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Review

Vascular Calcification: Molecular Networking, Pathological Implications and Translational Opportunities

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Abstract: Calcification is a process of accumulation of calcium in tissues and deposition of calcium salts by the crystallization of PO_4^{3-} and ionized calcium (Ca^{2+}). It is a crucial process in the development of bones and teeth. However, pathological calcification can occur in almost any soft tissue of the organism. The better studied is vascular calcification, where calcium salts can accumulate in the intima or medial layer or in aortic valves, and it is associated with higher mortality and cardiovascular events, including myocardial infarction, stroke, aortic and peripheral artery disease (PAD), and diabetes or chronic kidney disease (CKD), among others. The process involves an intricate interplay of different cellular components, endothelial cells (ECs), vascular smooth muscle cells (VSMCs), fibroblasts, and pericytes, concurrent with the activation of several signaling pathways, calcium, Wnt, BMP/Smad, and Notch, and the regulation by different molecular mediators, growth factors (GFs), osteogenic factors and matrix vesicles (MVs). In the present review, we aim to explore the cellular players, molecular pathways, biomarkers, and clinical treatment strategies associated with vascular calcification to provide a current and comprehensive overview of the topic.

Keywords: vascular calcification; signaling pathways; endothelial cells (ECs); vascular smooth muscle cells (VSMCs); biomarkers

1. Introduction to Pathological Calcification

Calcification is a process of accumulation of calcium in tissues and deposition of calcium salts by the crystallization of PO_4^{3-} and ionized calcium (Ca^{2+}). While it serves as an essential mechanism for bone and teeth development and maturation, pathological calcification can manifest in almost all soft tissues, which is associated with aging and a variety of diseases [1]. In bone physiology, calcification plays a pivotal role in skeletal growth and strength [2]. This process involves the deposition of calcium phosphate crystals within a matrix, transforming into a rigid and resistant skeletal framework, and it is finely regulated by a network of osteoblasts, osteoclasts, stem cells, and signaling molecules, ensuring the formation of robust bone tissue [3]. Proteins and inorganic crystals combine to form the composite tissues of mammalian teeth and bones. These tissues are made up of about 70% inorganic materials, 20% proteins, and 10% water by weight [4]. Inorganic crystals resist compressive stresses and increase tissue toughness by depositing on matrix proteins; type I collagen is the main protein component of the matrix, and hydroxyapatite is the main inorganic component of the tissues [5].

Aging is the primary cause of pathological calcification, although tumors, blood vessels, and joints are often associated with this process [6]. Pathological calcification occurs through various pathways with varying levels of cellular control in non-skeletal tissues such as the vasculature and neoplasms [7]. Calcified deposits can be adverse by causing mechanical stress or stiffness in affected tissues and have been linked to cellular damage and inflammation, although they are often used as a marker of disease [8]. Calcified deposits are calcium phosphate crystals anchored within the extracellular matrix.

Depending on the conditions leading to the development of calcium phosphate crystals, dystrophic calcification manifests as the accumulation of calcium in regions affected by trauma or necrosis, which is derived from various causes such as blunt trauma, inflammation, injections, and the presence of parasites, with normal plasma levels of calcium and phosphate [9]. In contrast, metastatic calcification occurs when abnormalities in the levels of calcium and phosphate ions in the blood serum, i.e., hypercalcemia, lead to calcification in previously normal tissue [10–12]. Furthermore, iatrogenic calcification can be caused by some medical procedures, such as surgery, radiation therapy, or the administration of calcium or phosphate-containing agents [13]. For example, the calcification of skin known as calcinosis cutis appears after patients with tuberculosis receive intravenous calcium gluconate, calcium chloride, and para-aminosalicylic acid [14]. Although pathological calcification can occur in almost all soft tissues of the body, the most prone areas are blood vessels, heart valves, brain, breast, kidneys, gastric mucosa, lungs, and tendons, and research has focused primarily on vascular calcification [15–22] (Figure 1).

The mineral produced in ectopic calcification is less ordered, has variable crystal sizes and shapes, and is composed of calcium phosphate crystals and other calcium salts in contrast to physiological mineralization [23]. Glycosaminoglycans are present on the surface of hydroxyapatite crystals in both blood vessel calcification and bone mineralization, according to chemical and nuclear magnetic resonance (NMR) investigations. In general, calcium is deposited in the form of fine white granules or gritty clumps [24]. Microscopically, in sections stained with hematoxylin and eosin, calcium appears as basophilic, amorphous granular, or agglomerated and appears in black with the von Kossa stain. Interestingly, the presence of ribosomal RNA (rRNA) derived from ribosome degradation and nuclear chromatin contributes to aortic valve calcification through the stimulation of hydroxyapatite nucleation capacity exerted by the cell membrane-derived substance containing acidic phospholipid substrates (PPM/PPLs) with their anionic charges [25].

The deposition of calcium salts in blood vessels is known as vascular calcification. The calcium phosphate deposits can be found in the medial and intimal layers and aortic valves [26,27]. The intimal calcification is associated with atherosclerosis. Normally, the calcium salts are formed in the ECM rather than intracellular in the intimal layer, which is associated with atherosclerotic plaques, medial layer, or aortic valves [28]. The mineral phases can be apatite, whitlockite, or octacalcium phosphate ranging in sizes from

submicron to larger than 0.5 mm [29]. Vascular calcification together with remodeling of the extracellular matrix (ECM) results in arterial stiffness and contributes to vessel rupture [30]. Recent findings with Raman spectroscopy indicate an interaction between intimal and medial calcifications related to atherosclerosis, demonstrating an elevation of the apatite/whitlockite ratio in the aortic media precisely beneath an atherosclerotic plaque [31]. Medial calcification, also termed Mönckeberg sclerosis, occurs preferentially along the elastic lamina and is usually identified in small and medium-sized arteries of the lower extremities. It is associated with advanced age, diabetes, and chronic kidney disease (CKD), and, unlike intimal calcification, it occurs independently of atherosclerosis [32–34]. It is a process similar to intramembranous bone formation, involving the transdifferentiation of vascular smooth muscle cells (VSMCs) to mineralizing cells. Arterial stiffness and calcification of the arterial media create a vicious circle, in which ECs are often the initiators [35]. The calcific aortic valve disease (CAVD) is the cause of the thickening, fibrosis, and mineralization of the aortic valve leaflets, which ranges from aortic sclerosis (mild valve thickening) to aortic stenosis (severe calcification with impaired leaflet motion) [36–39]. The valve endothelial and interstitial cells and immune cells promote the remodeling of aortic valve leaflets. Atherosclerotic risk factors contribute to the risk of CAVD, such as age, smoking, hypertension, hyperlipidemia, and diabetes [40].

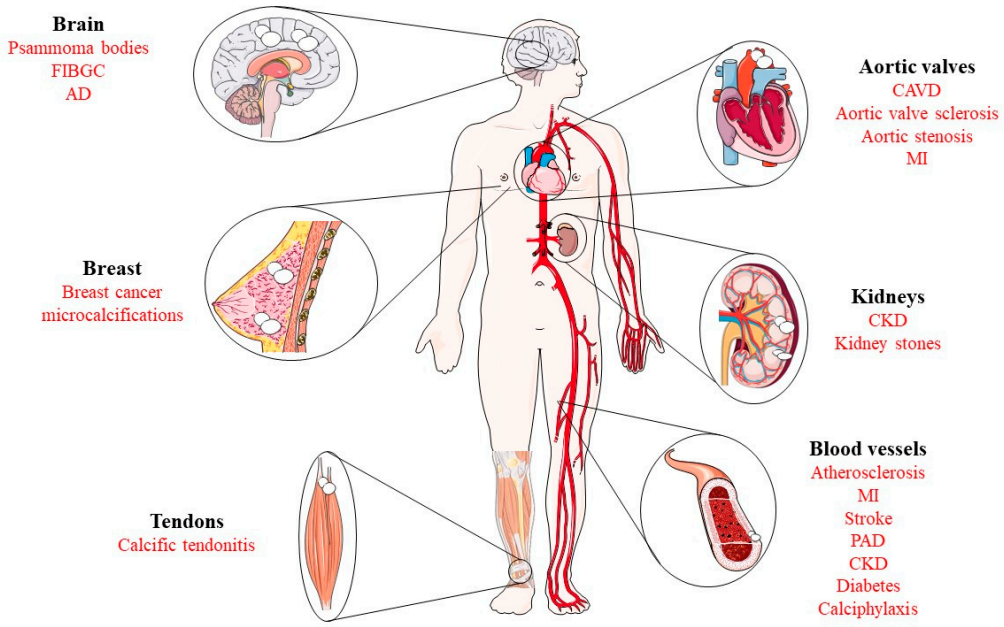

Figure 1. Schematic representation of the main anatomical locations susceptible to the deposition of calcium salts: blood vessels, aortic valves, breast cancer, brain, tendons, or kidneys. In black appear the anatomical locations of calcium deposits and in red the related diseases. FIBGC: familial idiopathic basal ganglia calcification, AD: Alzheimer's disease, CAVD: calcific aortic valve disease, MI: myocardial infarction, CKD: chronic kidney disease, PAD: peripheral artery disease.

On the other hand, the structure–function relationship of soft tissue calcification is a very dynamic and intricate process that still requires further understanding. Different cellular types play a role in the process and multiple signaling pathways have been recognized, including Ca^{2+} signaling, Wnt/β-catenin, BMP/Smad, and Notch pathways, and different molecular mediators are involved, such as osteogenic factors, growth factors (GFs) or matrix vesicles (MVs) [1,41–45]. The study of the molecular pathways may drive the exploration of potential therapeutic targets and the use of proper biomarkers. Therefore, the present review aims to explore the cellular players, molecular pathways, biomarkers, and clinical treatment strategies associated with calcification to provide a current and comprehensive overview of the topic.

2. Cellular Players in Vascular Calcification

Several actors are involved in soft tissue calcification: not only cells but also cell matrix components. In this section, we will center on the main cell types playing a role in the deposition of calcium, including specialized cells in which changing microenvironments led to an osteogenic-like phenotype. The study of the mechanisms governing vascular calcification is difficult, which is mainly due to the unresolved origin of calcified cells within cardiovascular lesions. These cells could originate from various sources: VSMCs undergoing dedifferentiation and proliferation, bone marrow-derived stem cells present from the bloodstream, or multipotent calcifying vascular cells resident in the vascular wall which can differentiate into osteoblasts and chondrocytes with the contribution of endothelial cells, macrophages, pericytes, and fibroblasts [46]. These cells are characterized by the expression of calcification markers, including some transcription factors such as Msx2 (Msh homeobox 2) and Runx2, which are both responsible for the osteogenic phenotype, Cbfa1 (core-binding factor α1), SP7/Osterix or SOX9, some proteins such as osteopontin (OPN), osteocalcin (OC), alkaline phosphatase (AP), bone sialoprotein and collagens II and X, while the contractile markers are decreased or lost [47,48]. Runx2 is the principal activator of mineralization, as it is the transcription factor that upregulates the expression of osteoblastic differentiation genes, e.g., osteocalcin and osteopontin.

2.1. Endothelial Cells

ECs are the vascular inner lining epithelium and have a critical role in the maintenance of blood flow. The EC plasticity is responsible for vascular calcification, specifically the endothelial-to-mesenchymal transition, which is a phenomenon also found in normal biological processes such as embryonic development. In both cases, ECs lose their cell features and acquire new markers that characterize the osteogenic lineage through the increase in the osteocalcin expression in areas where stretching occurs [49,50]. These places will be areas for ectopic calcification due to the EC dysfunction that affects endothelial secretory function and inflammatory regulation [51]. The human umbilical vein ECs (HUVECs) could suffer a mesenchymal transition through the overexpression of OCT-4 in the presence of some members of the transforming growth factor-β (TGF-β) family, specifically bone morphogenetic protein type 4 (BMP4), to induce osteogenesis in vitro experiments [52].

2.2. Vascular Smooth Muscle Cells

Vascular smooth muscle cells (VSMCs) form the middle layer of the vascular tree or the contractile layer of the digestive or genitourinary systems, contributing to the optimal functioning of the body. In the vascular system, the VSMCs are the major cellular component of the middle layer and are directly involved in the intimal metastatic calcification in several pathologies, because these cells can switch toward an osteogenic phenotype when the deposition of calcium increases in these tissues [53,54]. Sometimes, VSMCs can become foam cells changing the expression of SMCs markers as α-smooth actin and increasing macrophage markers as CD68+ and ABCA1 [55]. In addition, interaction between ECs and VSMCs can exacerbate arterial calcification in cases of hyperphosphatemia [56]. This occurs through the release of endothelial cell-derived exosomal miR-670-3p, which targets IGF-1 upon uptake by VSMCs. The osteoblastic/chondrogenic differentiation of VSMCs is regulated by various factors, including mechanical, biochemical, and molecular signals. Mechanical forces, such as transmural pressure, pulsatile pressure, and shear stress, promote dedifferentiation toward the synthetic phenotype through the mechanotransduction signals from the cytoskeleton [53,57,58]. Hypoxia within the vascular microenvironment can trigger signaling cascades that contribute to the differentiation of VSMCs toward a chondrogenic phenotype [59]. The mechanisms underlying VSMC calcification under hypoxic conditions require either hypoxia-inducible factor (HIF) activation or the production of mitochondrial-derived reactive oxygen species (ROS) [60,61]. High levels of inorganic phosphate (Pi) increase VSMC migration and calcification and are related to impaired

Pi/calcium balance and miR-223 involvement, whereas TGF-β/Smad3 signaling plays an inhibitory role in Pi-induced VSMC calcification [62–64]. Various cytokines and GFs regulate VSMC differentiation, such as TNF-α, IL-1β, IL-6 or TGF-β. The tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) enhance the VSMC calcification through their receptors [54,65,66]. M1 macrophages secrete TNF-α, and it is upregulated by miR32-5p in the microenvironment [67,68].

2.3. Macrophages

Macrophages are antigen-presenting cells (APCs) involved in inflammation processes releasing different cytokines; these cells detect phagocyte-damaged cells and pathogens, such as bacteria. Several subsets of macrophages are observed in tissues, depending on the lesion characteristics, being M1 and M2 as the predominant subtypes. M1 macrophages are considered classically activated and exhibit a proinflammatory profile, being responsible for removing pathogens and the release of ROS, and they are associated with the presence of microcalcifications; M2 macrophages or activated macrophages have an anti-inflammatory profile and promote tissue repair, being involved in the macrocalcification development [69,70]. Their plasticity is a key in some pathological processes to control the progression of abnormalities in tissues, as in the atherosclerotic plaque, where M1 is predominant in the lesion shoulder, M1 and M2 in the necrotic core, and M2 in the adventitia [71]. In addition, the calcium–phosphate imbalance impairs the phagocytic clearance of VSMCs-derived apoptotic bodies (ABs) by macrophages and promotes the release of pro-calcific MVs [72,73]. The accumulation of ABs serves as a nucleation site for the deposition of Ca/P nanocrystals, contributing to vascular calcification [74,75].

2.4. Pericytes and Fibroblasts

Pericytes are perivascular cells covering microvascular capillaries, and they are considered as a reservoir of precursor cells. These cells have been shown to have an osteogenic potential in a model of in vivo osteogenesis, forming mineralized nodules, and they have been able to express osteoblastic markers such as osteonectin, OPN, osteocalcin, and bone sialoprotein [76,77]. In addition, pericytes by the secretion of osteoprotegerin (OPG) may be involved in the formation of osteoid metaplasia [78].

Fibroblasts can acquire a "myofibroblast" phenotype, essentially muscle-fibroblast intermediate cells with contractile properties, which are connected to vascular calcification. These present the capacity to transition into calcifying osteoblast-like cells under specific conditions and mechanical and inflammatory stresses [79]. In these processes, TNF-α, elastin degradation products, TGF-β1 or ROS are involved [80,81].

3. Molecular Networking in Vascular Calcification

3.1. Signaling Pathways in Vascular Calcification

Different signaling pathways and molecular mediators lead to both physiological and pathological tissue calcification. Primarily, the activation of fundamental pathways such as calcium, Wnt/β-catenin, BMP/Smad, and Notch initiate this phenomenon [1,42,82,83]. In addition, molecular mediators regulate this process, including osteogenic factors, GFs, and MVs [84–86].

The calcium ion is the most versatile cellular messenger, and its functions are cellular communication, intracellular signaling, neuronal function, muscle contraction, cell cycle regulation, cell death, or enzyme activation [87,88]. Extracellular calcium serves as the primary initiator of vascular calcification. Dysfunctions in calcium regulatory proteins, including calcium-sensing receptors (CaSRs), calmodulin, and other associated molecules, lead to pathological calcification. It has been observed an increased CaSR expression in valvular interstitial cells (VICs) of calcified aortic valves and its modulation of CaSR expression in these cells has been shown to reduce calcium-induced valve calcification in vitro [89]. The upregulation of CaSR expression in VSMCs reduces cellular calcium deposition and difficulties in their transition to a calcifying phenotype [90]. VEGF-driven Runx2, a marker

of calcification whose expression in VICs operates through the IP3R/CaMKII/CREB axis and, in hypoxic conditions, miR-7-5p, facilitates osteoblastic differentiation and the calcification of human aortic smooth muscle cells (HASMCs) by activating calponin-3 and CaMKII [91,92]. Also, the calcium-dependent cytosolic phospholipase A2α (cPLA2α) contributes to aortic valve calcification in vitro [93].

The Wnt pathway plays diverse functions in embryonic development and adult homeostasis [94]. The activation of different Wnt signaling pathways promotes vascular calcification and valve sclerosis through different mechanisms [95]. The canonical Wnt/β-catenin functions by the binding of an extracellular Wnt ligand to a seven-pass transmembrane Frizzled (Fz) receptor and co-receptor low-density lipoprotein receptor-related protein (LRP5/6) activating β-catenin. Inside the nucleus, β-catenin forms a transcriptional complex with LEF-1/TCF DNA-binding transcription factors and induces the expression of specific target genes. In the case of pathological calcification, it upregulates markers of calcification, such as Runx2, BMPs, PPAR, or OPN [82,95]. On the other hand, the non-canonical Wnt signaling pathway stimulates pathological calcification through the Ror1/2 co-receptor and activates both branches, the Planar Cell Polarity pathway or PCP pathway and the Wnt/Ca^{2+} pathway [96,97]. The former triggers the activation of small GTPases Rho and Rac, leading to alterations in the cell cytoskeleton and lateral asymmetry. The latter implies the increase in cytosolic Ca^{2+} through phospholipase C (PLC), which activates both calmodulin-dependent protein kinase II (CamKII) and protein kinase C (PKC) [98]. These lead to the upregulation of target genes via the activation of transcription factors, such as nuclear factor-κB (NF-κB) or cAMP-responsive element binding.

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily essential for development, but they also participate in the regulation of cardiovascular structure and function. The extracellular binding of BMP to type I and type II receptors leads to the formation of heteromeric signaling complexes [99,100]. The canonical pathway induces the phosphorylation of Smad1/5/8 (R-Smad), which binds to Smad4 and the complex translocates into the nucleus to regulate gene expression [101]. The Smad-independent non-canonical pathway activates the ERK, JNK, and p38 MAPK signaling pathways, leading to the phosphorylation of several transcription factors (TF), such as serum response factor (SRF), ternary complex factor (TCF) family members, activator protein 1 (AP1) complexes and activating transcription factor 2 (ATF2) [102,103]. Several investigations have highlighted the ability of BMP-2, BMP-4, and BMP-6 to stimulate the development of vascular calcification in association with atherosclerosis [104,105]. These BMP ligands direct osteogenic programming and promote the expression of Runx2 and Msx2. The receptor activator of nuclear factor κB ligand (RANKL) increases VSMC calcification by promoting BMP-4 expression [106]. Altered blood flow in large vessels downregulates KLF2 expression, leading to endothelial–mesenchymal transition (EndoMT) and increased vascular calcification through BMP-4/Smad1/5 signaling [107]. BMP-6 and ox-LDL synergistically induce osteogenic differentiation and mineralization, highlighting an important connection between BMP signaling, oxidative stress, and inflammation in vascular calcification associated with atherosclerosis [108]. Conversely, BMP-7 exogenous administration has demonstrated anti-calcifying effects in rodent models of uremia, where it attenuates vascular calcification together with the downregulation of Runx2 and osteocalcin [109,110].

Lastly, Notch signaling is crucial for development, differentiation, and tissue homeostasis. It consists of a juxtacrine signaling between Notch transmembrane receptors making contact with Notch transmembrane ligands in neighboring cells. This interaction triggers the proteolytic cleavage of the Notch receptor, resulting in the release of the Notch intracellular domain (NICD). This acts as a transcription regulator of CSL, HES/Hey, and Runx2 [111,112]. The Notch/RBP-Jk signaling directly upregulates *Msx2* gene expression in VSMCs [83]. On the other hand, Notch presents inhibitory effects of vascular calcification. The activation of Notch1 induced by USP9X S-nitrosylation inhibits the calcification of porcine aortic VIC regulated by nitric oxide (NO) [113]. Elevated Wnt16 levels stimulate Notch activity and counteract TGFβ3-induced Notch suppression in VSMCs [114]. This

process leads to reduced chondrogenesis in VSMCs exposed to TGFβ3, suggesting that the absence of Wnt16 plays a pivotal role in TGFβ3-induced chondrogenic change in VSMCs. Moreover, exosomal Notch3 released by endothelial cells, when present in high glucose environments, decreases vascular calcification by inhibiting mTOR [115]. Lastly, the induction of Notch1 and matrix γ-carboxyglutamate (Gla) protein (MGP) by shearing results in the downregulation of osteoblast-like genes in human aortic valve endothelial cells [116].

3.2. Molecular Mediators of Vascular Calcification

In addition to the above-mentioned signaling pathways, multiple molecular mediators play an important role in the process of vascular calcification, including GFs, osteogenic factors, and MVs. Within the GFs, some of the ones that stand out are TGF-β, in particular the aforementioned BMPs, platelet-derived growth factor (PDGF), fibroblast growth factor 21 and 23 (FGF21/23), and vascular endothelial growth factor (VEGF). PDGF accelerates vascular calcification through different mechanisms that combine the induction of inflammation (IL-1β, IL-6, MCP-1, and ICAM-1), oxidative stress (ROS), phenotype transition (BMP2, Pit-1, OPG, and CNP), and mesenchymal stem cells (MSCs) migration A [117]. Elevated levels of circulating FGF23 and its overexpression in blood vessels are associated with vascular calcification [85]. Contrary to this, FGF21 has gained special attention due to its anti-calcifying effects [118,119]. In rats, it alleviates the endoplasmic reticulum stress-mediated apoptosis pathways. Lastly, it has been proposed that VEGF induces Ca^{2+}/CaMKII activation through VEGF receptor 2, leading to the upregulation of Runx2 and therefore vascular calcification [92].

The osteogenic factors are those that regulate the process of calcification, both promoters and inhibitors. Among these promoters, cadherin-11 (Cad11) is a cell–cell adhesion protein related to inflammation in rheumatoid arthritis. Cad11 induces aortic valve calcification through Rac1, and its overexpression leads to the upregulation of RhoA and Sox9 and extracellular matrix remodeling [120,121]. Calcific nodule morphogenesis by valvular myofibroblasts requires robust cell–cell connections regulated by Cad11 [122,123]. In contrast, the several osteogenic factors that inhibit vascular calcification include MGP, OPN, OPG, fetuin-A, and also small molecules, such as inorganic pyrophosphate (Ppi), bisphosphonates, and magnesium [124,125]. MGP has been proposed to act through the direct inhibition of calcium–phosphate precipitation, the formation of MVs, the formation of ABs, and the transdifferentiation of VSMCs, and it is an independent predictor of both intimal and medial vascular calcification in CKD [126–128]. Phosphorylated OPN acts as a physiological inhibitor of vascular calcification and reduces inflammation factors in osteoclast formation by the regulation of macrophage activation in hypertensive patients [129,130]. Endogenous arterial OPG shows a protective role against vascular calcification through different mechanisms in animal models, such as the inhibition of ALP-mediated osteogenic differentiation of vascular cells and downregulation of the Notch1–RBP–Jκ signaling pathway, while in humans, plasma OPG is probably produced as a response against vascular calcification [131–133].

MVs are tiny extracellular structures originating from chondrocytes and osteoblasts, essential in early mineralization, especially in the formation of hydroxyapatite. Ranging in size from 100 to 700 nm, MVs contain hydroxyapatite nanocrystals, which are observed in aortic and media valve calcification and atherosclerotic lesions [45]. Unlike spherical particles in aortic valve calcification, MVs are observed in vessel walls with a hollow core and amorphous minerals [86]. Their formation is linked to intercellular calcium signaling and contains calcium-binding annexins and alkaline phosphatase, which hinders the action of pyrophosphate and favors hydroxyapatite formation [134]. VSMCs regulate mineralization; under healthy conditions, inhibitors prevent their calcification [135]. VSMCs in atherosclerosis and CKD lack mineralization despite their presence in the tissue, which is associated with elastin and collagen fibrils [136]. Elevated extracellular calcium triggers VSMC responses, influencing MV-related calcification, but the exact mechanisms are under exploration.

Also, MVs released locally or the presence of circulating nucleation complexes serve as sites for calcium complex crystallization. Indeed, increased bone turnover, such as in the case of postmenopausal osteoporosis, is related to the release of circulating nucleation complexes that contribute to vascular calcification [137,138]. The process of bone remodeling regulates the calcium levels in the body and is masterly modulated by the OPG–RANK–RANKL system [139].

The vascular calcification process is influenced by many more factors, which present a balance between inhibitors and promoters (some of them are reviewed below). This is impaired under pathological conditions, leading to a decrease in protective inhibitors. Among the factors known to decrease calcification are osteopontin, OPG, BMP-7, magnesium ions (Mg^{2+}), osteonectin, vitamin K, high-density lipoprotein cholesterol (HDL-C), growth arrest-specific protein 6, albumin, parathyroid hormone, parathyroid hormone-related peptide, phosphonoformic acid, C-type natriuretic peptide, and adrenomedullin [130,133,140–151].

Conversely, factors that promote calcification include TNF-α, TGF-β, ROS, platelet-derived growth factor (PDGF), cadherin-11 (cad-11), the BMP-2/Smad pathway, klotho, IL-1, -4, and -6, oxidized and acetylated low-density lipoprotein cholesterol (LDL-C), C-reactive protein (CRP), leptin, advanced glycation end products, glucocorticoids, type I collagen, fibronectin, 25-hydroxycholesterol, 17β-estradiol, uremic serum, 1,25-dihydroxycholecalciferol, and cyclic adenosine monophosphate (cAMP) [117,120,152–169].

4. Pathological Implications

Pathological calcification does not usually cause clinical dysfunction, but significant deposits in organs can cause organ damage, such as nephrocalcinosis, leading to renal failure. Calcium deposition decreases the mechanical elastance of the arteries required for physiological functions; this is especially risky for the aorta, which has an impact on cardiovascular hemodynamics and contributes to significant morbidity and mortality [26]. Vascular calcification has been related to different cardiovascular diseases, including myocardial infarction (MI), stroke, aortic and peripheral arterial disease, heart failure, chronic kidney disease, diabetes, aortic stenosis, and calciphylaxis [48].

Vascular calcification, particularly in the coronary arteries, significantly influences the trajectory and outcome of myocardial infarction (MI) [170]. Coronary artery calcification (CAC) is a key marker of plaque burden, as elevated CAC, often identified by computed tomography, substantially increases the risk of future MI or coronary heart disease mortality. Although statins effectively attenuate overall plaque growth and subsequent events, they paradoxically accelerate CAC progression, raising difficulties in treatment monitoring [171]. This unique response of statins contrasts with that of other cholesterol-lowering drugs, such as PCSK9 inhibitors, which show less impact on CAC progression, especially when used concomitantly with statins [172]. Furthermore, during coronary stenting for acute MI, severe and moderate calcification in culprit lesions significantly increases complications such as thrombosis or restenosis, highlighting the complexities imposed by calcification in clinical interventions for MI [173]. Therefore, it is imperative to understand the relationship between vascular calcification, CAC, and treatment responses to optimize risk assessment, guide therapeutic decisions, and improve outcomes after MI.

Vascular calcification, especially in the intracranial, coronary, and carotid arteries, significantly influences stroke risk, prognosis, and response to treatment [174]. Vascular calcification also affects cerebral hemodynamics and contributes to small vessel disease [175]. Recent studies exploring intracranial artery calcification (IAC) to predict stroke occurrence and post-stroke mortality revealed that patients with IAC face an increased risk of stroke and recurrence but not necessarily post-stroke mortality [176]. Despite the frequency and detectability of IAC on computed tomography angiography (CTA), its role as a prognostic tool for stroke risk or recurrence remains limited. Although it is recognized that atherosclerosis in intracranial arteries frequently contributes to stroke, the possible predictive value of IAC on stroke incidence, recurrence, and response to treatment of acute ischemic stroke is an area under exploration that requires further investigation.

Vascular calcification is highly prevalent among people with chronic kidney disease (CKD), increasing the risk of cardiovascular morbidity and mortality. Its development involves an interaction between altered mineral balance, chronic inflammation, and cellular responses of VSMCs, macrophages, and ECs [177]. CKD risk factors accelerate and initiate calcification earlier, often earlier than in the general population [178]. Alterations in mineral metabolism, especially calcium and phosphorus homeostasis, play a central role in the initiation and promotion of CV, as observed in clinical and experimental studies [179]. Low vitamin K levels lead to inactive forms of MGP, a calcification inhibitor, which is associated with CV acceleration.

Vascular calcification is related to diabetes mellitus (DM), in which several signaling pathways, such as TNF-α, and ILs, contribute to the development of vascular calcification [180]. Despite advances in antidiabetic drugs, their effects on the regression of vascular calcification remain unexplored. Similarly, in type 2 diabetes mellitus (T2DM), there is a strong association with vascular calcification driven by the OPG/RANKL/TRAIL system, which is normally involved in bone remodeling [181]. RANKL promotes vascular calcification, while OPG acts as a counterbalance, deviating from its functions in bone metabolism. Arterial calcification, prevalent in metabolic syndrome and diabetes, impairs vessel function and increases the risk of adverse outcomes [182]. Preclinical models point to BMP–Wnt signaling and endothelial–mesenchymal transition guiding arterial calcification in these conditions. Furthermore, elevated levels of dp-ucMGP, an inactive form of vitamin K-associated MGP, are associated with below-knee arterial calcification in patients with T2DM, warranting further exploration of its clinical significance and possible reversibility [183].

The echocardiographic examination is often used to establish the diagnosis of aortic stenosis (AS), which provides a wealth of information on the structure of the heart valve and blood flow characteristics [184]. Most patients are referred for echocardiography because of the appearance of symptoms such as dyspnea, angina pectoris, syncope, and dizziness, or because a systolic murmur is auscultated.

The occlusion of blood arteries in the dermis and subcutaneous fat causes calciphylaxis, also known as calcific uremic arteriolopathy, which is a cutaneous ischemic infarction that evolves into ulcerative lesions at risk of superinfection and sepsis [185]. Calciphylaxis is extremely debilitating due to severe pain and predisposition to infection; the annual mortality rate ranges from 40% to 80% [186]. Primarily, calciphylaxis is a condition of renal failure, and most patients are on or about to receive dialysis [187]. When a patient with end-stage renal failure presents with painful indurated plaques or ulcers on the belly and/or legs, the diagnosis can be determined solely from clinical considerations [188]. Physicians with expertise in nephrology, dermatology, plastic surgery, nutrition, and wound care are needed to treat calciphylaxis in a multidisciplinary fashion.

5. Translational Opportunities

5.1. Biomarkers of Vascular Calcification

The list of proposed biomarkers of vascular calcification is so extensive that it includes MGP, osteoprotegerin, bone morphogenetic proteins, fetuin-A, fibroblast growth factor 23, osteocalcin, osteopontin, osteonectin, sclerostin, pyrophosphate, Smads, fibrillin-1 and carbonic anhydrase II [189,190]. Therefore, we will review a few of them in the next section.

Fetuin-A is a circulating negatively charged protein, known as α2-Heremans–Schmid glycoprotein, that is produced by the liver and functions as an important local and systemic inhibitor of vascular calcification. It eliminates ectopic vascular calcification through several mechanisms, including the prevention of the growth of hydroxyapatite crystals outside cells and reducing OS and inflammation [191]. Fetuin-knockout mice showed severe and lethal extraosseous calcification in the heart, lungs, kidneys, and skin after treatment with a diet rich in vitamin D and phosphorus, confirming its crucial role as an inhibitor of calcification. It is intriguing to speculate that the measurement of serum fetuin-mineral complexes may function as a potential biomarker of ectopic calcification in addition to

serum and hepatic fetuin-A levels [192]. Also, MGP is a vitamin K-dependent protein that is expressed locally by VSMCs and is considered inhibitory of vascular calcification. However, its corresponding inactive form, the uncarboxylated MGP (ucMGP), accumulates at sites of calcification [128,193]. Moreover, vitamin K deficiency, commonly found in CKD, is associated with higher levels of ucMGP. Therefore, the regulation of MGP is involved in the pathogenesis of vascular calcification and may be employed as a useful biomarker for risk assessment [127].

Smad proteins are vital for signal transduction from the receptor to the nucleus within the cell when the type I receptor is activated. The BMP-2/Smad signaling pathway is crucial for the osteoblastic differentiation of VSMCs, resulting in vascular calcification [164]. Osteoblast development is inhibited by a reduction in Smad11/5/8 expression when the BMP-2 signaling pathway is inhibited [194].

Fibrillin-1 is a 350 kDa glycoprotein rich in cysteines that produces elastic fibers and microfibrils in connective tissue. The flexibility of arterial walls is facilitated by elastic fibers, and vessel diseases are a consequence of the rupture of these fibers [195]. The control of elastic fiber homeostasis and cellular repair, two processes involved in matrix remodeling, depend on FBN-1 [196]. It is likely that FBN-1 is involved in VC through these pathways and is possibly a therapeutic target.

Two phosphate ions combine to generate PPi, which are tiny molecules that attach to hydroxyapatite and prevent further crystallization. They are believed to be potent inhibitors of medial vascular calcification. VSMCs secrete PPi. Three factors regulate local PPi concentrations: ectonucleotide pyrophosphatase phosphodiesterase (ENPP1), the multi-pass transmembrane protein encoded by the progressive ankylosis locus (ANK), and nonspecific tissue alkaline phosphatase (TNAP) [197]. The limiting enzyme ENPP1 controls the intracellular production of PPi, whereas ANK regulates the proper transport of PPi out of the cell. TNAP breaks down excess extracellular PPi into phosphate ions, which aids local defense against vascular calcification [198]. In humans, severe calcification, heart failure, and early mortality in the arteries of neonates and infants are the hallmarks of a debilitating disease known as generalized arterial calcification of infancy [199]. Mutations in ENPP1 induce the disease and cause its dysfunction.

FGF-23 is a protein that has 251 amino acids, a molecular weight of 32 kDa, and two distinct regions. Bone osteocytes release FGF-23 in response to an increase in dietary phosphate load, resulting in phosphaturia and a decrease in calcitriol levels [200]. For FGF-23 to be activated, it must bind its receptor and the co-receptor Klotho, which facilitates the binding of FGF-23 to FGFR [201]. Both FGF-23 and Klotho are important players in the pathophysiology of vascular calcification complications in CKD and can be used as early biomarkers as well as potential targets for vascular calcification and CKD therapy.

Carbonic anhydrase II (CA II) is a class of zinc-containing proteins that catalyze the reversible conversion of carbon dioxide to bicarbonate. It plays a role in gluconeogenesis, lipogenesis, osteoclast differentiation, acid–base balance, and volume contraction in humans. Proton generation in osteoclasts is facilitated by CA II, which in turn causes the acidification of resorption lacunae and ultimately dissolves bone. Macrophages also show significant levels of CA II expression [202]. In a genome-wide microarray analysis investigating the differential transcriptional pro-life for vascular calcification, CA II overexpression was found in human atheroma plaques compared with normal arterial tissue from the same individual [203]. Compared with normal tissue, atheroma plaque was found to overexpress CA II by a factor greater than 1.7 [68]. In conclusion, given that vascular calcification is linked to CA II production, this enzyme can be employed both as a potential therapeutic target and as a biomarker of calcification.

In the case of patients with CKD, serum calciprotein particles and serum calcification propensity are hallmarks of vascular calcification [204]. Serum calciprotein particles (CPPs) are colloidal nanoparticles composed of a combination of proteins, including fetuin-A, albumin, and Gla-rich protein (GRP), and calcium phosphates. We can distinguish two differentiated states regarding their level of maturation and aggregation: the primary CPPs

(CPP I), which are small and spherical, and the secondary CPPs (CPP II), which are larger and have a damaging needle-shaped structure [205,206]. The serum calcification propensity is the intrinsic ability of serum to facilitate or inhibit the precipitation of calcium phosphate complexes. It is quantified by a recently developed in vitro assay termed T_{50} based on the half-transformation time from CPP I to CPP II. T50 is currently employed in research projects only. However, T_{50} is associated with cardiovascular events, mortality, and kidney disease progression, underscoring its importance as a prognostic marker, as a potentially therapeutic target, and as a management parameter of vascular calcification in patients with CKD, including those undergoing hemodialysis [207]. The presence of vascular calcification contributes significantly to increased mortality rates in patients with CKD due to cardiovascular complications, making these biomarkers of high clinical importance.

Lastly, PET-MDCT is a practical and repeatable technique that combines the anatomical images of MDCT with the molecular images of PET, which is used to identify biomarkers of aortic valve biology and flow patterns [208,209]. PET-MDCT measurements of valvular ^{18}F-sodium fluoride (^{18}F-NaF) uptake serve as a marker of the active mineralization process taking place within the valve.

5.2. Therapeutic Approaches

Modulation of the main regulators of vascular calcification is the basis of vascular calcification treatment. However, effective drugs or non-drug therapies against vascular calcification have not yet been found due to the complex underlying molecular regulation. The options better studied try to reduce the amount of calcium consumed (Table 1). Most of these therapeutic alternatives are being studied in clinical settings.

Elevated phosphate values are indicative of vascular calcification. Phosphate binders have been shown to reduce serum phosphorus levels by decreasing FGF23, which accelerates phosphorus excretion and prevents vascular calcification. Compared to calcium phosphate binders, a recent randomized study showed that the phosphate binder sevelamer reduced mortality more in elderly hemodialysis patients [210,211].

Ppi analogs and bisphosphonates have been used to treat osteoporosis by interfering with hydroxyapatite nucleation and development. In more recent research, when pamidronate and etidronate were administrated at the same dose, uremic mice also demonstrated the prevention of vascular calcification independent of bone resorption [212,213]. Exactly how bisphosphonates work is currently unknown; these drugs have different effects in different people, and there is a lack of information on patient safety with long-term use.

Vitamin K supplementation is necessary to maintain balance in calcium formation and blood clotting. Menaquinones (vitamin K2) and phylloquinone (vitamin K1) are the two naturally occurring forms of vitamin K. According to previously published research, the very active process of vascular calcification is partially controlled and prevented by MGP, which in turn is triggered by the carboxylation of glutamic acid residues in hemodialysis patients who are dependent of vitamin K [214,215]. Oral vitamin K2 administration to hemodialysis patients reduced serum uc-MGP levels but did not influence the progression of aortic calcification.

Sodium thiosulphate (NaTS) functions as an antioxidant and chelating agent, it is used as a treatment for cyanide poisoning and as a preventive measure against cisplatin toxicity. Compared to calcium oxalate or calcium phosphate, NaTS can chelate calcium to create very soluble complexes in the human body. It has been possible to treat individuals with nephrolithiasis, vascular calcification, and skin necrosis with NaTS [216,217]. Although clinical data are scarce, it is hypothesized that the antioxidant activity may function as an adjuvant in repairing damaged endothelial cells.

To evaluate the effects of drugs that allosterically activate calcium-sensing receptors to mimic the impact of calcium on cells, clinical studies on calcimimetics are ongoing. Regarding vitamin D and calcimimetic-guided therapy for the treatment of mineral bone disease and life-limiting cardiovascular disease in the CKD population, a few sophisticated and well-planned controlled studies are available. It has been documented that calcimimetics

such as R-568 and AMG-641 can inhibit vascular calcification [218]. This action is also related to the increased sensitivity of calcium-sensing receptors to extracellular calcium [219]. However, due to the evidence, their ability to prevent calcification remains in question.

The natural substance myo-inositol hexaphosphate (IP6), which is an endogenous intracellular phosphate storage molecule in plants and mammals, prevents the production and development of hydroxyapatite microcrystals without directly affecting blood levels of calcium and phosphate [220,221]. Due to its short plasma half-life (minutes) and poor oral absorption, its infusion is administered intravenously.

The human monoclonal antibody denosumab can bind to and inhibit human RANKL like the inherent bone-protective properties of OPG. Clinical studies with patients with breast cancer or bone metastases from multiple myeloma demonstrated that an injection of denosumab suppressed bone turnover markers rapidly and persistently. Based on calcium measurements, research in human RANKL knock-in (huRANKL-KI) mice revealed that huRANKL-KI mice treated with prednisolone had a 50% decrease in aortic calcium deposits when treated with denosumab [222,223].

Table 1. Main therapeutic opportunities against different targets of vascular calcification. Ppi: inorganic pyrophosphate, MGP: matrix Gla protein, NaTS: sodium thiosulphate, IP6: myo-inositol hexaphosphate, RANKL: receptor activator of nuclear factor κB ligand.

Compound	Target	Mechanism of Action	References
Sevelamer	Phosphate	Phosphate binder	[210,211]
Ppi analogs	Hydroxyapatite	Disruption of hydroxyapatite nucleation	[212,213]
Vitamin K2	MGP	Activation of MGP	[214]
NaTS	Calcium	Chelation of calcium	[216]
Calcimimetics	Calcium-sensing receptors	Increase the sensitivity of calcium-sensing receptors	[219]
IP6	Hydroxyapatite	Disruption of hydroxyapatite nucleation	[220]
Denosumab	RANKL	Inhibition of RANKL signaling	[223]

6. Conclusions

Cellular interactions, signaling pathways, and molecular mediators are involved in vascular calcification. Cells such as VSMCs, ECs, and macrophages play critical roles in vascular calcification through phenotypic changes and the expression of mineralization-related proteins. Calcium, Wnt, BMP, and Notch signaling pathways are involved in promoting or inhibiting calcification in a complex interplay, suggesting possible therapeutic targets to mitigate vascular calcification. Some studies, such as those investigating vitamin K2 in hemodialysis patients or the interaction between intimal and medial calcifications, provide clinical information. However, gaps remain, such as the limited effect of certain interventions on disease progression. Despite advances, the mechanisms driving vascular calcification remain incompletely understood. Future research should deepen into molecular networking and cellular interactions to identify specific therapeutic targets and interventions.

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