic hyperphosphataemia continues to stimulate PTH secretion. In CKD both negative feedback mechanisms of PTH are lost due to vitamin D deficiency and klotho deficiency resulting in ineffective FGF23; therefore a chronic secretion of PTH persists. Excessive PTH secretion contributes to excessive bone turnover, reducing bone density and leading to CKD-BMD [17]. The excessive resorption of Ca and P from bone leads to chronic hypercalcaemia and chronic hyperphosphataemia, which is a direct stimulus for vascular calcification and other forms of ectopic calcification [18].

Dysregulated Mineral Metabolism: The Risk of Ectopic Calcification and Cardiovascular Disease

Disrupted mineral metabolism leads to a multitude of risk factors for ectopic calcification in CKD. High levels of serum P even within the normal range have been associated with increased risk of cardiovascular events and death [19]. Patients in the early stages of CKD may develop hyperphosphataemia, and its prevalence was over 50% in a study of over 25 thousand haemodialysis patients in ESKD [20]. In

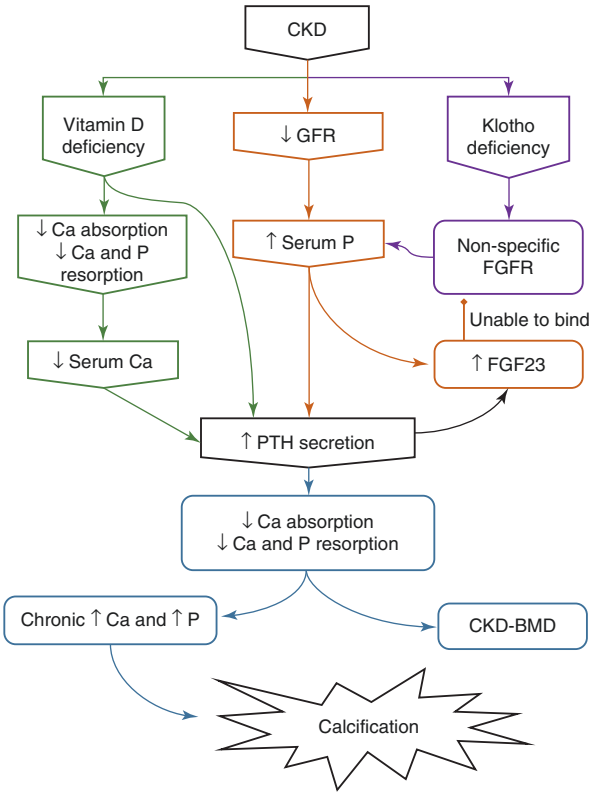

Fig. 7.1 Overview of dysregulated mineral metabolism in CKD. Multiple factors in CKD contribute to dysregulated mineral metabolism. The orange boxes indicate the decline in GFR and impaired P excretion that leads to raised serum P and triggers the PTH and FGF23 negative feedback loops to reduce serum P. As shown in the purple boxes, CKD is a state of klotho deficiency, and the FGFR requires klotho to confer it specific to FGF23; therefore serum P and PTH continue to increase despite increased FGF23 levels. The green boxes show that CKD is also a state of vitamin D deficiency, leading to reduced Ca absorption from the small intestine and reduced Ca and P resorption from bone; there is an initial decrease in serum Ca which also stimulates PTH secretion. As indicated in the blue boxes, high levels of PTH drive Ca reabsorption in the kidney and increase both Ca and P resorption from bone leading to CKD-BMD, a chronic increase in serum Ca and P and increased risk of ectopic calcification

both children and adults on dialysis, hyperphosphataemia was associated with the progression of vascular calcification [21].

The direct effect of SHPT observed in CKD on calcification is not known; however high PTH levels have been linked to an increased prevalence and severity of abdominal aortic calcification in patients with primary hyperparathyroidism [22].

The effect of vitamin D status on calcification in the CKD population has been studied, and both low and high levels of vitamin D have been associated with an increased risk of vascular calcification in children with CKD [23]. This suggests that vitamin D deficiency observed in CKD requires careful management.

The Alternative CKD Risk Factors for Calcification: The Uraemic Milieu

Disrupted mineral metabolism is not the only risk factor for vascular calcification in CKD, and uraemic serum has been shown to induce calcification independently of P concentration [24]. The systemic dysregulation in CKD can enable additional uraemic toxins to accumulate, which in healthy conditions would be excreted by the kidneys [25]. Patients in ESKD receive dialysis either in the form of haemodialysis or peritoneal dialysis to filter some of these uraemic toxins; however this can also be associated with problems. Some proteins may be excessively filtered immediately post-dialysis, and large fluctuations are observed [26].

The high turnover of bone in patients with CKD-BMD leads to elevated levels of alkaline phosphatase (ALP) [27]. ALP is an osteoblast marker mainly expressed by the liver and bone. High ALP levels are correlated with increased risk of cardiovascular disease, calcification and mortality [27]. Several large observational studies have found that ESKD patients had elevated serum ALP levels and that this was an independent risk factor of mortality [28, 29].

Experimental models suggest that impaired NaCl excretion in CKD increases the risk of hypertension. This has a detrimental positive feedback effect as sustained hypertension is a strong independent risk factor of ESKD [30]. Furthermore, systolic hypertension has been associated with faster progression of aortic valve calcification, and 5-year coronary artery calcification was accelerated even in pre-hypertensive patients [31, 32].

To compensate for impaired excretion and increased serum levels of P and NaCl, patients with CKD are advised to limit their intake of certain foods. Dietary restriction of P protects against conditions such as hyperphosphataemia, hypertension, proteinuria and other heart and bone problems [33]. However, vitamin K consumption is also reduced, a study of 172 CKD patients found that over 50% consumed less than the recommended adequate intake for vitamin K, and CKD patients are known to suffer from subclinical vitamin K deficiency [34, 35]. To confound this further, the uraemic environment is known to reduce vitamin K activity and increase the risk of ectopic calcification. It was demonstrated in rats that uraemia leads to a functional vitamin K deficiency; this was accompanied by increased renal and aortic Ca content [36]. Furthermore, loss of vitamin K activity is associated with calcification in CKD patients; haemodialysis patients prescribed warfarin (a vitamin K antagonist) had increased prevalence of vascular calcification [37].

Inflammation is another risk factor that has received much attention in the context of CKD, cardiovascular disease and its role in promoting vascular calcification [38]. The inflammatory markers C-reactive protein (CRP) IL-6, IL-1, and TNFα have been found to be elevated in CKD patients and associated with increased coronary artery calcification and mortality [39–44].

What Is Ectopic Calcification?

The dysregulated mineral metabolism and the uraemic milieu observed in CKD predispose patients to ectopic calcification. Ectopic calcification is the inappropriate biomineralisation of soft tissue that usually involves the deposition of calcium phosphate salts, including hydroxyapatite (HA). HA is formed from the crystallisation of Ca ions and inorganic P (Pi) ions; it has a mineral composition similar to that found in bone [45]. At physiological pH of 7.4, Pi exists predominantly as $H_2PO_4^-$ and HPO_4^{2-} in a 1:4 ratio and is neutralised by $Ca2^+$ ions to produce HA $Ca_{10}(PO_4)_6(OH)_2$ [46].

Vascular Calcification: CKD Is Associated with Arteriosclerosis

Vascular calcification is the deposition of HA crystals in the extracellular matrix (ECM) of the vessel wall. Once considered a passive degenerative process that occurs in ageing, vascular calcification has now been recognised as a highly regulated, cell-mediated process similar to bone ossification [4].

There are two distinct types of vascular calcification (see Chap. 2 for histopathological characterization of different vascular calcification types). In atherosclerosis, calcification occurs in lipid-rich plaques at damaged patches of the tunica intima. Atherosclerosis is associated with traditional cardiovascular risk factors including age, obesity, dyslipidaemia and smoking [4].

Arteriosclerosis (also known as Monckeberg's sclerosis) is associated with CKD and diabetes, it is characterised by calcification of the vascular smooth muscle cells (VSMCs) in the tunica media. Sheet like calcification forms in the tunica media layer resulting in a concentric thickening of the vessel wall and increased vascular stiffness that leads to systolic hypertension and left ventricular hypertrophy [47].

Although distinct diseases, atherosclerosis and arteriosclerosis can coexist in various combinations particularly in older diabetics and adults with CKD. These patients have been exposed to traditional cardiovascular risk factors for atherosclerosis and disease-specific risk factors for arteriosclerosis [48].

Coronary autopsy samples from renal patients had comparable tunica intima calcification to non-renal patients (atherosclerosis) but a higher proportion of tunica media calcification (arteriosclerosis) [49]. In young dialysis patients and those without comorbidity, calcification is exclusively in the tunica media [50]. From here onwards, vascular calcification in CKD will refer to arteriosclerosis.

Mechanisms of Vascular Calcification and the Impact of CKD

Vascular calcification is a highly regulated process that occurs in the matrix surrounding VSMCs. The molecular structure of Ca and P biominerals in an ectopic calcified human plaque in part resembles that of bone. Common features include the localisation of glycosaminoglycans and collagen with mineralisation, suggesting that similar mechanisms regulate physiological and pathological calcification [51].

Both processes require a microenvironment that enables extracellular crystal growth; this is formed by the accumulation of extracellular vesicles (EVs) in the ECM, where mineral nucleation and calcification can then occur [52]. To initiate ectopic vascular calcification, several molecular processes must occur simultaneously; this includes osteochondrogenic differentiation, downregulation of mineralisation inhibitors and the release of pro-calcific EVs [53] (Fig. 7.2).

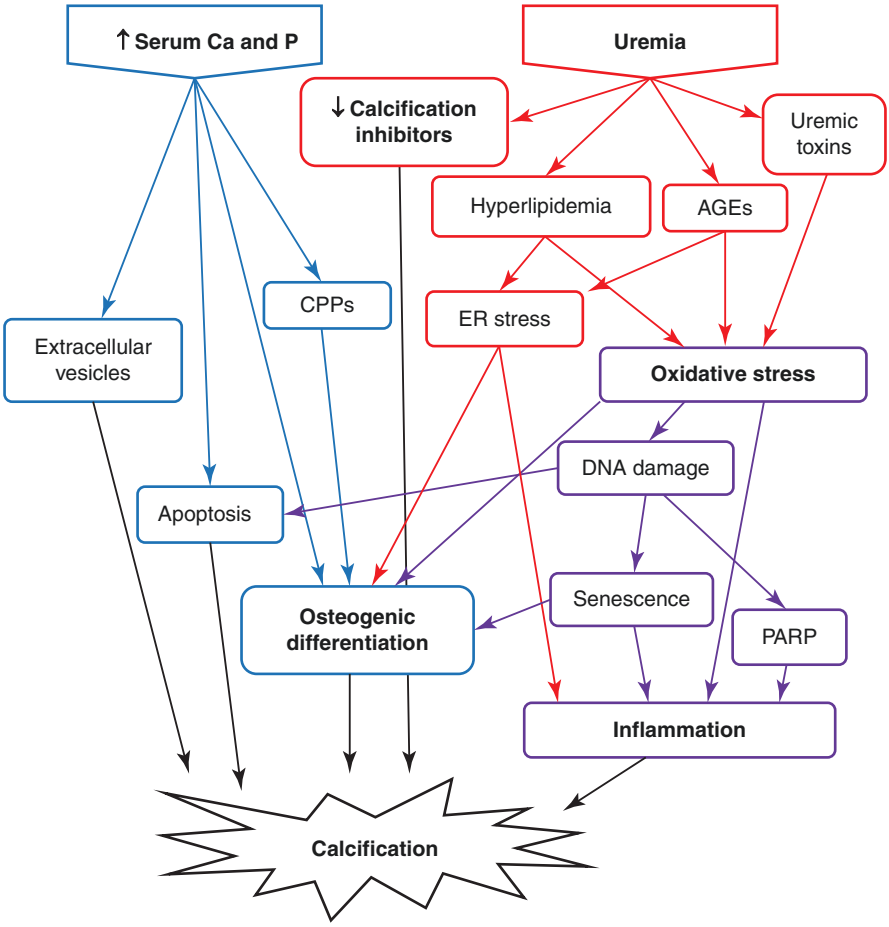

Fig. 7.2 Mechanisms of calcification in CKD. In CKD a multitude of mechanisms are affected that contribute to drive calcification. As shown in the blue boxes, high serum Ca and P drive apoptosis, osteogenic differentiation and the accumulation of both extracellular vesicles and CPPs. The red boxes indicate that the uraemic environment is characterised by a loss of calcification inhibitors as well as the accumulation of uraemic toxins, AGEs and hyperlipidaemia. The purple boxes show that this environment drives oxidative stress leading to DNA damage; the cellular responses to DNA damage include apoptosis, senescence and activation of the DDR pathway with increased PARP. This leads to an increased inflammatory response which directly promotes calcification as well as increasing osteogenic differentiation. Furthermore, the uraemic environment drives ER stress, contributing further to inflammation and osteogenic differentiation

Dysregulated mineral metabolism in CKD plays a key role in driving calcification; however, high levels of Ca and P alone do not result in the passive deposition of HA crystals in the vasculature [4]. A combination of risk factors found in the uraemic milieu is required to enable this pathological calcification to develop [21].

Apoptosis of Vascular Smooth Muscle Cells

Apoptosis of VSMCs play a key role in promoting calcification. Apoptotic bodies in the ECM provide a nidus for the accumulation of HA crystals and the initiation of calcification they are in part accountable for the increased ectopic calcification observed in CKD [4]. Apoptosis was shown to drive calcification in a VSMC model of calcification. VSMCs undergo apoptosis prior to calcification, and inhibition of apoptosis reduces calcification by 40% [54]. Indirect evidence linking apoptosis to calcification was also found in vivo, histological analysis of vessels from CKD dialysis patients found areas of apoptosis adjacent to calcified areas [50]. Ex vivo culture of these vessels in high Ca and P media mimicking the dysregulated mineral metabolism observed in CKD has been shown to drive apoptotic cell death and reduce VSMC density by 30% [55].

Extracellular Vesicle Release

EVs are small extracellular membranous particles, which, contrary to apoptotic bodies, are released by living cells. The release of mineralisation competent EVs of 100–300 nm in diameter into the ECM provides a nucleation site for HA crystals to form and is important in both physiological and ectopic calcification [56, 57]. Healthy VSMCs release EVs into the ECM; however they do not support mineralisation as they do not contain HA and are loaded with mineralisation inhibitors; matrix gla protein (MGP), prothrombin, osteopontin and fetuin-A that prevent mineral nucleation and crystal growth [56, 58].

High Ca and P conditions not only increase the rate of apoptosis in human VSMCs but also increase EV release [59]. Initially, this may be a defence mechanism to extrude excess HA; however accumulation of EVs can drive calcification. VSMCs persistently exposed to the high Ca and P levels observed in CKD release EVs that contain preformed calcium phosphate $Ca(H_2PO_4)_2$, were depleted of MGP and enabled HA crystal growth [59]. Raised extracellular Ca as observed in CKD was required for release of calcific EVs from VSMCs; these EVs shared properties with chondrocyte matrix vesicles [56]. This includes expression of Ca-binding annexins and exposed phosphatidylserine on the surface of EVs providing a site for HA nucleation, therefore supporting the early stages of ectopic calcification. Detailed information on the role of EVs in calcification is provided in Chap. 5.

Perturbation in the Level of Physiological Calcification Inhibitors

The expression of calcification inhibitors MGP, pyrophosphate, fetuin-A and osteopontin in healthy arteries plays a key role in preventing calcification.

MGP is endogenously expressed in both VSMCs and chondrocytes with local expression of MGP in the vessel wall required for inhibition of vascular calcification. This was shown in an experiment on MGP knockout mice which develop spontaneous vascular calcification. Re-expression of MGP in VSMCs prevented calcification, but high circulating levels of MGP did not [60]. MGP is expressed in its inactive form as dephosphorylated-uncarboxylated MGP (dp-ucMGP) and requires serine phosphorylation and γ-glutamate carboxylation to form active p-cMGP [61]. UcMGP has five glutamic acid residues which require vitamin K for their γ-carboxylation to form five γ-carboxyglutamate (GLA) residues and produce carboxylated MGP (cMGP).

As shown in Fig. 7.3, CKD patients are often deficient in vitamin K; the prevalence and severity of vitamin K deficiency is higher in CKD than the general population for two reasons. The first, dietary restrictions in CKD that limit P intake also reduce vitamin K consumption. The second, during γ-glutamate carboxylation, vitamin K is oxidised and must be recycled by reduction for subsequent carboxylase activity; CKD patients are often prescribed warfarin (a vitamin K antagonist), which blocks the reductase pathway and prevents vitamin K recycling [62]. In addition, uraemia was shown to reduce vitamin K γ-carboxylase activity in a rat model leading to accumulation of ucMGP and calcification that was reversed by vitamin K treatment [36].

In CKD, the local expression of ucMGP in VSMCs is increased [60], however, in a state of vitamin K deficiency which often occurs in CKD, γ-glutamate carboxylation is limited, and therefore ucMGP cannot be activated and accumulates at sites where calcification has been able to proceed (Fig. 7.3) [63]. This functional vitamin K deficiency affects several mineralisation inhibitors discussed below [37].

Fetuin-A is synthesised in the liver and bone, and in healthy individuals, it is present in high levels in the circulation and plays a key role in bone remodelling [64]. Fetuin-A is taken up by VSMCs where it reduces apoptosis and is concentrated in EVs to reduce HA crystal formation [65]. The protective effect of fetuin-A against calcification was demonstrated in deficient mice which showed an increased susceptibility to widespread calcification [66]. The key role of fetuin-A is to act as a circulating calcification inhibitor by binding Ca ions and HA with high affinity to remove excess mineral from the circulation. Binding of fetuin-A and HA forms fetuin-mineral complexes, also known as calcium phosphate-containing particles (CPPs) [66]. CPPs remove Ca and P from the serum. At low concentrations, CPPs decrease inflammatory cytokine secretion, therefore protecting against ectopic calcification [66]. CPPs are quickly cleared from the blood and are not detected in serum of healthy individuals [67]. Elevated Ca and P levels in CKD provide the perfect environment for CPPs to form, and they are present at high levels [68]. As fetuin-A forms CPPs with Ca and P, a decline in GFR is correlated with decreased serum fetuin-A [69]. In CKD, clearance of CPPs from the blood is reduced, and high concentrations of CPPs remain in the circulation. High concentrations of CPPs

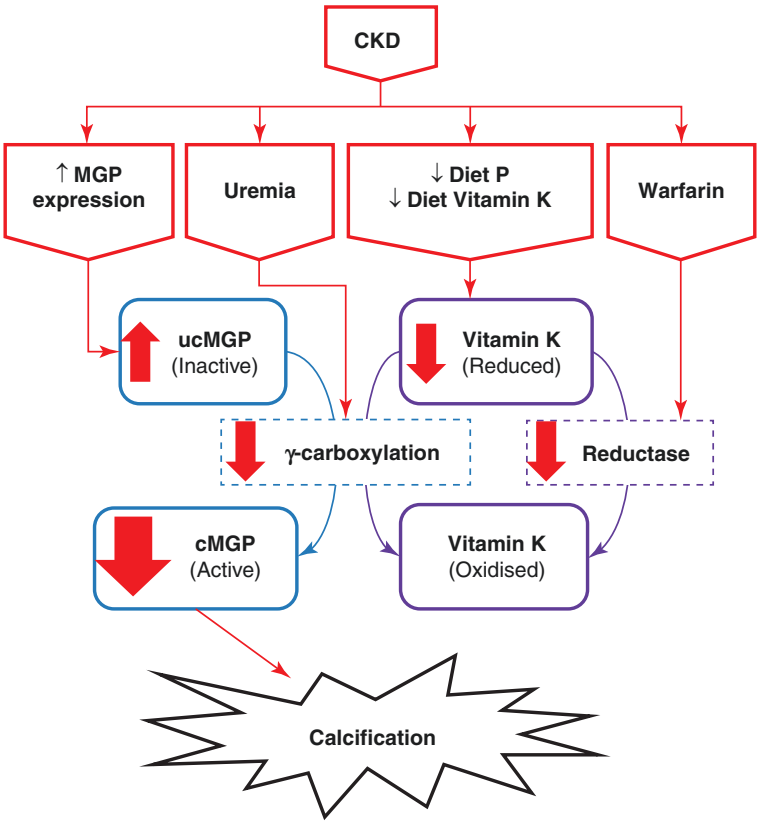

Fig. 7.3 The effect of CKD on the activation of MGP. MGP is expressed in its inactive, uncarboxylated form (ucMGP); as shown in blue, it is γ-carboxylated to active carboxylated MGP (cMGP) which inhibits calcification. The purple boxes show that vitamin K is required for γ-carboxylation; during this process vitamin K is oxidised and must be recycled by reduction for subsequent carboxylase activity. The red boxes and arrows indicate the effect of CKD on the activation of MGP. CKD increases the expression of ucMGP; however it remains and accumulates as inactive ucMGP for several reasons. The uraemic environment reduces γ-carboxylase activity. A reduced P diet advised in CKD also reduces intake of vitamin K which is required for γ carboxylation of ucMGP. Warfarin is often prescribed in CKD and impedes reductase activity and prevents the recycling of vitamin K. These factors lead to reduced levels of active cMGP and calcification ensues

stimulate inflammatory cytokines and cause apoptosis in macrophages in a dose-dependent manner; these conditions could exacerbate ectopic calcification [68, 70]. CPPs have been shown to correlate with calcification in rats with renal failure [71]. Additionally, levels of CPPs correlate with coronary artery calcification [72] and predict all-cause mortality in predialysis CKD patients [73].

Pyrophosphate is a potent endogenous inhibitor of calcification; the main source of pyrophosphate in VSMCs is the hydrolysis of adenosine triphosphate (ATP) generating AMP and pyrophosphate [46]. Pyrophosphate can also be released into extracellular fluid to inhibit mineralisation; physiological levels of 3–5 μM pyrophosphate completely inhibited VSMC calcification in rat aortas both in vitro and in vivo [74]. Pyrophosphate can be dephosphorylated and inactivated by alkaline

phosphatase (ALP), and the high levels of ALP observed in CKD catalyse the breakdown of pyrophosphate [75]. Plasma levels of pyrophosphate were found to be lower in patients on haemodialysis (2.26 + 0.19 μM) as compared to healthy patients (3.26 + 0.17 μM), and pyrophosphate was reduced by a further 32% immediately post-dialysis [26]. ALP activity and pyrophosphate hydrolysis were upregulated in aortic rings from uraemic rats as well as in aortic rings from normal rats incubated with uraemic rat plasma, suggesting that circulating factors present in uraemic plasma lead to pyrophosphate deficiency [76].

Osteopontin is another calcification inhibitor important for bone remodelling that is normally expressed in mineralised tissues such as bone and teeth [77]. Osteopontin was shown to accumulate in calcified vessels and reduces the extent of calcification in vivo, as MGP/osteopontin double-deficient mice had more extensive calcification than MGP single-deficient mice [78]. Post-translational phosphorylation is required for osteopontin to have an inhibitory effect on calcification [79]. As with pyrophosphate, the upregulated ALP activity observed in CKD leads to dephosphorylation and inactivation of osteopontin, reducing its effect on inhibiting calcification.

Osteoprotegerin (OPG) is a protein whose deficiency in mice has seemingly contradictory effects because it causes both osteoporosis and soft tissue calcification. OPG-deficient mice exhibit a decrease in bone density and mass and at the same time medial calcification of the aorta and renal arteries [80]. OPG is a secreted factor that decreases osteoclast activity by inhibiting receptor activator of nuclear factor kappa B ligand (RANKL) activation of its receptor RANK. This signalling is essential for the maturation of osteoclast progenitors [81], thus the increased bone resorption and osteoporosis in its absence. OPG is endogenously expressed in the media of the aorta [80], but the mechanisms by which it inhibits vascular calcification are yet unknown. However, increased levels of serum OPG were shown to correlate with vascular calcification and associate with negative cardiovascular outcomes in CKD patients [82–84]. Circulating levels of OPG are increased in predialysis, dialysis and post-transplant CKD patients, suggesting that OPG is upregulated in vascular injury in kidney disease [85].

Bone morphogenetic protein 7 (BMP-7), a member of the TGFβ superfamily, is an example of an inhibitor of vascular calcification. BMP-7 deficient mice, however, do not have a vascular or soft tissue calcification phenotype. Mice lacking BMP-7 show skeletal abnormalities, delayed ossification of bones as well as kidney and eye defects [86]. Polymorphisms in the BMP-7 gene have been linked to inverse relationships between bone mineralisation and vascular calcification in the coronary and carotid arteries and abdominal aorta in diabetes patients [87]. BMP-7 has also been shown to play a role in VSMC differentiation and maintaining their differentiated phenotype in vitro [88]. Moreover, intraperitoneal injection of BMP-7 prevented vascular calcification in a mouse model of CKD [89]. Interestingly, BMP-7 has been shown to be decreased in the kidneys of CKD patients leading to aggravation of fibrosis. Exogenous administration or transgenic overexpression of BMP-7 has been shown to have beneficial anti-fibrogenic effects in rodent models of CKD suggesting that there is therapeutic potential for BMP-7 treatment in CKD patients [90].

Several other factors (SMAD6, fibrillin-1 and carbonic anhydrase II) have been linked to preventing soft tissue mineralisation [91–93]. However, their effects have not been studied in the context of vascular calcification in CKD.

Osteo/Chondrogenic Differentiation

VSMCs, osteocytes, chondrocytes and adipocytes, are all derived from mesenchymal stem cells. The terminal differentiation of these stem cells is dependent on the paracrine and autocrine factors in the microenvironment [94]. VSMCs have great phenotypic plasticity and can dedifferentiate into mesenchymal-like cells; this is important during cell stress and in vascular repair. VSMCs may either proliferate and aid in repair or in pathological conditions, reach senescence or undergo an osteo/chondrocyte phenotypic change [95].

The high levels of P observed in CKD drive cellular stress and osteo/chondrocytic differentiation; the type III sodium-dependent P transporters, PiT-1 and PiT-2, play a key role in modulating this in VSMCs. It has been demonstrated in mouse models that PiT-1 promotes vascular calcification by both P uptake-dependent and P uptake-independent functions and PiT-1 played a key role despite the required P concentration to induce cellular stress being well above the maximal P intake [96]. On the other hand, PiT-2 protects against P-induced vascular calcification; PiT-2 deficient VSMCs were found to have lower levels of OPG and increased calcification [97].

Osteo/chondrocytic differentiation of contractile VSMCs involves the downregulation of VSMC markers; α-SMC actin, SM22α and myocardin along with upregulation of osteo/chondrocytic genes; including osterix, ALP, osteopontin, type 1 collagen and osteocalcin [53]. Expression of these genes are regulated by osteogenic transcription factors Runx2 and Sox9, which are also upregulated in calcified vessels and in VSMCs that spontaneously calcify [98]. A cell lineage study in mice lacking the calcification inhibitor MGP found that 97% of calcifying cells in the tunica media were derived from VSMCs, which had early upregulation of Runx2 and downregulation of myocardin [99]. Notably the osteo/chondrocytic differentiation preceded calcification. Similarly, in human arteries with arteriosclerosis, evidence of osteo/chondrocytic differentiation was observed; this included upregulation of ALP along with other osteogenic markers such as bone gla protein (BGP), bone sialoprotein (BSP) and collagen II [100]. As discussed above, ALP is a hydroxylase enzyme that dephosphorylates calcification inhibitors, pyrophosphate and osteopontin, deeming them inactive and promoting calcification [46, 79]. In vitro co-expression of ALP and collagen I was sufficient to induce mineralisation in high P medium [101]. The tunica media has a collagen-rich matrix; therefore this suggests that the dysregulated mineral metabolism and increased ALP activity observed in CKD would be enough to enable ECM mineralisation in the vasculature.

Ageing-Related DNA Damage and Senescence

As discussed above, Klotho is a cofactor of FGF23, and lower levels of Klotho observed in CKD contribute to disrupted mineral metabolism and aggravate vascular calcification [102, 103]. Importantly, mice deficient in Klotho, FGF23 and Memo, another regulator of FGF23 signalling, exhibit symptoms resembling accelerated ageing, including a short lifespan, infertility, skin atrophy, osteoporosis and calcification of the aorta and other arteries, accompanied by intimal thickening [6, 104, 105]. In addition, ectopic calcification of various organs is observed [104]. In humans, a homozygous Klotho mutation causes tumoural calcinosis, which manifests itself with carotid and dural artery calcifications and ectopic calcifications of soft tissues [102]. Decreased Klotho levels have been observed in calcified arteries showing its important role in inhibiting vascular calcification [106]. Additionally, elevated P is associated with increased cardiovascular calcification and mortality in ageing populations [107]. These studies show a link between ageing and increased calcification, and increased P is the common denominator. Increased P has been shown to contribute to ageing-related processes [108]; it is therefore no surprise that excess P in CKD has been found to promote premature ageing [109, 110].

Ageing is a series of time-related, degenerative processes beginning in adulthood that eventually end life [111]. It is now accepted that ageing is caused in part by the accumulation of genetic damage throughout life [111, 112]. The DNA damage response (DDR) signalling network is essential in the maintenance of genomic stability, via the initiation and coordination of DNA repair mechanisms with appropriate cell cycle arrest checkpoints. This evolutionarily conserved signalling cascade has two distinct but coordinated functions: it prevents or arrests the duplication and partitioning of damaged DNA into daughter cells to impede the propagation of corrupted genetic information, and it coordinates cellular efforts to repair DNA damage and maintain genome integrity [113–115].

DNA damage has been shown to accumulate during ageing both in humans and rodents [116–119]. Aside from increased occurrence of DNA lesion development, elevated levels of DNA damage are also a consequence of a decline in efficiency of DNA repair pathways [112]. Collectively, spontaneous mutations and DNA damage gradually impair the function of genes involved in stress responses and DNA repair. DNA repair becomes less efficient and more error-prone leading to cascading accumulation of DNA damage and mutations, which further exacerbate age-related physiological decline.

The most prominent examples of how accumulated DNA damage and defective DNA repair pathways affect the organism are segmental progeroid syndromes, diseases in which multiple phenotypes generally associated with normal ageing appear prematurely. It now appears that most, if not all, human premature ageing diseases are caused by heritable mutations in genes affecting genome maintenance either directly or indirectly: Hutchinson-Gilford progeria [116], Werner syndrome [117], ataxia telangiectasia [120], Nijmegen breakage syndrome [118], trichothiodystrophy [119], Bloom syndrome [121] and Cockayne syndrome [122].

Unsuccessful or insufficient DNA damage repair can have two consequences for the cell: apoptosis or cellular senescence. Senescent cells cease dividing, are resistant to apoptosis and undergo distinctive phenotypic alterations [111, 123], such as expression of $p16^{INK4a}$ [124], increased senescence-associated β-galactosidase activity [125] and secretion of a bioactive senescence-associated secretory phenotype (SASP) consisting of inflammatory cytokines, chemokines, growth factors and proteases [126, 127]. Unlike apoptotic cells, which are rapidly removed, senescent cells remain viable and continue to contribute to tissue stress responses long after the onset of senescence [128].

Vascular ageing lies at the heart of vascular calcification, and recent evidence points to the role of DNA damage in vascular ageing pathologies. Many cancer treatments, which are known to induce DNA damage, carry a risk of late effects including cardiovascular diseases [129]. The segmental progeroid syndromes, Hutchinson-Gilford progeria syndrome (HGPS) [130] and Werner syndrome [131] exhibit age-related phenotypes in the vasculature. HGPS is caused by a mutation in the lamin A/C (*LMNA*) gene that leads to the accumulation of a truncated form of prelamin A, referred to as progerin [132], which leads to the accumulation of DNA damage and senescence [133]. As a result, HGPS patients show premature atherosclerosis/arteriosclerosis, characterised by VSMC degeneration and calcification [134]. The same mechanism involving prelamin A and senescence has been shown to promote calcification in vessels of children on dialysis [133], as uraemic conditions promote DNA damage via oxidative stress [135]. Senescent VSMCs from children and adults on dialysis have been shown to have all the hallmarks of aged cells, with DNA damage accumulation and the SASP, which promotes calcification [109, 110, 133]. Moreover, elevated levels of SASP factors such as BMP2 and IL6 have been detected in the serum of both children and adults on dialysis correlating with calcification [109]. The link between CKD and DNA damage-induced senescence is further illustrated by studies showing that uraemic toxins; indoxyl sulphate and p-cresyl sulphate induce VSMC senescence (increased $p16^{INK4a}$ and prelamin A expression) and calcification, in vitro and in a rat model of CKD [136, 137].

DNA damage and senescence also drive osteogenic differentiation of VSMCs, and inhibition of DNA damage signalling can block calcification [133, 138]. Intriguingly, poly ADP ribose polymerases (PARP), components of the DDR, have been shown to promote calcification, as poly ADP ribose (PAR), their product, whose synthesis is increased with DNA damage, has the ability to concentrate Ca and P forming the direct nidus for mineralisation [139]. PARP inhibitors can block VSMC calcification in a rat model of CKD [140] which further supports the conclusion that vascular calcification in CKD is linked to premature ageing [141, 142]. What remains unclear is how the presence of uraemic toxins and increased P leads to DNA damage. The most likely culprit is oxidative stress, as it is induced both by uraemic toxins and high P, it has also been shown to induce DNA damage in the vasculature [108, 143, 144]. Oxidative stress is discussed further in the following section.

Inflammation

The hallmarks of inflammation in the vessel wall include increased expression of TNFα, IL1β, IL6, IL8, monocyte chemoattractant protein 1 (MCP1), OPG and intracellular adhesion molecule-1 (ICAM-1) by VSMCs [109, 133] and recruitment of leukocytes [136]. Although the acute release of pro-inflammatory cytokines is beneficial, sustained release is detrimental and leads to vessel remodelling. TNFα can induce mineralisation of calcifying VSMCs in vitro [145, 146]. Pro-inflammatory cytokines also increase the synthesis of CRP from the liver [147]. CRP may also be a direct vascular toxin, as CRP and complement activation have been detected in atherosclerotic lesions [148].

Numerous factors have been identified in CKD that promote vascular calcification by triggering inflammatory responses; this includes senescence and CPPs (as discussed above), as well as advanced glycation end products (AGEs), lipids, oxidative stress, ER stress bacterium and HA itself.

AGEs are formed via a non-enzymatic glycosylation reaction between glucose and proteins; they usually form as a result of hyperglycaemia; however, they are also increased in nondiabetic patients with uraemia [149]. AGEs have many detrimental effects, including accelerating atherosclerosis. AGEs trigger an inflammatory response by interacting with their receptors, which are expressed by a wide range of tissues. This interaction leads to oxidative stress and increased secretion of cytokines and inflammatory factors, such as ICAM-1, TNFα, IL-6 and others, the exact response depending on the cell type and receptors involved. Therefore, unsurprisingly, AGEs have been shown to induce vascular calcification [150].

Patients with CKD are at risk of metabolic syndrome and dyslipidaemia. Hyperlipidaemia is a major cardiovascular risk factor [136, 151, 152]; it can induce oxidative stress and lends itself to loading of cholesterol (in the form of LDL) into VSMCs. Cholesterol promotes both VSMC osteogenic differentiation and VSMC differentiation into macrophage-like cells; therefore it directly contributes to vascular calcification and amplifies the inflammatory responses in the vasculature [153–156].

Oxidative stress is another inducer of inflammation in VSMCs; as mentioned above, oxidative stress can be triggered by AGEs and hyperlipidaemia as well as by uraemia itself; therefore it is a common occurrence in CKD [157, 158] [157]. Oxidative stress is caused by an imbalance between enzymes producing and scavenging reactive oxygen species (ROS) leading to accumulation of ROS. Oxidative stress has been shown to induce TNFα and activate NFκB signalling, which leads to the production of further inflammatory factors such as MCP-1, IL-1β and TGFβ [157]. Oxidative stress-induced inflammation ultimately leads to osteogenic differentiation and calcification of VSMCs.

The common occurrence of oxidative stress along with the perturbations in Ca homeostasis that are observed in CKD can activate ER stress. ER stress is a cellular stress response that leads to activation of the unfolded protein response, a set of signalling pathways starting with three ER-resident ER stress transducers, IRE1,

PERK and ATF6, which sense ER stress [159]. Activation of the unfolded protein response leads to increased transcription of genes that help resolve ER stress, such as chaperones Grp78, Grp94 and proteins involved in ER biogenesis. It can also lead to apoptosis if the ER stress is persistent and unresolved [160]. ER stress has been shown to mediate vascular calcification [161] by promoting apoptosis and osteogenic differentiation of VSMCs [162, 163]. Importantly, ER stress has been shown to mediate calcification induced by uraemia-related factors in vitro such as lipids [164–167], AGEs, hyperphosphataemia and TNFα [145, 168] and in a rat model of CKD induced by 5/6 nephrectomy [145]. ER stress is also implicated in metabolic syndrome [169].

Finally, recent evidence suggests that sterile inflammation might not be the whole story. *Porphyromonas gingivalis*, a bacterium behind periodontal disease, has been implicated in many diseases of ageing, including CKD [170]. Although the exact mechanism of this is unclear, *P. gingivalis* can enter the bloodstream and increase the inflammation load in the body, thus contributing to disease processes that are worsened by inflammation. Recent studies suggest that this pathogen can promote VSMC calcification in vitro [171–173].

After calcification has occurred, the resulting HA has been shown to further increase inflammation by inducing IL1β and TNFα secretion in macrophages [174–176]. HA also induces apoptosis and promotes osteogenic differentiation further contributing to a pro-calcific environment in the vessel wall and exacerbating calcification [177–180].

Treatment Strategies

Treatments to Reduce the Risk Factors of Calcification

A specific therapy to prevent vascular calcification has not yet been found, and current treatment strategies focus on regulating the Ca and P balance and reducing secondary hyperparathyroidism (Fig. 7.4). Patients with CKD are often advised to take a low P diet; they may be treated with P-binders or vitamin B3 derivatives that modulate intestinal PiT-2 to reduce P absorption [181]. Current therapies to treat SHPT and reduce the risks of CKD-BMD also include vitamin D receptor activators (VDRAs), calcimimetics and parathyroidectomy [182].

P-binders are routinely prescribed to patients with ESKD. There are many different types of P-binders; they bind P in the gastrointestinal tract and reduce P absorption. Ca-based P-binders such as calcium acetate and calcium carbonate are effective in lowering phosphataemia; however, they are associated with raised serum Ca and increased risk of cardiovascular calcification [183, 184]. Meta-analysis showed that non-Ca-based P-binders such as sevelamer showed a significant decrease in hypercalcaemia compared to Ca-based P-binders; however, they were less effective at lowering P and PTH levels, they had increased risk of gastrointestinal adverse events and there was no difference in all-cause mortality [185].

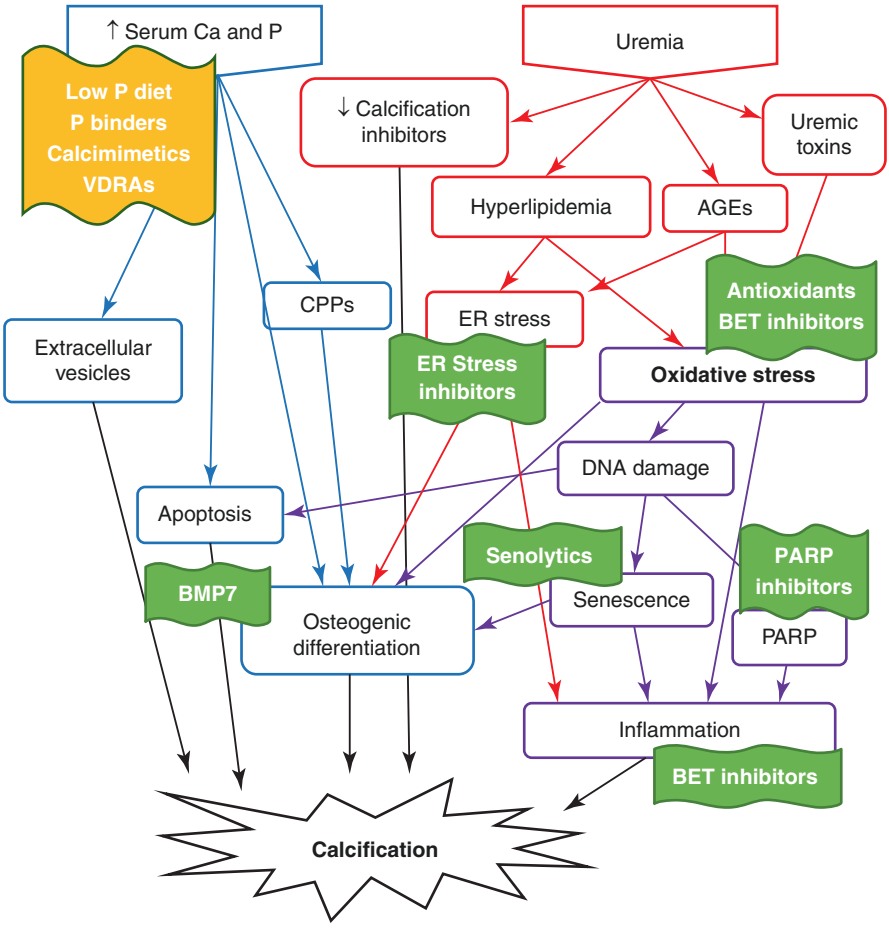

Fig. 7.4 Treatment strategies for calcification in CKD. Treatments shown in the yellow shape are in clinical use and all potential treatments are shown in green shapes. CKD patients are often advised to have a low P diet along with P binders, calcimimetics or VDRAs to prevent secondary hyperparathyroidism and prevent elevated Ca and P levels which are key risk factors for calcification. The individual mechanisms of calcification can also be directly targeted, for example by antioxidants, ER stress inhibitors, BET inhibitors, PARP inhibitors, senolytics or BMP-7

New non-Ca-based P-binders with improved safety and tolerability are required. Several studies both in rats and CKD patients have shown that bixalomer is as effective as sevelamer at correcting hyperphosphataemia but has fewer gastrointestinal side effects [186–189]. P-binders are not routinely used in the early stages of CKD where FGF-23 is increased to compensate for dysregulated mineral metabolism and prevent hyperphosphataemia; however, a rise in FGF-23 is associated with progression of CKD-BMD and calcification [184]. A randomised control trial to investigate the effectiveness of P-binders in patients with moderate to advanced CKD and normal phosphataemia found that all three P-binders reduced serum P and slowed

down progression of secondary hyperparathyroidism; however, they also increased vascular calcification highlighting the potential time-dependency of any treatment strategy [190].

In patients with ESRD, hyperphosphataemia is also managed by dialysis treatment, which helps to remove uraemic toxins including excess P. P-clearance can be improved by extending dialysis treatment times, increasing frequency of treatment or improving dialysis technique such as with haemodiafiltration [191]. Preserving residual renal function (RRF) has been shown to reduce cardiovascular events and improve long-term survival in CKD patients. Peritoneal dialysis is associated with a slower decrease in RRF than haemodialysis; in fact loss of RRF is 24–80% higher in haemodialysis than in peritoneal dialysis; therefore peritoneal dialysis is thought to provide better P control [192, 193].

Cinacalcet is a type II calcimimetic; it is an allosteric activator of the Ca receptor and increases sensitivity to extracellular Ca; therefore it decreases secretion of PTH. Multiple studies have shown that in CKD patients on dialysis, cinacalcet reduces circulating levels of PTH, Ca, P and HA and prevented the progression of vascular and cardiac valve calcification [194, 195]. VSMCs also express the CaR, and studies in vitro suggest the drug may also directly impact on cell phenotype.

Vitamin D deficiency in CKD exacerbates the disrupted mineral metabolism; many patients particularly children are prescribed VDRAs to prevent secondary hyperparathyroidism and CKD-BMD [14]. The direct effect of VDRAs on vascular calcification is controversial in the literature, and in children on dialysis both high and low levels of vitamin D were associated with increased carotid intima thickness and vascular calcification [23]. This suggests that vitamin D has a bimodal effect on calcification and there is a narrow physiological range where VDRAs are beneficial to vascular calcification in CKD patients.

Treatments to Directly Target Calcification

There is an extensive and growing understanding of the mechanisms involved in the development of vascular calcification; this opens the door to new therapies that directly target mechanisms of calcification such as inflammation and senescence (Fig. 7.4).

Bromodomain and extra-terminal (BET) proteins play a key role in epigenetics; they bind acetylated lysines on chromatin to regulate gene transcription. A clinical study in CKD patients treated with the BET inhibitor apabetalone found that a single dose countered the activation of multiple risk factors associated with calcification in CKD, including inflammation, oxidative stress and endothelial dysfunction [196]. CKD patients treated with apabetalone also had reduced expression of the calcification risk factor ALP, were less likely to experience cardiovascular events and had an improved GFR. BET proteins are a potential novel therapeutic for CKD that would target multiple systems that are disrupted and contribute to ectopic calcification in CKD [197].

The association of senescence with age-related diseases suggests it may be beneficial to specifically target senescent cells therapeutically. These strategies consist of senolysis, immune-mediated senescent cell clearance and SASP neutralisation. Senolytics are drugs that can specifically eliminate senescent cells. Senolytics have been shown to eliminate senescent cells in vivo and have beneficial effects in models of ageing and age-related disease [198, 199]; however, whether they inhibit vascular calcification has not yet been examined. In addition, drugs that target the DDR may also be effective in treating calcification, in particular PARP inhibitors [139].

Summary

There are a multitude of risk factors for ectopic calcification in CKD that arise from the uraemic environment and build-up of uraemic toxins; a progressively disrupted mineral metabolism develops, which is further complicated by dietary restrictions and prescribed medication. Vascular calcification is well studied in the CKD cohort as these patients are at risk via a multitude of detrimental mechanisms. This growing body of knowledge continues to improve the understanding and management of the condition and paves the way for the development of new treatment strategies.

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