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Calcifying circulating cells: an uncharted area in the setting of vascular calcification in CKD patients

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Abstract

Vascular calcification, occurring during late-stage vascular and valvular disease, is highly associated with chronic kidney disease-mineral and bone disorders (CKD-MBD), representing a major risk factor for cardiovascular morbidity and mortality. The hallmark of vascular calcification, which involves both media and intima, is represented by the activation of cells committed to an osteogenic programme. Several studies have analysed the role of circulating calcifying cells (CCCs) in vascular calcification. CCCs are bone marrow (BM)-derived cells with an osteogenic phenotype, participating in intima calcification processes and defined by osteocalcin and bone alkaline phosphatase expression. The identification of CCCs in diabetes and atherosclerosis is the most recent, intriguing and yet uncharted chapter in the scenario of the bone–vascular axis. Whether osteogenic shift occurs in the BM, the bloodstream or both, is not known, and also the factors promoting CCC formation have not been identified. However, it is possible to recognize a common pathogenic commitment of inflammation in atherosclerosis and diabetes, in which metabolic control may also have a role. Currently available studies in patients without CKD did not find an association of CCCs with markers of bone metabolism. Preliminary data on CKD patients indicate an implication of mineral bone disease in vascular calcification, as a consequence of functional and anatomic integrity interruption of BM niches. Given the pivotal role that parathyroid hormone and osteoblasts play in regulating expansion, mobilization and homing of haematopoietic stem/progenitors cells, CKD-MBD could promote CCC formation.

Key words: atherosclerosis, calcifying circulating cells, chronic kidney disease-mineral and bone disorders, mineral metabolism, vascular calcification

Introduction

Cardiovascular disease (CVD) is the leading cause of death among patients with chronic kidney disease (CKD). The clinical and pathologic background of CVD in CKD patients is schematically represented by left ventricular hypertrophy (LVH), cardiac electrical remodelling (often connected to LVH), arteriosclerosis, atherosclerosis and valvular disease, the presence of which is often correlated to the degree of coronary atherosclerosis [1, 2].

Vascular calcification occurs during the late stage vascular pathology (arterio/atherosclerotic) and valvular disease. Additionally, vascular calcification is one of the independent risk factors associated with cardiovascular morbidity and mortality of CKD patients.

Three types of vascular calcifications have been described in uraemia: (i) classical calcified atherosclerotic plaque, (ii) arterial media calcification (mediacalcinosis or Monckeberg's disease) and (iii) cardiac valvular calcifications. However, this does not exclude the possibility of distinct types of vascular calcifications concurring in the same patient. Indeed CKD patients have been shown to have vascular calcifications involving not only the media but also the intima, the latter being associated with reduced renal function [3].

A crosstalk exists between the bone and the vasculature, which is commonly referred to as the bone–vascular axis, the pathological expression of which is the close association between bone turnover, vascular calcification and cardiovascular events either in general population or in patients with CKD [4–6].

Several studies suggest that in CKD patients, the disorders of bone and mineral metabolism, commonly known as CKD-mineral and bone disorder (CKD-MBD), together with inflammation and oxidative stress are implicated in the pathogenesis of vascular calcification [7]. For decades, vascular calcification has been considered a passive phenomenon, intrinsically connected with ageing and atherosclerotic vascular degeneration.

However, a series of clinical and basic science studies performed in the last several years underscored the biological complexity of the processes driving ectopic mineralization, the main hallmark of which is the activation of an osteogenic programme with the acquisition by the cells involved of an osteogenic phenotype. Vascular calcification is thought to develop inside the vascular wall with two seminal events leading to their formation: (i) a disequilibrium between protective and promoting factors of calcification pathways and (ii) the differentiation of parietal cells into chondrocyte/osteoblast-like cells.

However, while activating a common osteogenic programme, the factors involved in intima and media calcification are not identical.

It is well known that resident vascular cells produce local mediators, such as pyrophosphate and matrix-Gla protein that, in cooperation with circulating molecules (i.e. fetuin-A), protect the arteries from vascular calcification [8–10]. Several factors regulate, either promoting or inhibiting, vascular calcification pathways, namely imbalances in serum calcium and phosphate, parathyroid hormone (PTH), FGF23, Klotho, systemic inflammation, RANK/RANKL/OPG signalling, Wnt inhibitors and osteocalcin (OC) [11–13].

In addition, cells showing morphological and biological features close to chondrocyte/osteoblast-like cells have been identified in intimal and medial calcifications, of both mouse models of atherosclerosis and human atherosclerotic samples, although with a different impact depending on the location of calcification (intima, media and valvular) [14, 15]. These cells might originate from several sources, including vascular wall-resident mesenchymal stem cells (MSCs), transdifferentiation of mature vascular smooth muscle cells (SMCs) or circulating calcifying cells (CCCs), even though there is poor evidence for a contribution of CCCs in medial calcification [16].

A growing number of studies are shedding light on how CCCs, cells deriving from bone marrow (BM) differentiated towards an osteogenic phenotype, engraft into the pathological tissue and participate in calcification processes that take place in the intima. An important issue is to fully elucidate the role of CCCs in the pathogenesis of vascular calcification. Therefore, the term CCC identifies several osteogenic cell subsets, expressing different but interrelated phenotypes, sharing a common origin from BM progenitor cells and able to promote intima calcification, so representing a further and complex aspect of the bone–vascular axis.

The role of BM in vascular health

An understanding of the pathophysiological processes linked to the presence of the CCCs cannot be separated from an analysis of the factors involved in regulating the passage, from BM into the bloodstream, of haematopoietic stem/progenitor cells (HSPCs) that are committed or not towards different cell lineages.

In adulthood, BM is the major reservoir for HSPCs since a specialized microenvironment (niche) hosts and modulates their renewal and egress in the bloodstream. Inside the niche, a complicate interplay that involves soluble mediators and surface cell receptors regulates the HSPCs number, fate and location.

Moreover, current data suggest the existence of specialized niches for distinct types of haematopoietic stem and progenitor cells [17].

The BM niche consists of two major elements. The first is the osteoblastic niche that maintains HSPCs, keeping them quiescent for its self-renewal. The second element is the vascular niche, composed of vascular sinuses, lining endothelial cells, nestin-positive CXCL12-abundant reticular (CAR) cells, sympathetic neurons and haematopoietic stem cells. Inside the vascular niche, HSPCs are activated for proliferation and vascular/tissue repair [18, 19].

Under normal and pathological conditions, there is continuous egress out of the BM into the bloodstream of HSPCs, including those committed towards a specific lineage: endothelial and monocytic, osteoprogenitors (MSC), perivascular cells (i.e. pericytes and SMCs), endothelial progenitor cells (EPCs) and precursors of interstitial valve cells: this process is termed mobilization [20, 21]. Besides, homing is a set of complex mechanisms that modulate the mobilization towards distant BM niches in the periphery and peripheral (vascular) tissues. Mobilization and homing are mirror processes depending on the interactions between cytokines, erythropoietin, growth factors, hormones and match receptors cells inside the niche [20].

While osteoblastic cells regulate the haematopoietic stem cell frequency and renewal, inside the niche PTH plays the role of pivotal director of the HSPCs microenvironment through activation of PTH/PTHrP receptors (PPRs) [22]. PPR-stimulated osteoblastic cells produce high levels of the Notch ligand jagged 1 (a Notch ligand), and activated Notch signalling elicits a further increase in the number of HSPCs [23]. PTH-driven HSPCs expansion is also mediated by the upregulation of cytokines like IL-6, IL-11 and granulocyte colony-stimulating factor (GCSF) that controls, in its turn, the CXCL12 expression in different cell types. CXCL12, also called SDF-1, is the key chemokine in mobilization and homing processes. CXCL12 expression in osteoblasts, endothelial cells, BM, heart, skeletal muscle, liver and brain is regulated by PTH, the sympathetic nervous system and GCSF [17, 24]. The interaction between CXCL12 and the homing receptor CXCR-4, which is expressed on many progenitors, circulating or not, is the most important mechanism for both retaining HSPCs within the BM and their mobilization [25–27]. Inside the niche, osteoblasts regulate and ensure the renewal of HSPCs since they express CXCL12, so binding HSPCs that are primarily quiescent [19]. PTH drives the expansion of HSPCs in the BM either directly or through stimulation of GCSF, which in turn, through the loss of osteoblasts and lowering of CXCL12 expression by the cells inside the niche, fosters the transmigration of HSPCs into the vascular sinuses (Figure 1). PTH promotes the homing in both normal and pathological peripheral tissue by inducing an increased expression of CXCL12 through the downregulation of dipeptidylpeptidase IV [21]. Indeed endosteal calcium concentration (near to resorbing osteoclasts), active on calcium sensing receptor
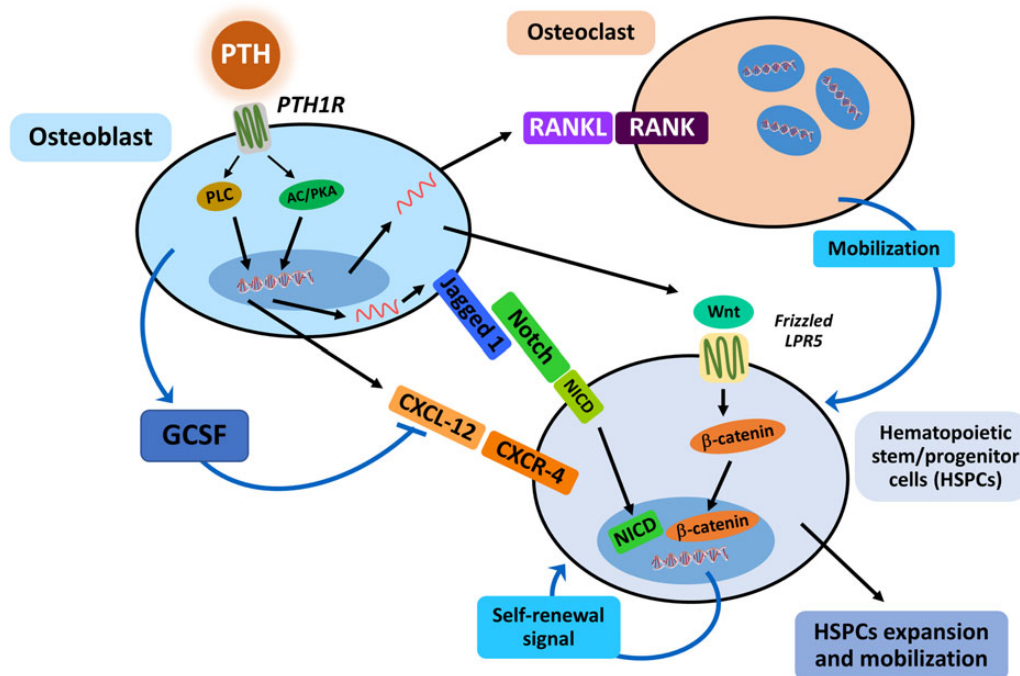
Fig. 1. PTH is a pivotal regulator of BM niche and hematopoietic stem cells/circulating precursor cells expansion and mobilization. In the BM, PTH binds to parathyroid hormone 1 receptor (PTH1R) on osteoblasts and drives the expansion of HSPCs. In addition, GCSF inhibits CXCL12 expression, thus promoting HSPC transmigration into the vascular sinuses and so into the bloodstream.

(CASr) expressed on HSPCs, regulates their lodging in the BM, but little is known about the effect of calcium serum levels [28].

Therefore, any factor able to deplete osteoblasts, impair bone metabolism and/or reduce the expression of CXCL12 results in impairment of transmigration of HSPCs and derived cell lineages into the vascular sinuses and then into the circulation [19, 21].

It is through this complex regulatory network that the BM ensures mobilization into the bloodstream of BM-derived cells, such as EPCs or precursors of resident interstitial valve cells, in order to maintain the morphological and functional integrity of the vessels and valves.

In this framework, it is not surprising that interruption of the dynamic anatomy of the niche as well as any changes in BM microenvironment or in HSPCs function can result in a failure of their mobilization ('mobilopathy') or in an alteration of cellular differentiation processes. These modifications are observed especially in the course of diabetes. In the BM of diabetic patients, GCSF lowers osteoblast number and their CXCL12 expression, leaving unchanged the CXCL12 expression in CAR cells, resulting in a decreased mobilization of haematopoietic stem cells [19].

The 'mobilopathy' can extend to the reduction of HSPCs until the appearance of progenitors or cell subsets endorsed of an osteogenic phenotype, which is the hallmark of the CCC [18].

The circulating calcifying cells

The BM mesenchymal and haematopoietic compartments represent the sanctuaries that may give rise to HSPCs from which the pool of CCC originates. Regardless of the type of BM progenitor cell, CCCs are defined by OC and bone alkaline phosphatase (BAP) expression.

OC is a noncollagenous bone protein implicated in bone mineralization and calcium homeostasis, and BAP is a glycoprotein

found on the surface of osteoblasts that is essential to the mineralization process [12, 29].

The pool of CCCs includes circulating (mesenchymal) osteoprogenitor cells, circulating calcifying EPCs and myeloid calcifying cells [16] (Figure 2).

Circulating (mesenchymal) osteoprogenitor cells

Although the presence of CCCs has been documented in patients with diabetes and vascular disease, these cells can be also found in healthy subjects and may therefore be regarded, within certain limits, as a 'physiological phenomenon' related to ageing, bone remodelling or bone healing after fractures.

The precursors of osteoblasts can be found as circulating osteoprogenitors in the bloodstream, and they consist of two populations, one related to MSCs and the other to haematopoietic stem cells/EPCs. These cells contribute to bone health by participating in bone remodelling. The appearance of MSC-derived circulating osteoprogenitors takes place after fracture. Their mobilization into the circulation in response to fracture occurs at any age, but with a higher frequency in young patients [16].

Circulating osteoprogenitors may also arise from haematopoietic stem cells expressing CD34 and CD133 antigens (markers of stemness) and able to differentiate into endothelial cells and osteoblasts *in vitro*. Similarly, in addition to improving bone vascularization, EPCs can undergo procalcific differentiation. This cell plasticity may ensure an adequate blood supply to bone fracture sites that is crucial for healing process. This finding, supported by the recruitment of CD34+ progenitors and endothelial cells to the fracture sites, reinforces the idea of a tight link between osteogenesis and vasculogenesis and raises the possibility that under pathological conditions, haematopoietic stem cells or
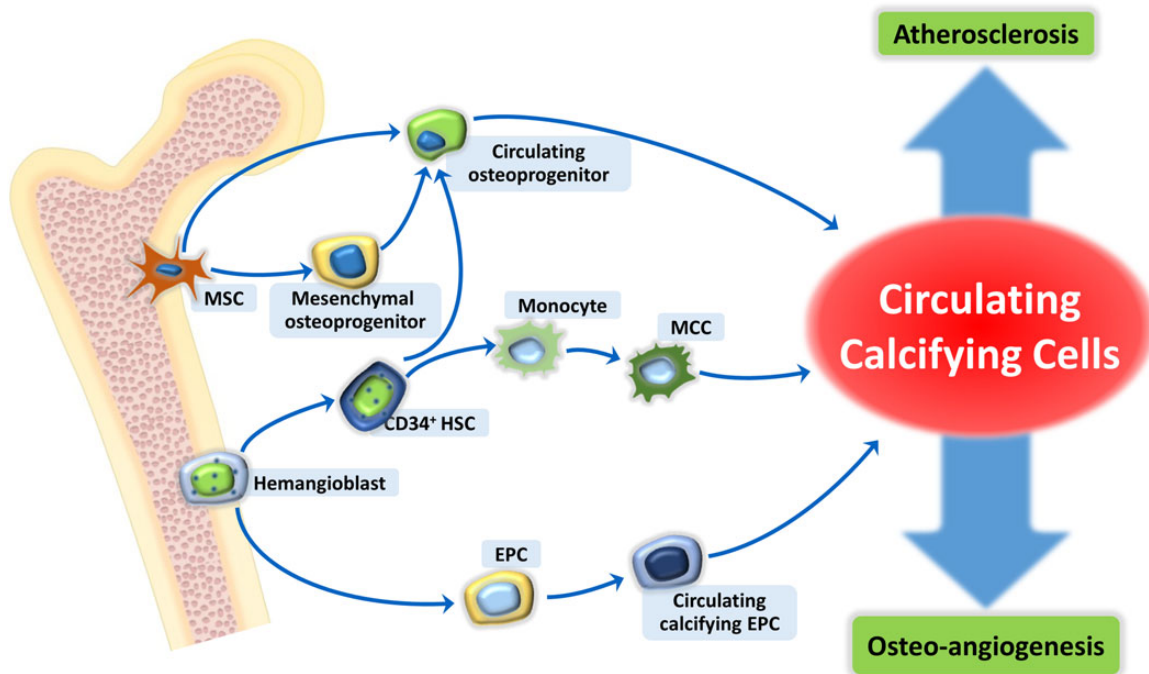
Fig. 2. The pool of CCCs includes osteoprogenitor (mesenchymal) cells, calcifying EPCs and myeloid calcifying cells. The term CCC identifies several osteogenic cell subsets expressing different yet partly interrelated phenotypes that have a common origin from BM progenitor cells. CCCs are involved in osteogenesis/angiogenesis and in intimal calcification.

EPCs may be activated, disclosing a procalcific differentiation capacity [30, 31].

Calcifying endothelial progenitor cells

EPCs are a subgroup of blood mononuclear cells derived from BM, which circulate, proliferate and differentiate into mature endothelial cells. They are involved in angiogenesis and vessel repair. While several putative EPC phenotypes with different lineages and function have been identified, true EPCs are supposed to derive from HSPCs. EPCs descending from this lineage should express both stemness markers, CD34 and CD133, and endothelial markers such as vascular endothelial growth factor receptor-2 (VEGFR-2). Coexpression of the stem cell antigen CD133 increases specificity for EPCs because it is not expressed by mature endothelial cells [32–34]; additionally, the lack of CD45 (CD45−), which is generally considered a specific pan-leukocyte marker, identifies cell phenotypes restricted to endothelial lineage [32].

Flow cytometry is the gold standard for the classification of EPC subsets (based on expression of the surface markers) and for their assessment as cardiovascular biomarkers [32]. As described earlier, EPCs are able to undertake differentiation towards both vascular and bone phenotypes. Recent data have demonstrated that circulating CD34 progenitor cells and CD34+/VEGFR-2+ EPCs can express bone-related proteins, in particular OC and BAP, the markers of osteogenic phenotype. Calcifying circulating EPCs have been associated with coronary artery disease, coronary endothelial dysfunction, calcific aortic stenosis and diabetes, although their presence in the bloodstream is minimal (0.01%). An increase of calcifying circulating EPCs is associated with the reduction of circulating CD34+/VEGFR-2+ EPCs in Type 1 diabetes but not in CAD [35–37].

In CKD, many factors have been identified as potential triggers of endothelial dysfunction [38], but poor data are currently available on the quantitative and qualitative changes in EPC as well as on the presence of circulating calcifying EPC in CKD patients. Although there is a general consensus that CKD-MBD, inflammation and hyperglycaemia all exert pivotal roles, the presence of additional factors potentially promoting an osteogenic shift are still undefined. It is well established that osteoblasts and PTH are critical regulators of HSPCs expansion and mobilization into the bloodstream and that EPCs and endothelial cells can express vitamin D receptor (VDR), PTH receptor and calcium-sensing receptor. Moreover, vitamin D has been proven to have a beneficial effect in restoring EPC number and function impairment observed in diabetic and CKD patients [39–41]. The role of FGF23 and Klotho on these cells is not known: an evaluation of the effects of these molecules would be appropriate given their putative impact on the vascular calcification process.

We have previously reported that CKD patients have an higher relative count of CD34+/CD133−/VEGFR-2+/CD45− cells expressing OC compared with healthy subjects [42].

The presence of circulating EPC with an osteogenic phenotype provides insights into the paradox of a calcifying stimulus originating from cells that normally exert a vasoprotective role.

Circulating myeloid calcifying cells

Cells belonging to the myeloid lineage (monocytes–macrophages), are characterized by an extreme phenotypic variability. These cells can exist in different states of activation: pro-inflammatory (M1) and anti-inflammatory (M2), with opposite effects on inflammation, tissue remodelling and angiogenesis.

In particular, M2 monocytes–macrophages support angiogenesis and are identified by expression of the angiopoietin receptor TIE2 [43, 44].

Fadini et al. [45] demonstrated that a fraction of circulating monocytes (~1% in healthy adults) express BAP and OC driven

by Runx2, a master regulator of osteogenesis, and proposed the term 'calcifying myeloid cells' (MCCs) for this cell population. Human MCCs also possess anti-angiogenic properties mediated by upregulation of the thrombospondin-1, a protein that inhibits VEGF signalling and angiogenesis, as well as endothelial cell migration, proliferation and survival. More generally, calcification and inhibition of angiogenesis displayed by MCCs may be part of a late attempt to control inflammation [46].

MCCs were found to be significantly increased in the presence of either CVD or diabetes (Type 2) [44]. In addition, MCC numbers were higher regardless of the coexistence of CVD in diabetic versus non-diabetic patients, accounting for up to 3–4% of blood cells, and were also expanded in the BM (2- to 4-fold higher in diabetic BM than in control BM) and atherosclerotic plaques.

However, the study does not prove a direct participation of MCC in intimal calcification, but their detection in carotid atherosclerotic specimens from diabetic patients supports this hypothesis.

The mechanisms that trigger MCCs remain to be defined. Their appearance in diabetic patients is promoted by intima hypoxia and especially by hyperglycaemia. In this regard, the levels of circulating MCCs in diabetes mellitus are reversible after optimization of glycaemic control.
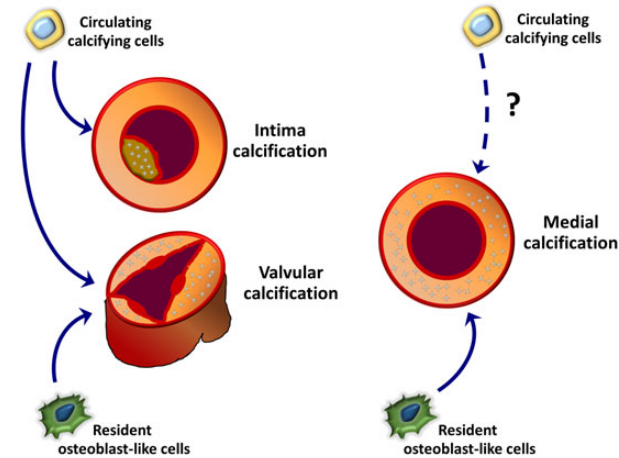
Potential role of CCCs in CKD patients

The identification of CCCs is undoubtedly the most intriguing, yet relatively uncharted area in the multifaceted scenario of the bone–vascular axis. Although their role is not yet defined, the detection and recognition of CCCs is a landmark in the comprehension of vascular calcification pathogenesis. Whatever their origin and phenotype, CCCs may engraft to sites of vascular disease to further promote ectopic calcification. However, up to now, there is no clear proof of CCCs actively participating in medial calcification (Figure 3). This is particularly important considering that the derangement of the bone–vascular axis is amplified by ageing, CKD, diabetes and atherosclerosis, the incidence of which are constantly rising in the general population.

There are still limited data available concerning the factors related to the presence of CCCs, as well as on the districts where the osteogenic shift of the involved cell subsets occurs: in the BM or bloodstream or both.

However, even in various clinical settings, it is possible to speculate inflammation as the shared pathogenetic link, also bearing in mind that diabetes, atherosclerosis and CKD often coexist. CCCs are mainly involved in atherosclerotic and valvular lesions whose progression is interrelated to inflammatory mechanisms. It is conceivable that, at least in the early stages, CCCs might be recruited along with resident cells endowed of osteogenic phenotype in order to deposit calcium in the tissue in an effort to resolve inflammation in the vascular/valve wall. In diabetes, the degree of metabolic control may have a significant role in regulating the osteogenic shift.

Studies conducted to date (not including CKD patients) failed to find relationships between the presence of CCCs and markers of bone metabolism. Hypothetically, MBD could play a more active role in CKD patients where they are associated with a well-known disruption of bone microarchitecture perturbing the functional and anatomic integrity of BM niches.

Given the crucial role that PTH, osteoblasts and MBD play in regulating HSPCs expansion, mobilization and homing of HSPCs, the impairment of bone remodelling that is inherent in CKD-MBD may also foster the development of cell subsets expressing an osteogenic phenotype (Figure 4). Additional factors may involve specific MBD-related receptors (e.g. VDR, PPR and
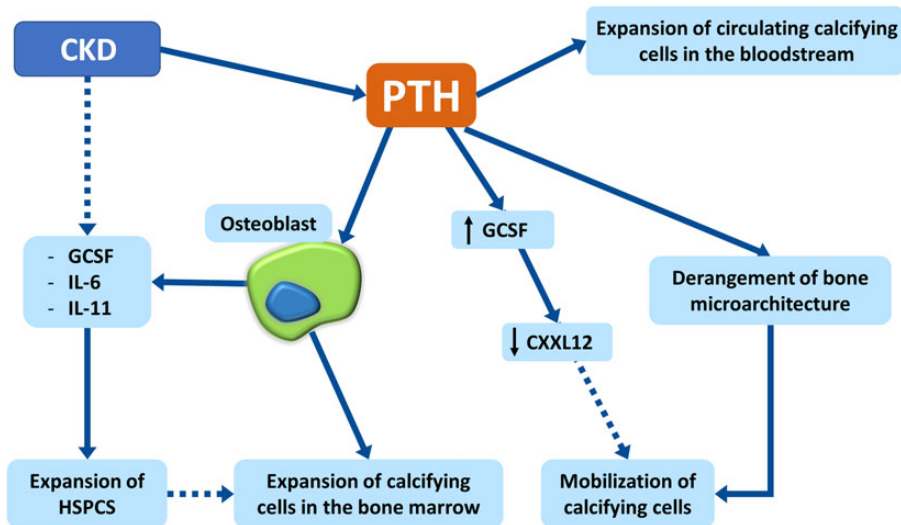
Fig. 3. Putative role of CCCs in intimal, valvular and medial calcification.

Fig. 4. PTH and CCCs in CKD. The putative link between CKD, CCCs and intimal calcification.

CaSR) on HSPCs and/or their derangement, that have either committed or not towards different cell lineage [37–41].

Moreover, even though OC function is still poorly understood, gene deletion studies seem to indicate a possible contribution in bone remodelling [12]. OC is produced by osteoblastic cells and circulating osteoprogenitor cells (i.e. cells of haematopoietic and mesenchymal origin), and its expression is regulated by a number of calcitropic hormones and growth factors, including 1,25-dihydroxy vitamin D3, PTH, bone morphogenetic proteins, tumour necrosis factor α and transforming growth factor β. In addition, PTH, signalling through PPRs and Runx2, might induce OC/BAP expression on several cell populations originating from the BM.

Defining the role of CCCs in the bone–vascular axis will be important for CKD patients, given the high cardiovascular morbidity and mortality, the increasing prevalence of diabetes and vascular disease as causes of CKD, and the marked changes in bone metabolism.

Conflict of interest statement

The results presented in this paper have not been published previously in whole or part.

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