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Risk Factors, Biomarkers, Molecular and Cellular Mechanisms of Vascular Calcification Genesis and its Methods of Detection

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ABSTRACT

Vascular calcification has traditionally been a passive process. However, in the last years, it has been proven to be an actively regulated biological process that is associated with crystallization of hydroxyapatite in the extracellular matrix and in cells of the media (VCm) or intima (VCi) of the arterial wall. Corresponding to bone mineralization, different pro and anti-calcifying mechanisms play an active role in mineral deposition in vascular cells. Evidence from clinical observations, animal models, and molecular studies suggest factors that regulate bone cell differentiation and mineralization, including fetuin A, BMP-2, matrix gla protein, osteopontin, osteoprotegerin, and inorganic pyrophosphate are used as biomarkers of vascular calcification. Under normal conditions, there is a balance between all calcification promoters and inhibitors, and it is possible that each pathological condition such as age, diabetes, dyslipidemia and hypertension, disrupts the balance with its own approach. The initiating factors and clinical consequences depend on the underlying disease state and the location of the calcification. Thereby, the pathogenesis of vascular calcification is a complex mechanism and not completely clear. On the other hand, diagnosis of the calcified arterial injury depends up on functional characteristics and imaging methods. In this article, the current knowledge of molecular and cellular mechanism, risk factors, biomarkers, and methods of detection of vascular calcification were reviewed.

Keywords: Vascular Calcification; Calcification Promoters and Inhibitors; Risk Factors; Clinical Assessment

Abbreviations: CT: Computed Tomography; VSMCs: Vascular Smooth Muscle Cells; IEM: Internal Elastic Membrane; CAD: Coronary Artery Disease; OPN: Osteopontin; OPG: Osteoprotegerin; TRAIL: TNF-Related Apoptosis-Inducing Ligand; OC: Osteocalcin; TNF: Tumour Necrosis Factor; ALP: Alkaline Phosphatase; Cbfa1: Core-Binding Factor Alpha 1; BMP: Bone Morphogenic Proteins; MBD: Mineral and Bone Disorders; VDN: Vitamin D Plus Oral Nicotine; AOPPs: Advanced Oxidation Protein Products; AV: Aortic Valve; MMP-9: Matrix Metalloproteinase 9; TGF-β: Transforming Growth Factor-β; STC: Stanniocalcin; VC: Vascular Calcification; Cbfa-1: Core Binding Factor Alpha-1; RUNX2: Runt-Related Transcription Factor 2; MSX-2: Msh Homeobox 2; MGP: Matrix Gla Protein; MVSC: Multipotent Vascular Stem Cells; CASR: Calcium-Sensing Receptor; DXA: Dual-Energy X-Ray Absorptiometry; EBCT: Electron Beam Computer Tomography; MSCT: Multi-Slice Computer Tomography; MRI: Magnet Resonance Imaging; IVUS: Intravascular Ultrasound; OCT: Optical Coherence Tomography; AS: Arterial Stiffness

Introduction

Calcium is the most common element in bone, and 99% of the total body calcium is in bone in the form of a calcium phosphate crystalline structure called hydroxyapatite $[Ca_{10}[PO_4]6[OH]_2]$ [1]. Calcium is also found outside of bone in a variety of tissues, broadly termed extra-skeletal calcification. In these sites, the calcium can be in multiple forms- including hydroxyapatite, magnesium whitlockite, and amorphous calcium phosphate [2]. In vertebrates, all extracellular body fluids are supersaturated with respect to calcium and phosphate, resulting in a tendency for spontaneous calcium phosphate precipitation, which is often expressed as the calcium 3 inorganic phosphate product [3]. Vascular mineralization is a process in which mineral is pathologically deposited in blood vessels, mainly in large elastic and muscular arteries such as the aorta, coronaries, and carotid and peripheral arteries [4-7]. Arterial calcification can occur in both intimal (VCi) and medial layers (VCm). The two forms of vascular calcification are distinct in their morphology and pathology [8]. Intimal calcification is exclusively associated with atherosclerosis and morphologically appears as punctate and disorganized mineral deposition in the arterial wall intima. Intimal calcification forms an important part of atherosclerotic plaques, which constitute VSMCs, macrophages, lipid, connective tissue, and necrotic debris [9]. Intimal calcification is ubiquitously associated with atheroma, can be used as a surrogate marker of atherosclerosis [10,11], and is predictive of future cardiovascular events [12,13]. Calcification had been thought to occur late in the disease course. Non-contrast computed tomography (CT) is the most sensitive method to quantify vascular calcification, although it measures total vessel calcium content and does not distinguish between intimal and medial mineralization.

However, the wider consensus is that calcification in coronary arteries in the general population (non-diabetic and non-chronic kidney disease) predominantly affects the intima [14,15]. In contrast, the medial layer may also be affected, leading to thickening of the medial layer of larger elastic arteries resulting in arteriosclerosis of smaller elastic arteries classically described as Mönckeberg's calcification, or medial calcinosis [16,17]. VCm confirmed by histology were observed in large elastic type arteries (ascending aorta), medium sized visceral and kidney arteries, and small transitional arteries (coronary, temporal, uterine, ovarian, parathyroid, mammary gland, and other) with diameter of at least 0.5 mm [18,19]. The four stages of lesion progression distinguish the extent and severity of Mönckeberg's calcification (Table 1) [20-22]. In stage 1, calcifications appear on haematoxylin–eosin (H&E) staining as irregular blue or violet deposits embedded within the media [23]. On a high-resolution light microscopy using H&E, Elastica-van-Gieson, von Kossa, or Alizarin-staining deposits consisting of fine granulations, which increase in size and become confluent, are revealed. Both intra- and extracellular deposits are present. Intracellular deposits are in vascular smooth muscle cells (VSMCs); extracellular deposits are largely associated with damaged and fractured elastic fibres embedded within the extracellular matrix. In muscular and transitional arteries, with H&E-staining granular calcifications develop alongside the internal elastic membrane (IEM) and nearby VSMC.

Table 1: Stages of medial calcifications and histological aspects.

Stage	Histological aspects of vascular calcifications; MMS type
I	Granular calcifications alongside the internal elastic membrane
	Calcification nearby vascular smooth muscle cells
II	Calcifications increasing in size and becoming confluent Solid plates distorting the media spanning up to the incomplete circumference
	Association of subendothelial hyperplasia in the intima
III	Calcifications distorting the media spanning the entire circumference
	Association of subendothelial hyperplasia in the intima
IV	Calcifications and foci of bone formation (osseous metaplasia)

In experience, the involvement of the IEM is common. These bands of calcium-rich deposits may thicken, becoming solid plates extending deep into the inner layer of the media. With further progression of the disease calcifications may distort the junctions of the innermost and outermost layers of the media, spanning up to three quadrants of the cross section (stage 2) or it may involve the entire circumference (stage 3). In stages 2 and 3, large conglomerates of calcifications may form solid plates or sheaths, progressively distorting the architecture of the media; intrusions upon the intima are then common [24].

In the absence of atherosclerotic lesions, the intima shows a subendothelial hyperplasia. In stage 4 of VC m foci of bone formation within the arterial media may be found, calcifications may undergo osseous metaplasia giving rise to true bony trabeculae. These structures delineate true medullary spaces harbouring haematopoietic cells interspersed with adipocytes [25]. In the arterial wall, calcification deposits associated with VCm may be perceived as foreign bodies and induce granuloma formation within the media; these structures often contain multinucleated giant cells. Other inflammatory components such as foam cells, lymphocytes, and mast cells may also be present.

In advanced stages, large calcifications may induce secondary changes in the intima such as subendothelial hyperplasia characterized by an increase in cellularity (e.g., myofibroblasts, fibroblasts, fibrocytes) and ulcerations characterized by infiltrations of the intima or even protrusions into the lumen.

It should be noted that VCm lesions do not spontaneously regress, and the clinical complications may vary according to the site and the amount of calcification. This disease of small vessels is also more common in patients with diabetes, renal failure and advanced aging [26]. The clinical manifestations of vascular calcification depend on the location within the arterial wall and the tissue perfused [27]. Intimal, atherosclerotic calcification can lead to myocardial infarction from stenosis and acute thrombus, or ischemia in both coronary and peripheral arteries. Medial or circumferential calcification can lead to reduced compliance due to arterial stiffening, resulting in an impaired vasodilation during ischemia that, in theory could lead to arrhythmias and sudden death. With medial calcification (arteriosclerosis) of the aorta, there will be increased pulse wave velocity, elevated pulse pressure, and systolic hypertension. Lastly, calcification of the arterioles of the skin and other organs can lead to calciphylaxis and ischemic gut [28]. Therefore, coronary calcium quantified by non-contrast CT can be taken as a measure of intimal calcification in the general population [14,15]. Numerous studies have investigated the association of cardiovascular risk with mVC in the general population as well as in diabetic, hypertensive and ESRD patients [29-31]. These studies have established in the general population, the amount of vascular calcification, as measured and quantified by multi-slice computed tomography, is an important predictor of all-cause mortality, vascular complications, and myocardial infarction [32].

On the epidemiological scenario, vascular calcification increases with age, atherosclerosis, renal failure, diabetes mellitus, hypercholesterolemia, osteoporosis, obesity, smoking, menopause, and lack of physical exercise [33,34]. Calcification of the aorta may affect 65% of people in a general population with a mean age of 60 years and correlates with coronary calcification identified by multi-detector computed tomography, with a positive predictive value to increase cardiovascular morbidity and mortality in asymptomatic patients at intermediate risk [35]. Additionally, calcification of the abdominal aorta is associated with increased cardiovascular mortality, even when adjusted for age [35]. Conversely, calcification of the coronary arteries is associated with a greater risk of myocardial infarction and with increased incidence of adverse events during percutaneous and surgical myocardial revascularization [36]. Aortic valve sclerosis has a 40% prevalence in octogenarian patients [34], and initiates the process of calcific aortic valve stenosis, in which mineralization of the cusp has a pathophysiological mechanism like vascular calcification [37]. Calcific aortic valve stenosis is a predictor of cardiovascular risk in the elderly [38]. In opposition to control valves, calcified aortic valves express more alkaline phosphatase and matrix metalloproteinase 2 [39]. Despite the calcified degeneration of the aortic valve being associated with atherosclerosis and its risk factors, studies that used statins to treat patients with aortic valve stenosis did not demonstrate decreased aortic valve stenosis progression [40].

Calcification Promoters and Inhibitors

Calcification Inhibitors

Under normal conditions blood vessel cells express mineralization-inhibiting molecules [41]. The loss of their expression, as happens in CKD, causes what is known as "loss of natural inhibition", giving rise to spontaneous calcification and increased mortality [41]. A list with these calcification inhibiting molecules has been drawn up after mutation analysis on mice, including among others:

Fibroblast Growth Factor-23 (FGF-23): FGF-23 is an approximately 30 kDA protein released by bone that requires the presence of the cofactor Klotho for its classical effects [42]. FGF-23 promotes phosphate excretion by reducing its proximal reabsorption by reducing the expression of NPT2a and NPT2c mRNA, sodium/phosphate transporters [43]. FGF-23 also decreases conversion of calcidiol into its active form by reducing 1α-hydroxylase activity [44]. Thereby, gastrointestinal absorption of calcium and phosphate is reduced. In parathyroid glands, FGF-23 decreases PTH secretion and parathyroid cell proliferation [45]. FGF-23 null mice develop hypercalcitriolemia and VC [42]. Although the mechanistic link remains to be explained, FGF-23 may serve as a novel risk marker for the cardiovascular mortality in CKD [44]. In patients with coronary artery disease (CAD), the same independent link between FGF-23 and mortality has been demonstrated [46]. In contrast to FGF-23, Klotho excess has never been shown to be noxious [47]. Interestingly, Klotho levels are up regulated by vitamin D receptor agonists (calcitriol or paricalcitol) in CKD mice submitted to a high phosphate diet. These mice show half less calcification than those who did not receive therapy. Phosphaturia is increased whereas phosphatemia and FGF-23 levels are lowered [48]. In contrast, vascular Klotho deficiency favors the development of arterial calcification and mediates resistance to beneficial vascular effects of FGF-23 [47].

Fetuin-A (FET-A): Fet-A is a serum 59-kDa glycoprotein that inhibits ectopic vascular calcification [41] and produced by the liver that possess a systemic action [49,50]. It is a powerful inhibitor of hydroxyapatite formation, reducing the formation of crystals in *in vitro* solutions containing calcium and phosphorus without affecting those that are already formed [41,51]. Mice that are deficient in this protein develop extensive calcifications in soft tissue such as the myocardium, kidneys, tongue, and skin [52]. Fet-A is thought to inhibit calcification by binding early calcium phosphate crystals and by inhibiting crystal growth and mineral deposition [42]. This could be facilitated by the formation of large calciprotein particles

[51,53]. Indeed, the accumulation of naked calcium phosphate crystals is responsible for extraosseous calcification and causes inflammation. These crystals are usually digested by the cells of the reticuloendothelial system such as macrophages. In contact with the crystals, macrophages secrete proinflammatory cytokines and undergo more apoptosis [42]. The formation of fetuin-A calciprotein particles (CPP) facilitates the clearance of these crystals and thereby reduces their negative impact. Fet-A likely plays a very important role in the stabilization of these complexes and reduces the inflammatory response [42]. Fet-A binds and sequesters insoluble mineral nuclei, forming soluble colloidal CPP, thereby inhibiting crystal growth and aggregation [42]. Macrophages secrete less cytokines and undergo less apoptosis phenomenon compared to reactions caused by naked crystals. This property of Fet-A to decrease inflammation may be influenced by the phosphorylation degree of the glycoprotein [54]. In these studies, lower serum Fet-A concentrations have been associated with increases in calcification scores, arterial stiffness, mortality and incidence of cardiovascular events [55-58].

Osteopontin (OPN): Osteopontin (OPN) is a phosphoprotein that is usually found in mineralized tissue such as bones and teeth [41,59]. It inhibits mineralization by blocking hydroxyapatite formation and activating osteoclast function [60]. Although it is not found in normal arteries, its expression is detected in atherosclerotic plaques and calcified calcified vessels. OPN knock-out mice do not develop VC but, when these mice are bred with MGP knock-out mice, the VCs are more important than in simple MGP knock-out mice [61]. OPN must be phosphorylated to act as a calcification inhibitor [42,62]. OPN inhibits mineralization of VSMC by binding to the mineralized crystal surface [63]. On the contrary to the fully phosphorylated OPN, cleaved OPN could act as a proinflammatory cytokine and a proangiogenic factor facilitating vascular mineralization [60,64]. The possibility that OPN could serve as a calcification serum marker is controversial [42]. Berezin et al showed that OPN was a good predictor of coronary calcification in type two diabetes mellitus patients [65]. Tousilis et al found a positive association between OPN and arterial stiffness in coronary artery disease [66]. Indeed, the discrepancy between the different studies may perhaps be explained by the differences in patient populations. It is thought OPN plays a key role in inflammatory process [42]. Its relationship with diseases related to inflammation such as atherosclerosis, obesity and autoimmune diseases has already been shown [67-69]. It has also been suggested that hyperglycemia could up-regulate OPN and thereby lead to VSMCs proliferation [70].

Osteoprotegerin [O]: Osteoprotegerin [OPG] is a member of the tumour necrosis factor receptor family that has been identified as a regulator of bone resorption [71]. OPG is produced by many tissues, including the cardiovascular system, lungs, kidney and immune system [72]. OPG is a regulatory factor produced by bone marrow derived stromal cells [42]. OPG plays a pivotal role in the regulation of the bone turnover, inhibiting osteoclast differentiation and acting like a decoy receptor for the receptor activator of NF-KB ligand (RANKL system) [73]. It interferes with the interaction between RANK (expressed by osteoclast-like cells) and RANKL (expressed by osteoblast-like cells). OPG is also thought to inhibit alkaline phosphatase activity [74]. OPG levels are significantly higher in CKD patients, in relation with the severity of renal failure. Although OPG is known to impede osteoclast differentiation in bone, OPG is usually considered as a protective factor against VC as it blocks the bone remodeling process in the vascular tissue [42]. OPG is also a neutralizer of the pro-apoptotic actions of TRAIL (TNF-related apoptosis-inducing ligand), which strongly activates vascular cells apoptosis [75]. Apoptotic bodies can also lead to mineralization. In support of that, it has been observed that OPG deficient mice do develop both severe aortic calcifications and osteoporosis [76,77]. Interestingly, OPG seems to be a marker of VC onset rather than a severity or progression predictor [42,78].

Osteocalcin (OC): OC, a vitamin-K dependent matrix protein that inhibits calcium salt precipitation *in vitro* [79], shows a strong affinity for hydroxyapatite [42]. OC has been found in calcified atherosclerotic plaques and calcified aortic valves [80]. It was generally thought that OC inhibits crystal growth [81] and limits bone formation [82]. Nonetheless, its utility as serum marker is still discussed in conflicting studies. Aoki, et al. [83] did not show any relationship between OC and VC in type 2 diabetes mellitus patients whereas Kim, et al. [84] found an inverse correlation between OC and Agatston calcification score in Asian women, even after adjusting for age [42]. To define if OC can be used as a diagnostic or a screening tool, the role of OC in the pathogenesis of VC clearly remains to be clarified.

Pyrophosphate (PPi): PPi is a small molecule made of two phosphate ions [42]. It acts as a calcification inhibitor by inhibiting hydroxyapatite crystal formation [85]. Once again, knock-out mice (in fact, knock-out mice for a precursor) develop VCs [86]. Absence of PPi would promote VSMC differentiation but the mechanism is not fully understood [87,88]. O'Neill, et al. demonstrated the negative association between PPi and VC in CKD [89]. Although the short half-life of PPi limits the possibility for improving VC by bolus injections, daily peritoneal dialysis achieved with a solution which contains PPi in CKD mouse model do succeed in inhibiting calcification [90]. O'Neill et al demonstrated that daily intraperitoneal injections in rats could also reduce both incidence and amount of calcification [91]. PPi has been shown to inhibit mineralization on rat aortic VSMCs cultures too [92]. Furthermore, biphosphonates, non-hydrolysable analogs of PPi, have also proved their ability to inhibit aortic calcifications in experimental renal failure rats. Calcification was stopped in cultures of rat aortas as well as *in vivo* model [42]. It supports the idea that biphosphonates have direct effects on VC, independent of bone [93], maybe via a down regulation of Notch1-RBP-Jk signaling pathway and MsX2 gene induction [94]. ATP, which is a polyphosphate associated

with nucleoside, might also act as calcium phosphate deposition inhibitor, not only as the source of PPi but also as a direct inhibitor [95]. Even if PPi seems to be a promising marker, its determination has been performed in a single center only and the transferability to other centers should be validated.

Matrix Gla Protein (MGP): MGP is a vitamin K, 14-kDa γ-carboxylated protein expressed by chondrocytes, VSMCs, endothelial cells and fibroblasts [42]. Its role as a calcification inhibitor has been illustrated by MGP knock-out mice who develop extensive arterial calcifications [96,97]. In 2002, Moe et al demonstrated a correlation between vascular MGP expression and the calcification of epigastric arteries in dialysis patients [98,99]. MGP-deficiency in humans leads to Keutel syndrome, a rare genetic disease hallmarked by abnormal soft tissue calcification [96]. MGP binds calcium crystals, inhibits crystal growth, and plays a role in the normal phenotype of VSMCs in preventing the osteoblastic differentiation [100,101]. MGP also binds and inactivates a pro-mineralization factor, bone morphogenetic protein-2 (BMP-2) [102]. Among other effects, BMP-2 promotes osteogenic conversion of VSMCs via MSX2 transcription factor [42]. MGP could also protect mineral nucleation sites on elastin and thereby prevent spontaneous calcification of the elastic laminae [42]. In support of that, the irregular calcification of the thoracic and abdominal aorta segments in MGP −/− mice correlates with the local variations of the elastin content [96]. Parallel to this study, other authors hypothesized a mineralization process by size exclusion, in which MGP proves to be essential to prevent mineralization within fibrils [42].

Calcification Activators

There are studies that speculate that, as well as hyperphosphataemia and hypercalcaemia, there are substances present in the blood serum of patients with CKD capable of stimulating calcification [103]. Bovine VSMC in the presence of uraemic serum increases the expression of calcification-related proteins. Many uraemic factors have been identified that can induce osteogenic genes, transforming osteoblasts and secreting some bone matrix proteins in the walls of blood vessels and soft tissue. Some of these factors are tumour necrosis factor (TNF) [104], inflammatory cytokines [105], fibronectin [106], type-I collagen [106] and 25-hydroxycholesterol [107]. These uraemic serum substances stimulate the expression of molecules essential to vesicular calcification.

Alkaline Phosphatase: Alkaline phosphatase (ALP) is one of the osteoblastic phenotype markers and is considered essential in the vascular calcification process [41] t has been detected in vascular and heart valve calcifications. ALP expressed on the surface of cells can act on phosphate liberators, releasing inorganic phosphate [108] Inflammatory cytokines and vitamin D induce its up-regulation and mineralization [109].

Core-Binding Factor Alpha 1: Core-binding factor alpha 1 (Cbfa1) is the main regulator of bone cell differentiation [41]. fa1-deficient mice have problems with cartilage formation and bone mineralisation [110]. acts as a transcription factor that accelerates the expression of important osteoblast lineage genes such as osteocalcin, osteopontin, ALP or type-I collagen [111]. s expression is up regulated by phosphate43 and uraemic toxins [103].

Bone Morphogenetic Protein – 2 (BMP-2): Bone morphogenic proteins (BMP) are a group of, at least, 30 proteins that receive their name from their osteoinductive properties [41]. Bone morphogenetic proteins (BMPs) belong to a subdivision of TGF-β like growth factors family. BMPs regulate growth, differentiation, and development in the embryo as well as during tissue remodeling processes in the adult organism. BMP-2 is an important molecule iVC [he regulation of bone formation as well as in VC [41,42]. In bone, it promotes osteoblast differentiation and mineralization [112]. Inhibition of BMP-2 inhibits osteoblast differentiation and bone formation *in vivo* and *in vitro* [113] and protects against atherosclerosis and VC [114]. They act by binding to a heterodimeric system of transmembrane receptors (BMP-1 and BMP-2 receptor) that trimerises upon binding. The binding of a BMP to its specific type II receptor results in the type 1 receptor being activated. This causes phosphorylation and nuclear translocation of the Smad transcription factors thus modifying the transcription rate of target genes [115]. They then induce ectopic bone formation [116].

Sclerostin: Sclerostin is an osteocyte-specific glycoprotein and is considered as a potent inhibitor of bone formation [117,118]. It inhibits specific co-receptors needed for β-catenin-dependant signaling activation [119]. This pathway is involved in osteoblast-mediated bone formation [120]. It is thought that sclerostin plays a role in bone mechanosensibilisation [42]. When bone undergoes a substantial strain, sclerostin production would be decreased and bone could thus increase its formation in response to mechanical stress [121]. As β-catenin belongs to Wnt cascade signaling and as Wnt pathway is thought to be implicated in development of VC, it is interesting to investigate a potential association between sclerostin levels and VCs [42]. In non-CKD patients, some studies have demonstrated a positive association between sclerostin levels and VC [122,123] whereas in other ones, there was not a significant correlation between the two parameters [124,125].

Rankl: RANKL (also known as OPGL) is a protein consisting of 316 amino acids with a molecular weight of 38kD. Its expression is also modulated by several cytokines, glucocorticoids and PTH [126]. RANKL is produced by osteoblast lineage cells and activated T cells. It promotes osteoclast formation, fusion, differentiation, activation and survival, leading to increased bone resorption and bone loss [127]. RANKL stimulates its specific receptor RANK, which is expressed in fewer cells such as progenitor cells and mature osteoclasts, activated T cells and dendritic cells [128-130]. The activation of RANK by

RANKL triggers the NF-KB intracellular signalling cascade. The final stage of RANK activation is the NK-κB translocation into the nucleus, which can take place by the classical or alternative pathway [41]. Both pathways are regulated by their kinases which are, respectively, IKK, and IKKα. The NK-κB translocation to the nucleus modulates the expression of different genes, e.g., BMP4 [131]. The biological effects of OPG are the opposite of RANKL-mediated effects, since OPG acts as a soluble inhibitor that prevents RANKL interaction and the subsequent stimulation of its RANK receptor [132]. Many trials have shown that VC as well as arterial stiffness and cardiovascular events are inversely related to serum RANKL [133-135] and positively related to serum OPG [136-138].

Risk Factors for Vascular Calcification

The risk factors for VC are divided into traditional: involving advanced age, hypertension, diabetes, smoking, dyslipidemia, and others; and the non-traditional ones: including inflammation, oxidative stress, and mineral and bone disorders (MBD) of CKD, among other factors [139].

Age

Age is the strongest predictor of coronary artery disease [140] but multiple other clinical risk factors have been implicated in the pathogenesis of arterial calcification. Coronary artery calcification is also more prevalent and more severe among CKD patients than in the general population, and studies in CKD patients offer insight into the pathogenesis. In patients not yet on dialysis, over 50% have coronary artery calcification [141] whereas 70–90% of prevalent dialysis patients have significant coronary artery calcification [142,143]. Histologic studies comparing dialysis patients to non-CKD patients who died of a coronary event showed that dialysis patients had more calcification in the atheromatous plaques, but not more plaque. Dialysis patients also had a thicker medial layer [144]. Studies evaluating distal segments of the coronary arteries found medial calcification adjacent to the internal elastic lamina in dialysis patients [14] and in patients with advanced CKD [15]. Moe et al had found isolated medial calcification in the absence of intimal calcification in the inferior epigastric artery of patients undergoing a renal transplant [98]. Thus, calcification can occur both in intimal and medial arterial layers and in different vascular beds. In a study of 4544 patients, the presence of calcification in the thoracic aorta, carotids and iliac arteries were associated with all-cause mortality with hazard ratios of 2.1, 1.6, and 1.67, respectively, whereas coronary artery calcification was associated with a hazard ratio of 3.4 for cardiovascular mortality [145]. At the present time, it appears that there may be different initiating factors in different vascular beds and in the intima and media, but a common downstream process of de-differentiation to an osteoblast like phenotype.

Hypertension

Hypertension is associated with vascular remodelling and arteriosclerosis. In clinical studies, hypertension is not a commonly cited risk factor for calcification, perhaps because most subjects with calcification have hypertension as a clinical manifestation of the arteriosclerosis. The renin-angiotensin system is known to be a major pathogenic factor in VSMC apoptosis, growth, and differentiation, and therefore it likely plays a role in calcification [146]. Armstrong et al fed rabbits an atherogenic diet with high dose vitamin D to induce calcification along the internal elastic lamina and the media layer. There was upregulation of BMP-2 and down regulation of alpha-smooth muscle actin suggesting a dedifferentiation from a vascular smooth muscle cell phenotype to an osteoblast like phenotype. Furthermore, calcified arteries had upregulation of angiotensin 1 receptor and treatment with an angiotensin receptor blocker prevented the calcification [147]. In contrast, in $5/6^{th}$ nephrectomized rats (a model of CKD), treatment with enalapril improved myocardial hypertrophy and progression of renal disease but had no effect on vascular calcification [148]. In a rat model of arterial calcification induced by intramuscular administration of vitamin D plus oral nicotine (VDN), increased calcium content of arteries was associated with increased levels of angiotensin II and adlosterone in the tissue; treatment with captopril or spironolactone reduced the calcification [149]. Thus, the renin-angiotensin and aldosterone pathway appear to play a role in arterial calcification. Whether this is due to the reduction of underlying remodelling (arteriosclerosis), or a direct inhibition of the osteogenic transformation will require additional studies.

Diabetes

Patients with diabetes had increased calcification compared to non-diabetic patients and there was increased expression in the medial layer of bone matrix proteins in the arteries such as osteopontin, type I collagen and alkaline phosphatase [150]. In vitro, there is a study that found VSMC incubated with high glucose led to an increase in the expression of the osteoblast transcription factor RUNX2, BMP-2 and osteocalcin and enhanced calcification in bovine VSMC. The protein kinase C signaling pathway was involved in this high glucose-induced expression of RUNX2 and bone matrix proteins [150]. Another group found that when fed high fat diet, the Ldlr−/− diabetic mouse develops hyperglycemia, dyslipidemia and aortic calcification with concomitant upregulation of aortic BMP2 and Msx2 gene expression [151]. Increased glucose increased the BMP-2/Msx2-Wnt pathway, leading to an osteogenic phenotype in a subset of the myofibroblasts; inhibition of the BMP-2 pathway reduced arterial calcification [152]. Interestingly, the location of BMP2 and BMP4 differed in diabetic aortas in that BMP-4 was found in the endothelium and BMP2 throughout the vascular wall [152]. These results suggest that the increased vascular calcification in diabetes is at least partially

due to the direct effects of hyperglycemia on transforming the VSMC to osteoblast like phenotype via multiple mechanisms.

Dyslipidemia

Although clinically the role of lipids in vascular calcification is unclear, during osteogenic differentiation, calcifying vascular cells (CVCs, a clone of VSMC that readily calcify) accumulate not only minerals but also lipids such as triglycerides [153]. In vitro, HDL inhibits the osteogenic differentiation pathway [153]. In CVCs, stearate, compared to other fatty acids, promoted mineralization whereas inhibition of acetyl-CoA carboxylase or acyl-CoA synthetase reduced mineralization [154]. In these same CVC, n-3 unsaturated fatty acids play a protective role through a p38-MAPK (mitogen-activated protein kinase) and PPARγ (peroxisome proliferator activated receptor gamma) dependent mechanism [155]. Finally, oxidized lipids such as oxysterols and oxidized phospholipids illicit procalcific effects in vascular cells as detailed below [156]. Thus, dyslipidemia, rather than elevated LDL cholesterol appears to be a major causative factor in vascular calcification.

Inflammation

Inflammation is a known non-traditional risk factor for atherosclerosis and vascular disease in the normal population and in CKD and is associated with increased mortality [157]. Both CRP [158] and inflammatory cytokines [159] are associated with increased coronary artery calcification in patients with CKD. Interestingly, osteogenesis is associated with local inflammation and macrophage infiltration in atherosclerosis in ApoE−/− mice as revealed by molecular imaging *in vivo* [160]. Tumor necrosis factor alpha can induce mineralization of calcifying vascular cells *in vitro* [104] and co-culture of these cells with monocyte/ macrophages (the source of most cytokines) can accelerate mineralization [161]. In human VSMC, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway may inhibit inflammation induced calcification, perhaps by mediating alkaline phosphatase which is a 'marker' of osteoblast phenotype but also a potent inhibitor of a naturally occurring inhibitor of calcification, pyrophosphate [162]. Cytokine stimulation of alkaline phosphatase from VSMCs probably plays an important role also in calcification associated with diabetes, since the TNF-α inhibitor infliximab was shown to reduce the osteogenic phenotype of VSMC and the extent of medial calcification in LDLR−/− diabetic mice, without reducing obesity, hypercholesterolemia, and hyperglycemia [151].

Oxidative Stress

CKD is a state of increased oxidative stress due to impaired antioxidative mechanisms [163]. Elevations in asymmetric dimethylarginine, a naturally occurring inhibitor of NO synthase, are associated with increased intima-medial thickness in the carotid arteries, concentric left ventricular hypertrophy, and mortality in dialysis patients [164]. In a rat model of CKD, the antioxidant Tempol inhibited vascular calcification by reducing oxidative stress and inhibiting osteogenic transdifferentiation of vascular smooth muscle cells [165]. In the general population, there is growing evidence indicates that there is a correlation between oxidative stress and the development of vascular calcification [166-168]. Macrophages, endothelial cells, and smooth muscle cells produce reactive oxygen species such as hydrogen peroxide and superoxide anion in response to several stimuli. The free radical nitric oxide (NO) is generated from the endothelium from L -arginine by the enzyme NO synthase and leads to production of hydroxyl or peroxyl radicals. When VSMC are treated with β-glycerophosphate or uremic serum for 24 h, the production of H2O2 and early expression of NADPH oxidase sub-unit p22(phox) are increased. The elevated oxidative stress was associated with increased expression of RUNX2 and alkaline phosphatase and calcification of VSMC [166]. An important contributor to oxidative stress in atherosclerotic lesions is the formation of hydrogen peroxide from various sources in vascular cells [37]. A recent study by Byon et al [168] demonstrated that H2O2 induces a switch of VSMC from contractile to osteogenic phenotype associated with an increased expression of RUNX2 and calcification in VSMC. Furthermore, inhibition of H2O2-activated AKT signaling pathways blocked increased expression of RUNX2 and VSMC calcification [168]. A similar study has also demonstrated that advanced oxidation protein products [AOPPs] induce vascular calcification by promoting osteoblast differentiation of human vascular smooth muscle cells via the ERK signaling pathway [167]. In a rabbit model of atherogenesis fed high dose vitamin D, there was increased oxidative stress and aortic valve (AV) calcification/stenosis. The latter could be abrogated by the antioxidant lipoic acid [37]. Lipid oxidation products have direct effects on both bone forming and bone-resorbing cells. Oxidized LDL directly inhibits differentiation of osteoblasts [156] while directly inducing differentiation of osteoclasts [169]. Oxidized lipids also regulate osteoclastogenic cytokines produced by osteoblasts [170]. Thus, oxidative stress may be causative in vascular calcification, and may also explain the relationship between increased coronary artery calcification and osteoporosis found in both CKD and the general population [171].

Advanced Glycation End-Products (AGEs)

Proteins can be modified indirectly by reactive carbonyl compounds formed by auto-oxidation of carbohydrates and lipids, leading to eventual formation of AGEs. AGEs have been found in arterial and cardiac tissue as well as atherosclerotic lesions in dialysis patients [172]. Circulating AGEs such as pentosidine are elevated in patients on dialysis [173]. AGE-modified elastin and calcification has been found to co-localize in the aortic media of dialysis patients and binding of mineral to elastin is thought to be an important factor in the pathogenesis of medial calcification [174]. In cultured VSMC, AGEs can accelerate calcification of microvascular pericytes [175]. AGEs induced the expression of RUNX2 mRNA and alkaline

phosphatase activity and calcification [176]. The receptor for AGE (RAGE) is expressed in a variety of cells including VSMC [177] and these AGE mediated changes in VSMCs were partially attenuated by a neutralizing antibody to RAGE [178]. A study by Suga, et al. [179] demonstrated that activation of RAGE inhibited VSMC phenotypic gene expression and induces osteogenic differentiation of VSMC. This RAGE mediated effect was via Notch/Msx2 induction in VSMC. The results suggest that AGEs that accumulate in diabetes could elicit the osteoblastic differentiation of VSMCs, thereby contributing to vascular calcification via the RAGE pathway.

Abnormal Mineral Metabolism

6.8.1.Hyperphosphatemia: Abnormal mineral metabolism has been recognized as a nontraditional risk factor in the development of vascular calcification in CKD patients and is associated with increased mortality in both pre-dialysis and dialysis patients [27,180]. Hyperphosphatemia is associated with the prevalence and progression of vascular calcification in dialysis patients [181]. Several studies have demonstrated that the use of noncalcium-based as compared with calcium-based phosphate binders attenuated vascular calcification and mortality in dialysis patients [182,183]. In the general population, phosphorus levels in the upper quartile of the normal range are also associated with increased cardiovascular and all-cause mortality [184]. In vitro, phosphate increased the calcification of VSMC in dose-dependent manner [185]. High phosphate induced the loss of VSMC markers, such as smooth muscle (SM) α-actin and SM22α and increased the expression of the osteochondrogenic markers Runx2, osterix, osteopontin, and alkaline phosphatase [62,186]. A recent study has demonstrated that bovine VSMC incubated with calcification media (10 mM β-glycerolphosphate as a phosphate donor) generated cellular matrix vesicles that have high annexin II and VI content and the ability to mineralize extracellular matrix compared to that from bovine VSMC incubated without phosphate [187]. The matrix vesicles serve as nucleation sites for calcification, like the vesicles that bud from osteoblasts and hypertrophic chondrocytes in normal bone formation. Phosphate transport to cells is primarily mediated by sodium-dependent phosphate (NaPi) co-transporters [188] and treatment with phosphonoformic acid (PFA, a competitive inhibitor of NaPi transport) inhibits phosphate uptake and VSMC osteochondrogenic differentiation [185].

The type III NaPi co-transporters, PiT-1, is highly expressed in VSMC [189] and the knockdown PiT-1 with siRNA suppressed phosphate induced calcification and blocked induction of the osteogenic markers Runx2/Cbfa1 and osteopontin [189]. However, our group has shown that bovine VSMCs incubated with pooled uremic sera from dialysis patients had increased calcification, above that induced by phosphorus but only when phosphorus is available [190]. The addition of PFA (inhibitor of NaPi transport) or levamisole (inhibitor of alkaline phosphatase) only partially inhibited uremic serum-induced osteopontin upregulation. The cyclic adenosine monophosphate (cAMP)/protein kinase A signaling pathway was involved in uremic serum-induced upregulation of RUNX2 and alkaline phosphatase [191]. High phosphate may also regulate matrix mineralization through elastin degradation. A soluble elastin-derived peptide can induce mineralization of human VSMCs in the presence of high phosphorus concentration [192]. Treatment of rat VSMC with elastin peptide induced the expression of elastin–laminin receptors along with increased expression of osteoblastic transcription factor RUNX2 and alkaline phosphatase [193]. TGF-β which is known to upregulate RUNX2 [194], had synergistic effect on VSMC phenotypic change. In a rat aortic ring model, treatment with high phosphate and warfarin increased matrix metalloproteinase 9 (MMP-9) activity followed by transforming growth factor-β (TGF-β) signaling and aortic calcification [195]. One recent study demonstrated that MMP-2 and MMP-9 expression and activity are increased with progressive CKD, and blockade of MMP activity can inhibit arterial calcification [196]. This matrix degradation or alteration may be an initial step in calcification.

Hypercalcemia: There is an association with elevated serum calcium and the development of vascular calcification in the CKD population [180]. In addition, the use of calcium containing phosphate binders which induce positive calcium balance is associated with increased arterial calcification in the majority of studies [197]. In vitro, calcium alone can increase human VSMCs calcification [198]. Furthermore, calcium and phosphorus had synergistic roles in inducing mineralization of VSMC [199]. In an aorta ring culture model, elevated calcium was more potent than phosphorus to induce VSMC calcification for a given concentration of calcium and phosphorus, called the Ca × P product [200,201]. Calcium also stimulates VSMC matrix vesicle release [199]. Study by Chen et al have demonstrated that calcified VSMC derived cellular MV are enriched with annexin II and VI but with little fetuin-A [187]. Furthermore, blockade of annexin calcium channel activity with K201or the L-type calcium channel blocker verapamil significantly inhibit MV activity and the calcification of VSMC [202]. Shanahan and colleagues also demonstrated that blockade of intracellular calcium increase can inhibit MV calcification [1]. Abnormal mineral metabolism contributes to the development of vascular calcification by multiple mechanisms.

Fibroblast Growth Factor 23: The hormone fibroblast growth factor 23 (FGF-23) is predominately expressed in osteocytes and is involved in mineral homeostasis by inducing hyperphosphaturia, inhibiting calcitriol synthesis and inhibiting PTH secretion [203]. In the kidney it exerts its biological functions by binding to the FGF receptor in the presence of the cofactor Klotho [204]. Several studies have demonstrated that FGF-23 is associated with coronary artery and aortic vascular calcification in CKD and dialysis patients [205,206]. Targeted deletion of FGF-23 or Klotho in mice resulted in hyperphosphatemia and vascular calcification [204,207]. In

moderately uremic mice fed high-phosphate diets, elevated serum FGF-23 and osteopontin levels, but not serum phosphorus levels, were associated with extensive arterial-medial calcifications [208]. A recent study by Takei et al has demonstrated that the expression of stanniocalcin (STC) 2, a calcium/phosphate-regulating hormone, is increased and colocalized in calcified lesions of FGF-23 or Klotho null mice [209]. Although the mechanism by which FGF-23 affects vascular calcification is not clear at present, these data suggest that another mechanism by which phosphate affects vascular calcification may be through phosphorus-mediated elevation of FGF-23 levels.

Molecular and Cellular Mechanism of Arterial Calcification

Occurrence of vascular calcification (VC) has been discovered in the "Iceman" who lived 5000 years ago [210] and scientists had already paid attention to this phenomenon and to its relationship with renal disease in the 19th century [211]. Traditionally, two major forms of ectopic [pathologic] calcifications were distinguished; dystrophic refers to VC occurring in damaged tissues while metastatic was associated with systemic disorders of calcium and phosphate metabolism; these descriptions reflect the differences between vascular ossifications [active process] and petrifications [passive process] described by Virchow [212]. Initially VC was considered to be a passive process, the result of Ca2+ and phosphate ions exceeding solubility in tissue fluid, thereby inducing the precipitation and deposition of hydroxyapatite crystals [213]; however, VC is now considered as an active process that is complex, actively regulated via a variety of molecular signalling pathways and associated with crystallization of hydroxyapatite in the extracellular matrix and in cells of the media (VCm) or intima (VCi) of the arterial wall by involving the differentiation of macrophages and vascular smooth muscle cells (VSMCs) into osteoclast-like cell [214-216]. While considerable progress elucidating the signalling pathways regulating VC formation has been achieved, the exact molecular basis of VC remains elusive [217,218]. Within coming new research data, the already large number of molecular mechanisms suggested to contribute to VC formation continues to grow. The mechanism of arterial calcification is complex, but multiple investigators agree that the first step appears to be de-differentiation or transformation of vascular smooth muscle cells (VSMC) to an osteoblast/chondrocytic phenotype (Figure 1).

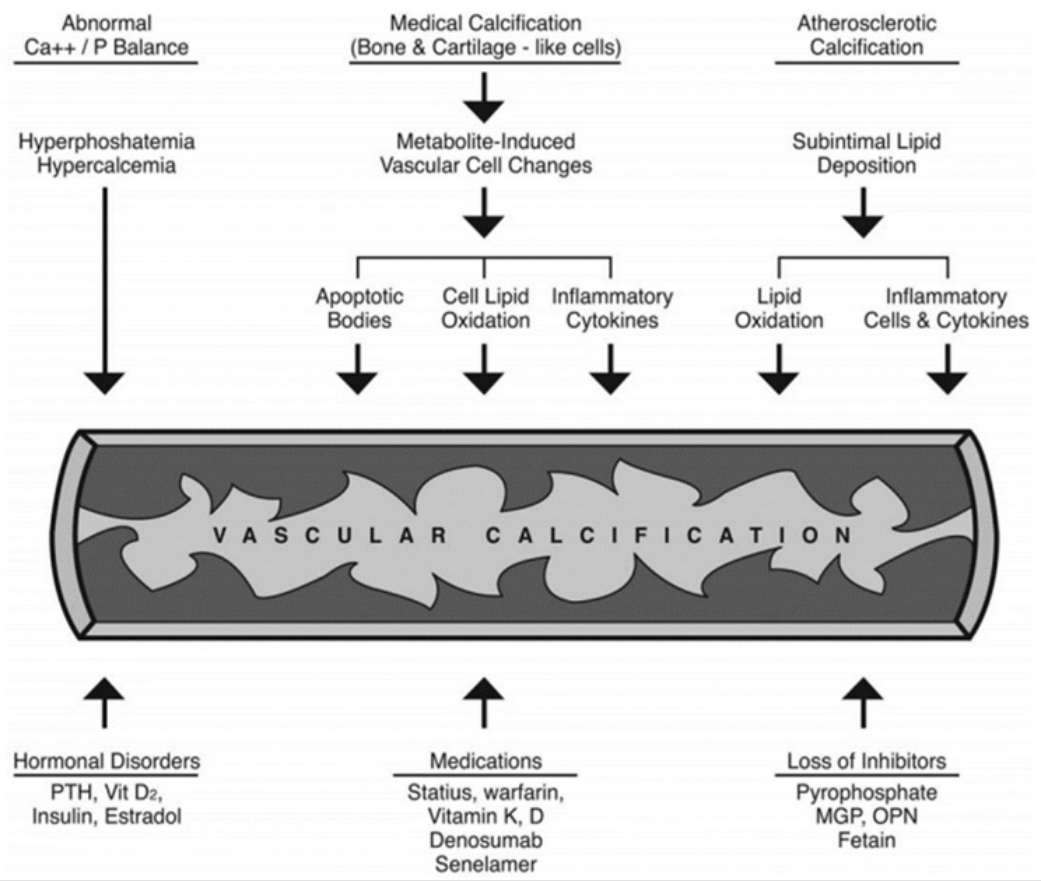

Figure 1: Schematic diagram depicting multiple mechanisms leading to vascular calcification.

VSMC originate from a similar mesenchymal stem cell as osteoblasts, the latter occurring with up-regulation of the transcription factor core binding factor alpha-1 (Cbfa-1) now called Runt-related transcription factor 2 (RUNX2) or msh homeobox 2 (MSX-2). These cells then do what a normal osteoblast does: secrete matrix proteins [98]. The signals that induce this transformation are multiple and once the matrix is laid down, these cells then mineralize the matrix through the secretion of matrix vesicles [187], or through apoptosis [219]. Phosphorus and calcium increase the mineralizing potential of these matrix vesicles [187,202]. It appears that while deposition of hydroxyapatite represents the resulting commonality of VC, different initiating and propagating molecular mechanisms, as well as diverse crystalline compositions of calcium apatite crystals may be present in various forms of VC [220-222]. The underlying pathophysiological mechanisms resulting in VC can be broadly described as: genetic predisposition certainly plays an important role in the genesis of this phenomenon [213]. According to Rutsch et al, 40 – 50% of cases of coronary calcification can be attributed to genetics [223]. Genes ENPP1 and NT5E are respectively implicated in infancy and idiopathic VC. The first one encodes a protein which transforms ATP to adenosine and pyrophosphate (PPi, inhibitor of calcification) whereas the second one converts AMP into adenosine and inorganic phosphate (Pi, accelerator of mineralization) [4,214].

The VC phenotype caused by mutations in these genes underlines the role of PPi and Pi in pathogenesis. Mutations in ABCC6, a gene encoding a nucleoside-sensitive transporter, have also been linked to hereditary calcification [42]. Alternative action of ABCC6 may include deficient hepatic production of inhibitory factor of matrix Gla protein (MGP), an important inhibitor of calcification [224]. Another major mechanism of development of VCs is looks like process through which bone formation occurs (Figure 1) [215,225]. First, vascular smooth muscle cells (VSMCs) undergo osteogenic differentiation into phenotypically distinct osteoblast-like cells [103,225]. In the case of renal failure, phosphate plays a key role in this mechanism [226]. In vitro, high extracellular phosphate concentrations induce a rise in intracellular phosphate concentration which is actively mediated by Pit-1, a sodium dependent phosphate co-transporter [185,189]. This increasing phosphate concentration in the VSMC induces a phenotypic switch of VSMCs into osteoblast-like cells [185,186,225]. The protein Cfba1/Runx2 [core-binding factor subunit 1α/runt-related transcription factor 2] is a specific and indispensable transcriptional regulator for this osteoblastic differentiation. Its expression is also enhanced with high extracellular phosphate [100,185,186]. These "new" cells will express alkaline phosphatase (ALP), secrete, under the control of Cfba-1, bone-associated proteins (such as osteopontin [227], collagen type 1, osteoprotegerin, bone morphogenic protein-2 and osteocalcin [228]) and release mineralization-competent MVs in the extracellular matrix [199,226]. VSMCs release MVs under normal physiological conditions and these MVs are protected from mineralization by the presence of calcification inhibitors [42]. Under pathological conditions, a combination of factors makes the MVs "mineralization competent" [229].

Moreover, an increase of intracellular phosphate level mediated by Na/Pi transporter is thought to induce VSMC apoptosis through an unclear process that possibly involves a disruption in mitochondrial metabolism [230]. Some studies suggest that apoptosis leads to calcification [231,232]. The MVs, in which proapoptotic factor BAX (BCL2-associated X protein) have been identified [233], may be remnants of apoptotic cells. As MVs have the capacity to concentrate and crystallize calcium, apoptosis could be a key regulator of VC [230]. More recently, a different point of view has emerged according to which phenotypically distinct osteoblast-like cells might originate from stem cells rather than VSMCs [42]. A new mechanism called "Circulating cell theory", suggesting an active role for circulating cells arising from sources such as bone marrow, has been postulated to contribute towards VC. It is well known that the extracellular fluid is a metastable soup about calcium and phosphate concentrations and that active inhibitors of calcification must be present, both circulating and locally, to prevent the spontaneous formation of apatite: a situation that certainly applies to the CKD population [23]. The active inhibition process involves vascular smooth muscle cells and several proteins, including some that are vitamin K-dependent. Under the influence of chemo-attractants [released by damaged endothelium for instance], these bone marker-positive cells may home to diseased arteries. Under pathologic conditions such as an imbalance between promoters and inhibitors of VC, this population may further undergo osteogenic differentiation in the lesions, which could promote vessel mineralization [42,234]. Another recent study has also claimed that multipotent vascular stem cells (MVSC) present in blood vessel wall might differentiate into osteoblast-like cells [235].

Nevertheless, this point of view is still very controversial. Although the role of phosphate is well established in osteoblastic differentiation process, many other factors can influence this conversion and accurate causal mechanisms remained not completely understood. Under normal conditions, VSMCs produce endogenous inhibitors of calcification such as matrix Gla protein (MGP), osteopontin, osteoprotegerin and pyrophosphate [6]. A long-term exposure of VSMCs to a variety of stresses can overwhelm the action of these inhibitors and induce differentiation [229]. Among these chronic stresses, ionic disorders (especially hyperphosphatemia and hypercalcemia) are incriminated but inflammation, hormonal perturbation, metabolic disorders, and oxidative stress can also lead to VC. Oxidative stress in VSMCs, generated by hyperlipidemia and oxidized lipoproteins or uremic milieu [166], causes the expression of runx2[168], osterix and govers Wnt signaling [236], leading to osteogenic differentiation. Inflammatory cytokines, such as TNF-α, can also induce calcification via Msx2/Wnt/β-catenin pathway [237].

In support of that, calcium deposits colocalize with inflammatory cells *in vitro* [161] and *in vivo* [160]. Moreover, it has been suggested that mineral crystals may themselves be pro-inflammatory, creating a vicious cycle of inflammation and calcification [220,238]. The receptor for advanced glycation end products (RAGE) endogenously expressed in endothelial cells and its ligands (in which S100 family proteins are found), are also known to be involved in atherosclerotic formation and VC [42]. It has been suggested that galectin-3 and RAGE modulate vascular osteogenesis in part via Wnt/β-catenin signaling [239]. Several trials have shown a raise in serum levels of S100/calgranulins in vascular disease [240,241]. Thereby, S100 proteins could be a potential biomarker and therapeutic target to develop [242].

Involved in the control of both parathyroid hormone (PTH) and calcitonin secretion, the calcium-sensing receptor (CaSR) is a G protein–coupled cell surface receptor that can sense extracellular calcium ions. Evidence have been provided to demonstrate that a decrease in the CaSR protein expression in the vasculature is directly involved in the development of VC [243,244]. It is of particular interest to note that calcimimetics, which are allosteric drug compounds that selectively target the CaSR, decrease VC at least in part through local control of the CaSR expression in VSMC [245,246]. However, so far, the mechanism whereby the CaSR exert its protective effect remains largely unknown. Hormones have pleiotropic effects on calcific vasculopathy. For example, the adipose-derived factor, leptin, promotes VC *in vitro* [247] and *in vivo* [248]. Adiponectin-deficient mice have increased vascular calcification [249]. The influence of PTH is part of bone turnover process. A disruption between promoters and inhibitors can also generate VC. Moreover, similar to bone formation, there might a balance between VC and its resorption. Indeed, monocytes and macrophages contained in the calcified wall can differentiate into an osteoclast-like phenotype and counteracts the action of VSMCs that have undergone osteoblast differentiation [250]. Hyperphosphatemia would disadvantage osteoclast phenotype by down-regulating RANK ligand-induced signalling [251] but this is not clear whether osteoclast-like cells can really counteract VC or solely witness vascular remodelling process. All these modifications will favour for an optimal microenvironment for hydroxyapatite formation and calcification. Similar osteogenic differentiation is also observed, *in vivo*, in animal and human uremic models [98,103,186].

Clinical Assessment Methods of Vascular Calcification

There are several methods to assess the amount of arterial calcification: conventional radiography, dual-energy X-ray absorptiometry (DXA), multi-slice computer tomography (MSCT), electron beam computer tomography [EBCT], magnet resonance imaging [MRI], ultrasound, intravascular ultrasound (IVUS) and optical coherence tomography (OCT) [252]. Unfortunately, except for intravascular ultrasound, none of these techniques can distinguish intimal from medial calcification [140,182]. In clinical practice, there are also other established methods for measuring arterial stiffness (AS) with functional measurements and diverse imaging methods. However, the direct prediction of mVC is not easy for all these methods.

Functional (Hemodynamic) Measurements

A clinically easily applicable method to assess mVC is the measurement of the ankle-/brachial index (ABI) with a high ABI (> 1.3) serves as marker of VC [253]. Conversely, the estimation of local stiffness is an established only by direct measurement of parameters strongly linked to stiffness. The pulse wave velocity (PWV) is a robust and reliable parameter that is considered as gold standard for AS assessment. The determination of PWV, is a one of the simplest ways to estimate the level of AS, includes the measurements of the distance covered by the wave and the time required to cover the distance (PWV = distance/time delay). Basically, the regional PWV of each vessel in the body can be measured. However, aorta and its major branches represent the main sites of interest and has clinical relevance, because they contribute to the larger part of the arterial buffering function [254] and responsible for most of the pathophysiological sequels of increased stiffness. Carotid-femoral PWV is very suitable and easily applicable in the clinic, as it assesses the aortoiliac pathway. There are also established methods for the measurement of the PWV in the upper [brachial PWV] and lower limbs (femoro-tibial PWV) [255]. The carotid-femoral PWV has predictive value for cardiovascular events in several epidemiologic studies in different populations [256-258] while PWV measurements outside the aortic track demonstrated no correlation with cardiovascular events [259].

Another method used to assess local stiffness is the measurement of arterial distensibility using ultrasound or echotracking devices for the detection of diameter changes of the vessel during systole and diastole [260]. The method's limitations are its dependence on high spatial resolution and the high degree of technical expertise required. Furthermore, only superficial arteries, such as the common carotid or femoral artery, are examined because its depth penetration is limited. The determination of pulse pressure (PP) and the augmentation index (Aix) is another method to assess AS. In case of stiff arteries, the reflected wave arrives earlier in the central arteries and augments the systolic pressure. As a result, the PP and AIx ((first systolic peak-second systolic peak)/ (pulse pressure increase)) [252]. The limitation of PP and AIx determination is their dependency on other conditions such as heart rate, ventricular contractility, duration and pattern of ventricular ejection, reflectance point and measured vessel segment. It has been elegantly shown by Scuteri, et al. [261] that PWV and therefore AS increase with age independently of blood pressure development. Furthermore, patients with ESRD on regular haemodialysis usually have higher PWV in comparison with healthy controls [262], and the PWV increase over time is higher than in the healthy population. Therefore, PWV seems to be a marker for time-dependent ageing processes in the vascular system and less sufficient as specific indicator for AS [255,263].

Imaging Methods

Conventional radiography is a semi-qualitative method with several established scores. The abdominal aortic calcification score was proposed by Kauppila, et al. [264] to assess the extent of calcification of the abdominal aorta in front of the lumbar vertebrae on a lateral X-ray of the lumbar spine. However, conventional radiography of peripheral vessels may be a useful marker to measure Vamp [265]. Conventional radiography can indirectly indicate the presence of VCm by a 'tram track' calcification pattern in comparison with a 'patchy' pattern typically when atherosclerotic plaques are calcified [266,267]. Linear 'tram-tracks' are a typical appearance of VCm on conventional radiography [268], whereas a 'patchy' pattern typically suggesting atherosclerosis [266,267]. However, the sensitivity and specificity of conventional radiographs for detecting VCm or discrimination between VCm and intimal calcification remain uncertain [269,270]. Dual-energy X-ray absorptiometry is also a well-established method usually used for the measurement of bone mineral density but can also be used for simultaneous semiquantitative assessment of VC [271]. Both MSCT and EBCT are also very sensitive and precise imaging techniques for the detection and quantification of calcification [272]. MRI is, in general, a superior method for imaging soft tissue while it is not suitable for reliable assessment of VC, due to very short calcium echo time [273]. During endovascular interventions, IVUS [274] and OCT [275] can be also used to detect and quantify the amount of VC. Although OCT provides higher resolution than IVUS, its penetration depth is not sufficient to evaluate the entire medial layer. In the clinical setting, multidetector computed tomography is often used and generates a quantitative calcium score [276,277], which is a potent predictor for cardiovascular events [278]. Most studies identified intimal calcification as predictor of a vulnerable plaque phenotype, the punctated "spotty calcification" [277,279].

Circulating Biomarkers

A comprehensive approach including gathering of exact patient history and performing hemodynamic measurements and imaging studies is needed to determine the presence of arteriosclerosis, to quantify its amount and to provide discrimination from atherosclerosis. Currently, the latter issue is not still difficult as hemodynamic measurements nor imaging studies can certainly exactly distinguish between intimal and medial calcification. Therefore, new specific probes imaging microcalcification can provide a platform to study the earliest events associated with VC at the molecular and cellular level. The use of circulating biomarkers such as MGP for detecting or screening VC is an attractive possibility. Vitamin K-dependent proteins have been associated with the earliest calcification areas in the plaque [280]. It was the uncarboxylated form of MGP that strongly correlated with both medial and intimal calcification [280,281]. By measuring circulating MGP isoforms it was shown that most of the healthy population have sub-optimal levels of vascular vitamin K [282,283]. Preliminary data confirmed MGP are associated with aspects of cardiovascular disease as patients with high VC scores display high levels of inactive MGP, especially dialysis patients [284-286].

Conclusion

Vascular calcification is recognized as an active cellular process that occurs in response to metabolic insults that is intimately entwined with aging, abnormal mineral metabolism, and other related chronic diseases (i.e., DM, CKD). Within vascular microenvironment itself, a dense and interconnected network of calcification inhibitors and promoters were highlighted. Under normal conditions, there is a balance between all these parameters. Currently, vascular calcification is regulated by a complex pathophysiological mechanism, primarily triggered when there is an imbalance between inhibitors and promoters, in favour of osteogenic proteins and transcription factors synthesis, in detriment of bone reabsorption mediators. According to active theory, VSMCs undergo differentiation into osteoblast-like cells, in great part because of an increased intracellular phosphate concentration that is likely mediated by the co-transporter Pit-1 in response to extracellular hyperphosphatemia. Other risk factors such as advanced age, smoking, inflammation, oxidative stress, mineral and bone disorders (MBD) are also known to be associated to VSMCs conversion. As evidenced by different clinical observations, animal models, and molecular studies, the exact molecular and cellular mechanism of vascular calcification is still far from being fully elucidated. Thereby, the challenge remains to understand which mechanisms are active and/or predominate under various disease states, and to develop effective therapeutic strategies that may prevent and potentially reverse vascular calcification. Correspondingly, qualities that would be appreciated for selecting a good vascular calcification biomarker depend on its capacities to achieve clinical goals, particularly its ability to select high risk patients for further investigation, to make a reliable calcification assessment, to provide a prognostic, to help in treatment choice or to follow up the treatment efficiency. However, functional characteristics and imaging methods are commonly used for the diagnosis of the calcified arterial injury.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent For Publication

Not applicable.

Availability of Data and Material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Authors' Contributions

LM had participated in the design of the study, data analyses, and manuscript preparation; and the authors could have read and approved the final manuscript.

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