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Role of the RANK/RANKL/OPG and Wnt/ β -catenin systems in CKD Bone and Cardiovascular Disorders.

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Abstract:	<p>In the course of chronic kidney disease (CKD), alterations in the bone-vascular axis augment the risk of bone loss, fractures, vascular and soft tissue calcification, left ventricular hypertrophy, renal and myocardial fibrosis, which markedly increase morbidity and mortality rates.</p> <p>A major challenge to improve skeletal and cardiovascular outcomes in CKD patients requires a better understanding of the increasing complex interactions among the main modulators of the bone-vascular axis. Serum Parathyroid hormone (PTH), phosphorus (P), calcium (Ca), fibroblast growth factor 23 (FGF23), calcidiol, calcitriol and Klotho are involved in this axis interact with RANK/RANKL/OPG system and the Wnt/β-catenin pathway.</p> <p>The RANK/RANKL/OPG system controls bone remodelling by inducing osteoblast synthesis of RANKL and downregulating OPG production and it is also implicated in vascular calcification. The complexity of this system has recently increased due the discovery of LGR4, a novel RANKL receptor involved in bone formation, but possibly also in vascular calcification.</p> <p>The Wnt/β-catenin pathway plays a key role in bone formation, when this pathway is activated bone is formed, when it is inhibited, bone formation is stopped. In the progression of CKD, a downregulation of the Wnt/β-catenin pathway has been described which occurs mainly through the not coincident elevations of sclerostin, Dickkopf1 (Dkk1) and the secreted Frizzled Related Proteins (sFRPs).</p> <p>This review analyses the interactions of PTH, P, Ca, FGF23, calcidiol, calcitriol and Klotho with the RANKL/RANKL/OPG system and the Wnt/β-catenin, pathway and their implications in bone and cardiovascular disorders in CKD.</p>	
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**Title: Role of the RANK/RANKL/OPG and Wnt/ β -catenin systems
in CKD Bone and Cardiovascular Disorders**

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ABSTRACT

In the course of chronic kidney disease (CKD), alterations in the bone-vascular axis augment the risk of bone loss, fractures, vascular and soft tissue calcification, left ventricular hypertrophy, renal and myocardial fibrosis, which markedly increase morbidity and mortality rates.

A major challenge to improve skeletal and cardiovascular outcomes in CKD patients requires a better understanding of the increasing complex interactions among the main modulators of the bone-vascular axis. Serum Parathyroid hormone (PTH), phosphorus (P), calcium (Ca), fibroblast growth factor 23(FGF23), calcidiol, calcitriol and Klotho are involved in this axis interact with RANK/RANKL/OPG system and the Wnt/ β -catenin pathway.

The RANK/RANKL/OPG system controls bone remodelling by inducing osteoblast synthesis of RANKL and downregulating OPG production and it is also implicated in vascular calcification. The complexity of this system has recently increased due the discovery of LGR4, a novel RANKL receptor involved in bone formation, but possibly also in vascular calcification.

The Wnt/ β -catenin pathway plays a key role in bone formation, when this pathway is activated bone is formed, when it is inhibited, bone formation is stopped. In the progression of CKD, a downregulation of the Wnt/ β -catenin pathway has been described which occurs mainly through the not coincident elevations of sclerostin, Dickkopf1 (Dkk1) and the secreted Frizzled Related Proteins (sFRPs).

This review analyses the interactions of PTH, P, Ca, FGF23, calcidiol, calcitriol and Klotho with the RANKL/RANKL/OPG system and the Wnt/ β -catenin, pathway and their implications in bone and cardiovascular disorders in CKD.

KEYWORDS: RANK/RANKL/OPG system, LGR4, Wnt/ β -catenin pathway, PTH, klotho, phosphorus.

RUNNING TITTLE: RANK/RANKL/OPG and Wnt/ β catenin systems in CKD

1. GENERAL ASPECTS: ROLE OF PARATHORMONE, PHOSPHORUS AND OTHER BIOMARKERS

The aging in the general population, which is markedly accelerated in chronic kidney disease (CKD) (1-8), is characterized by severe alterations in the bone-vascular axis that favour osteoporosis, fractures, vascular and soft tissues calcification as well as left ventricular hypertrophy (LVH), myocardial fibrosis and progression of renal damage.

In CKD patients, these abnormalities are associated to elevation in serum parathormone (PTH), phosphorus (P), fibroblast growth factor 23 (FGF23) and decrease in serum calcidiol, calcitriol and calcium (Ca), as well as a progressive reduction in renal Klotho and increase in the degree of systemic inflammation.

In the context of bone and mineral disorders, high serum P stimulates not only PTH synthesis and secretion but also parathyroid gland hyperplasia (9-12). These P/PTH interactions create a vicious bone-parathyroid gland circle, high PTH increases bone resorption releasing more P into the circulation and there is a trend to increase serum P levels because CKD patients cannot eliminate P efficiently, not only due to the reduced renal function, but also to the decrease in renal Klotho content that impair the phosphaturic response to FGF23.

High PTH induces high bone turnover and bone loss (13), but its effect in the vascular system is still controversial. While PTH 1-34 fragments inhibited vascular calcification in an atherosclerotic murine model (14), PTH 7-84 fragments increased vascular calcification in others (7, 13, 15). PTH seems to be not sufficient to directly induce vascular calcification (16). In fact, PTH can have synergistic effects with P, in addition to the well know capacity of PTH to increase osteoclast activity and bone turnover (16). A recent study has demonstrated in uremic rats and in cultures of vascular smooth muscle cells (VSMC), that high concentration of PTH increase the calcification induced by high serum P (17). Furthermore, the silencing of PTH 1 receptor (PTH1R), the most abundant PTH receptor in VSMC, partially abolished the pro-calcifying effect of high PTH, demonstrating an important PTH/PTH1R-driven induction of Ca deposition in the medial artery layer (17).

An additional critical consideration is that in advanced CKD, the serum levels of soluble Klotho cannot reflect the real reduction in renal Klotho content. In fact, soluble Klotho, due to its molecular weight cannot be filtered, so, its appearance in the urine to exert FGF23-independent

phosphaturic actions involves a process of transcytosis of soluble Klotho from the blood into the urine through renal tubular cells (18), a process that is impaired in a damaged kidney.

The decrease in urinary soluble Klotho could also partly explain the defects in renal tubular Ca reabsorption and its adverse impact on the skeleton. In fact, urinary soluble Klotho favors the anchoring of the Transient Receptor Potential Cation Channel Subfamily V Member 5 (TRPV5) Ca channel to the cell membrane through its sialidase/glucosidase activity, an action that attenuates the urinary excretion of Ca, preventing a negative Ca balance and, consequently, it could attenuate bone demineralization (19, 20).

This review will analyze the effect and interactions of the above mentioned factors with the two main bone pathways, Receptor Activator of Nuclear Factor (NF)- κ B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) and Wnt/ β -catenin, describing their implications in bone and cardiovascular disorders in CKD.

2. THE RANK/RANKL/OPG/ (LGR4) SYSTEM

PTH is the main regulator of the RANK/RANKL/OPG system that controls bone remodelling by inducing osteoblast synthesis of RANKL and downregulating OPG production. Both mechanisms favour osteoclastogenesis and bone resorption through a Protein Kinase A (PKA)-driven mechanism (21-23). PKA agonists mimic the PTH regulation of RANKL and OPG genes expression (22, 24).

The OPG/RANK/RANKL system was described in the mid 1990s as an essential regulator of bone modeling and remodeling (25). Its role in bone maintenance is well known, but some papers attribute to it an important role in vascular calcification (26, 27).

In bone, osteoblasts and osteocytes synthesize and secrete RANKL, which binds to its transmembrane receptor RANK in bone marrow-derived osteoclast progenitors, allowing the maturation, activation and survival of osteoclasts to initiate resorption. In summary, the RANKL action promotes and increases the osteoclastogenesis and bone loss. In addition, osteoblasts secrete OPG, a soluble decoy receptor for RANKL, which prevents the binding of RANKL to RANK, thus attenuating osteoclastogenesis (28) (Figure 1).

RANK, RANKL and OPG: The classical components of the pathway

RANK is fundamental for osteoclasts development. Also called Tumor necrosis factor (TNF)-related activation-induced cytokine (TRANCE) receptor, is a member of TNF receptor superfamily. It is a transmembrane receptor that consists in 616 aminoacids protein with 4 extracellular cysteine rich domains linked to a long C-terminal intracellular region (25, 28-30).

RANK is constitutively expressed in multiple organs and cells such us osteoclasts' precursors and mature osteoclasts, dendritic cells, mammary glands and vascular cells, among others. Its functions are associated with bone resorption, immune response, lymph node and mammary gland development and thermal regulation (31-35). RANK acts binding to the cytokine RANKL (31), however the RANK overexpression has shown to be enough to activate NF- κ B (36).

RANKL (also called OPG ligand) is the main stimulator of its specific receptor RANK (36-40). RANKL is a 316 amino acid protein with a molecular weight of 38 kDa. Its expression is also modulated by several cytokines, glucocorticoids, and PTH (41). RANKL is produced by osteoblasts, osteocytes and activated T cells. It promotes the formation, fusion, differentiation, activation and survival of osteoclasts, allowing increased bone resorption and mineral loss (42, 43) (Figure 1).

Activation of RANK by RANKL initiates the intracellular signaling cascade of NF- κ B (a protein complex that functions as a transcription factor modulating many cellular processes) (44, 45). Indeed, the final step in RANK activation is the translocation of NF- κ B to the nucleus, which can take place through the classical or the alternative route initiated by their respective kinases, named inhibitor of nuclear factor- κ B (I κ B) kinase (IKK) β and IKK α . The translocation of NF- κ B to the nucleus modulates the expression of different genes, such as c-Fos, Nuclear Factor of Activated T cells 1 (NFATc1) and some Bone morphogenetic proteins (BMPs) (25, 46).

OPG (also known as osteoclastogenesis inhibitory factor -OCIF-) is a decoy receptor for RANKL that regulates osteoclastogenesis disrupting the interaction between RANKL and its receptor RANK (28) (Figure 1). OPG is a 60 KDa glycoprotein, member of the TNF superfamily that is normally secreted by the osteoblasts, even though it has also been detected in association with the cell membrane in lymphoid cells (47). OPG consists of 7 structural domains. Domains 1

to 4 give OPG its osteoclastogenesis inhibitory activity, domains 5 and 6 are considered death domains and involved in apoptosis. Domain 7 harbors the heparin binding region, a common trait of growth factors and signaling molecules (48).

OPG is produced in a wide variety of tissues including the cardiovascular system (heart, arteries and veins), lungs, kidneys, intestine and bone, as well as in hematopoietic and immune cells (30, 47, 49-52). Its expression and production are regulated by several cytokines, peptides, hormones, and drugs. Cytokines, including TNF α , interleukins 1 α and 18, transforming growth factor β (TGF β), bone morphogenic proteins (BMPs), and steroid hormones, such as 17 β estradiol, regulate OPG mRNA levels (53-55). Conversely, glucocorticoids (known to promote bone resorption), immunosuppressant cyclosporin A (which causes osteoporosis and vascular disease), PTH, prostaglandin E2, and fibroblastic growth factor (FGF) decrease OPG expression (53, 56-60).

It is well established that decreases in OPG favor, not only increases in osteoclastogenesis and bone resorbing activity, but also the increases in vascular Ca deposition. Indeed, OPG reduction was identified as an independent variable for coronary artery calcification (61). Furthermore, a recent elegant work has linked the anti-calcifying effects of higher vascular Pit2 expression to increases in OPG levels as suggested by the low levels of OPG in the Pit2 deficient mice (62).

In healthy subjects over 70 years old, RANKL and OPG plasma levels were gender-independent (63, 64), but different serum OPG levels were found in men and premenopausal women (65). There is a discrepancy between OPG concentration and ageing, some studies did not observe variations with age (64, 66), while others have shown a positive correlation (63, 65, 67) mainly in subjects over 60 years old (63). In two age related disorders, such as rheumatic polymyalgia and osteoarthritis, circulating OPG levels did not differ from the age matched controls, but soluble RANKL levels were higher in both diseases (63).

The RANK/RANKL/OPG system is of high clinical relevance in osteoporosis treatment. OPG is not used in clinical practice but its discovery and the studies of its actions were the base of the development of Denosumab, a human monoclonal antibody against RANKL that mimics the actions of OPG (reducing osteoclastogenesis and bone resorption). Denosumab has been worldwide long-term used to prevent the reduction of bone mass and to decrease the risk of bone fractures (68).

LGR4: A New RANKL receptor

The recent discovery of a new RANKL receptor, the leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4) (69), also called G-protein-coupled receptor (GPR) 48, provided

1 a novel member of this system that regulates bone formation. This receptor counteracts RANKL-
2 driven osteoclastogenesis and also activates the Wnt/ β -catenin pathway (70), it acts on bone
3 formation but may also adversely promote vascular calcification.
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6 LGR4 is essential to increase bone formation by increasing osteoblast maturation and
7 mineralization (69). In addition, LGR4 inhibits osteoclast differentiation and maturation by the
8 competition with RANK to the binding to RANKL. In this sense, LGR4 knock-out mice developed
9 abnormalities in bone during embryonic and postnatal stages with delay in osteoblast differentiation
10 and mineralization, reductions in osteoid formation and increases in osteoclast activity (71). Also
11 in humans, a nonsense mutation in LGR4 has been strongly associated with low bone mineral
12 density and osteoporotic fractures (72).
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20 However, the effect of PTH on LGR4 expression and the likely mechanisms involved are
21 incompletely understood. A recent study has demonstrated that in uremic rats fed high P diet, LGR4
22 aortic expression markedly increased in response to high PTH. *In vitro*, the silencing of the LGR4
23 gene in VSMCs was capable to prevent PTH-induced vascular calcification without changes in
24 RANKL and OPG expression (73) (Figure 1). Due to its recent discovery, is still early to envision
25 if LGR4 will play a future role in the clinical management of bone and vascular disorders.
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34 **3. THE RANK/RANKL/OPG/(LGR4) SYSTEM. ROLE IN OSTEOPOROSIS AND** 35 **VASCULAR CALCIFICATION** 36 37 38

39 PTH indirectly regulates osteoclast differentiation and activity by increasing the production
40 of RANKL and decreasing OPG synthesis in osteoblasts (28).
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43 The biological effects of OPG are opposite to those mediated by RANKL, since OPG acts
44 as a soluble inhibitor that prevents the interaction of RANKL with its receptor RANK and
45 subsequently, its stimulation of osteoclastogenesis (74). The first evidence that this system was
46 involved in vascular calcification derived from the study of the OPG knockout mouse, which
47 presents osteoporosis and calcification of the aorta and renal arteries (51, 75).
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53 OPG expression can be found in the media of large arteries (51) and in different cells types
54 of the vessel wall such as VSMCs and endothelial cells (58, 76). In endothelial cells, OPG acts as
55 an autocrine survival factor (76). In contrast, RANKL and RANK have only been found in the
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1 calcified areas of aortas of transgenic mice prone to calcification, but not in the arteries of wild type
2 mice (77).
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4 The hypothesis that the RANK/RANKL/OPG system could establish a link between
5 osteoporosis and vascular calcification is clinically based on the increased risk of arterial
6 calcifications and cardiovascular disease in postmenopausal women and elderly people with
7 osteoporosis (78-80). Other studies have shown that OPG inhibits the extensive calcifications of the
8 aortic, carotid, femoral, mesenteric, hepatic, renal arteries induced by treatment with warfarin or
9 toxic doses of vitamin D (81). Moreover, VSMCs calcification induced by β -glycerophosphate or
10 RANKL was inhibited by OPG addition to the culture media (26).
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18 The discovery that the OPG knockout mouse develops osteoporosis and severe arterial
19 calcification (75) and the fact that RANKL expression increases in calcified arterial tissue (82), and
20 also induces calcification of VSMCs through its binding to RANK and increases in BMP 4
21 expression (26), suggests that the OPG/RANK/RANKL axis could be an important
22 autocrine/paracrine system involved in vascular calcification.
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28 In the vasculature, increases in RANKL and decreases in OPG favor vascular calcification
29 (26, 83). As it has been previously mentioned, LGR4 seems to promote vascular calcification in
30 experimental models. In addition, LGR4 has also shown to potentiate Wnt/ β -catenin pathway
31 through two mechanisms: the increase in Wnt receptors that involve a direct inhibition of the
32 ubiquitinases Ring Finger Protein (RNF) 43 and Zinc And Ring Finger (ZNRF) 3 that mediate their
33 degradation, and also through the recruitment of the guanosine triphosphate (GTP)ase-activating
34 protein Ras GTPase-activating-like protein (IQGAP1) to the Wnt/ β -catenin pathway complex,
35 which results in a potentiation of both, the canonical and noncanonical Wnt/ β -catenin pathways
36 (84). Recently, Apelin was identified as an important down-regulator of the activation of LGR4/ β -
37 catenin signaling, sufficient to ameliorate aortic remodeling and fibrosis in models of transverse
38 aortic constriction (85). As for most Wnt inhibitors, beneficial anti-calcifying actions in the
39 vasculature could adversely impact adequate bone formation.
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51 The role of RANKL and OPG as biomarkers of skeletal health has also been studied. In fact,
52 the RANKL/OPG ratio is a useful tool to indirectly determining the degree of bone remodeling
53 (86), however, its relationship with the degree of vascular calcification (87, 88) is still a
54 controversial issue.
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Denosumab has not shown any effect on vascular calcification. In the FREEDOM study (from Fracture Reduction Evaluation of Denosumab in Osteoporosis Every 6 Months), performed in osteoporotic women, the abdominal X-rays showed no differences in the progression of aortic calcification and the reported adverse cardiovascular events were found in the placebo and Denosumab groups (89).

4. WNT/ β -CATENIN PATHWAY

The Wnt/ β -catenin is an intracellular signaling pathway that plays a key role in bone formation, regulating the osteoblast activity (90-93). When the Wnt/ β -catenin pathway is activated bone is formed, when this pathway is inhibited, bone formation is stopped.

The activation of the Wnt/ β -catenin pathway start when the Wnt ligand bind to their receptors, Frizzled and Low-density lipoprotein receptor-related protein (LRP)5/6 inactivating the Glycogen synthase kinase 3 (GSK3) stabilizing β -catenin in the cytoplasm making possible the translocation of β -catenin into the nucleus initiating the transcription of bone forming genes, regulating the preosteoblast differentiation through Runt-related transcription factor 2 (Runx2) or/and Osterix induction, among others (94, 95). In the absence of the Wnt ligand, β -catenin is phosphorylated by GSK3, leading to its destruction avoiding its translocation to the nucleus and the osteoblast differentiation and osteocyte formation (Figure 2).

The Wnt/ β -catenin pathway has inhibitors such us the LRP inhibitors Dickkopf1 (Dkk1) and sclerostin (also called Sost), that bind to LRP5/6 receptor allowing its internalization into the cytoplasm; and the frizzled inhibitors called secreted Frizzled Related Proteins (sFRPs), which are able to block the Wnt/ β -catenin pathway, inhibiting an decreasing the osteoblast differentiation and survival, respectively (96-98) (Figure 2).

The Wnt/ β -catenin pathway interacts with several hormones and factors such as PTH, FGF23, calcitriol, Klotho, and LGR4. The latter it is not only a RANKL receptor, but also a key receptor for R-Spondin (R-spo) 2, which is a Wnt/ β -catenin pathway activator (99).

PTH is one of the main regulators of Wnt/ β -catenin pathway in bone. It is well known that PTH is an inhibitor of sclerostin, and this action is fundamental to increase bone formation (100-103); but PTH also affects the differential regulation of LRP5/LRP6 and the antagonist Dkk1 (100). The use of anti-sclerostin monoclonal antibodies has shown to be effective in preventing bone loss

1 in normal rats and rats with chronic renal failure (CRF) and low serum PTH (104), but not with
2 elevated serum PTH (105); which suggested that serum sclerostin values, could be even a more
3 sensitive and precise remodeling marker than circulating PTH. Both continuous and intermittent
4 PTH administration decrease sclerostin levels (102, 103). Also, continuous PTH, increase the
5 signaling receptor Frizzled 1 (103, 106) and co-receptors LRP5 and 6. However, there is not
6 consensus in the effect of PTH over the agonist Dkk1. Several studies have shown that PTH
7 decrease Dkk1 levels (100, 107-109) but others have found the opposite effect and PTH treatment
8 was associated with increases in serum Dkk1 levels (110, 111).

14 Although there is not much evidence about the direct interaction of FGF23/Klotho with Wnt
15 elements, it has been shown that the extracellular domain of Klotho binds to multiple Wnt ligands,
16 inhibiting their ability to activate Wnt signaling (112, 113). It is interesting to note that in CKD, in
17 parallel to the decrease of Klotho, and the increase of FGF23, there are also changes in the levels
18 of Wnt inhibitors, such as sclerostin or Dkk1 (109, 114). FGF23 directly inhibits the osteoblastic
19 Wnt/ β -catenin pathway through a soluble Klotho/mitogen-activated protein kinase (MAPK)–
20 mediated process that requires Dkk1 induction (109). However, the relationship between
21 FGF23/Klotho and Wnt / β -catenin pathway has not been sufficiently explored.

26 The soluble Klotho acts as an antagonist of Wnt/ β -catenin pathway activation through
27 protein-protein interactions between soluble Klotho and extracellular activators of the Wnt/ β -
28 catenin pathway (113). In CKD, the loss of kidney function is the most important cause of reduction
29 in renal Klotho gene expression. As Klotho downregulates renal calcitriol production, its reduction
30 could influence bone remodeling in CKD patients, acting through the complex PTH-calcitriol-
31 FGF23 axis modulating through a direct protein-protein mechanism the interaction between the
32 vitamin D receptor (VDR) and β -catenin (115, 116).

37 Since the activation of the Wnt/ β -catenin pathway is also involved in the progression of
38 kidney damage (117), part of the renal and vascular anti-aging effect of blood-soluble Klotho could
39 be explained by its ability to inhibit the Wnt/ β -catenin pathway (118, 119). The interactions between
40 soluble Klotho and the extracellular activators of the Wnt/ β -catenin pathway may have negative
41 effects in bone and positive effects in vessels, a matter of current research.

46 As mentioned earlier, LGR4 (and also LGR5 and LGR6) have been recently identified as
47 second class receptors for the R-spos family and it is another regulator of Wnt/ β -catenin pathway
48 (70, 84) through the formation of complexes with recognized Wnt/ β -catenin pathway modulators
49 action such as Frizzled/LRP (120). R-spos activates the Wnt/ β -catenin pathway through increasing

phosphorylation of the Wnt co-receptors LRP5/6. They cannot directly activate the Wnt/ β -catenin pathway, but they can do it indirectly acting as a key receptor for R-spo2, which activates the canonical Wnt/ β -catenin pathway promoting osteoblast differentiation and maturation (121-123). A similar effect may occur in osteoblast-like cells derived from VSMCs to initiate vascular calcification. Other member of the family, R-spo1, may also play a role in bone formation by synergizing with the Wnt ligand Wnt3A to induce osteoblast differentiation and OPG expression (99).

5. THE WNT/ β -CATENIN PATHWAY INHIBITORS AND VASCULAR CALCIFICATION

As it has been discussed, the Wnt/ β -catenin pathway is fundamental for bone formation, but it is also implicated in vascular calcification (17, 124-126). The pathophysiology of vascular calcification involves a transition of the VSMCs to a “osteoblast like” phenotype, afterwards, a process of mineralization takes place in the vessel (127-129). In this transformation, several hormones and proteins are involved as a promoters or inhibitors of the vascular calcification. It is beyond the scope of this review to list and discuss the role of these factors which have been analysed in detail in other publications (130-132) and also in other review of this special Calcified Tissue International supplement (133). Among them, the RANK/RANKL/OPG pathway, -already discussed in this review-, and the Wnt/ β -catenin pathway, play an important role in bone and vascular metabolism mainly through the inhibitory action of the Wnt/ β -catenin pathway through Dkk1, sclerostin and the sFRPs. As a result of the action of these Wnt/ β -catenin pathway inhibitors there is a decrease in the osteoblast differentiation and survival, reducing bone formation (96-98) (Figure 2).

During several decades, the excessive PTH suppression, mainly due to aluminum and Ca load and overdosing of active vitamin D, with the consequent abnormally low serum PTH values, have been considered the main responsible of the pathogenesis of low bone turnover, and low bone formation observed in CKD. However, in recent years, this paradigm has been challenged, as a result, the pathogenesis of low bone turnover is under reconstruction and the inhibitors of the Wnt/ β -catenin pathway have been blamed as responsible of the early low bone turnover observed in CKD patients. In fact, clinical and experimental data, both supported by bone biopsy, have shown that bone sclerostin is increased and bone formation decreased in early stages of CKD (17, 109, 134, 135). Unfortunately, these findings are difficult to translate in the clinical practice to guide

therapeutic decisions, due to the fact there is a poor or a lack of correlation between the bone and serum values of the inhibitors of the Wnt/ β -catenin pathway, mainly in sclerostin, the most studied molecule (136, 137).

As vascular calcification and bone loss are age-dependent, the association between serum sclerostin levels and age has been investigated. A study performed in healthy pre and postmenopausal women (aged 20 to 79 years), showed the serum sclerostin significantly increased in postmenopausal women after the menopause (138). Similarly, other study showed that serum sclerostin levels were 46% higher in old women (mean age, 72.9 years) compared to young women (mean age, 30.0 years), but in contrast, sclerostin mRNA levels measured in bone biopsies were no different in the two groups (139), suggesting the age-dependent decrease in glomerular filtration rate may play a role and it should be considered in the interpretation of serum sclerostin levels.

In experimental models of CRF, sclerostin increase at very early stages, before the increase in P, PTH, and FGF23 (109, 134, 140). Furthermore, studies in humans (134), in a mouse model of slow developing polycystic disease (134, 140) and in a model of CKD with hyperphosphatemia (109), have also shown that the increase in sclerostin in bone precedes the increase in serum P, PTH and FGF23. In fact, the increments of these three factors are a later event which coincides with the decrease in bone sclerostin and with the increments in other inhibitors of the Wnt/ β -catenin pathway (109, 134, 140). In fact, bone biopsies from CKD patients have shown the signals of inhibition in the Wnt/ β -catenin pathway were associated with low levels of sclerostin in osteocytes (134), suggesting there may be a contribution of the other inhibitors of the Wnt/ β -catenin pathway in the pathogenesis of low bone turnover (141).

In fact, *in vitro* studies have shown that although the decrease in one Wnt/ β -catenin pathway inhibitors, is associated with greater vascular calcification, the increase in other Wnt/ β -catenin pathway inhibitors may play a role counteracting the decrease and attenuating the effect on vascular calcification (142-144). A good example is a study in diabetic rats with CRF, in which the neutralization of Dkk1 with monoclonal antibodies, was sufficient to prevent both bone and vascular damage (145).

Recent studies analyzing the direct effect of PTH and FGF23 on osteoblasts have revealed that elevated PTH inhibits not only the increases in sclerostin, but also the increment of other Wnt/ β -catenin pathway inhibitors and that FGF23 may have a direct inhibitory effect on the Wnt/ β -catenin pathway in osteoblasts through the induction of Dkk1 (109). On the contrary, the action of FGF23 would be opposite to that of PTH, since high FGF23, through the induction of increases in Dkk1,

would inhibit Wnt/ β -catenin pathway in bone contributing to the bone loss, whereas in vessel could attenuate vascular calcification. In addition to these effects, sclerostin can influence serum concentration of calcitriol and FGF23, both implicated in the mineralization process (146).

The inhibition of sclerostin and other Wnt/ β -catenin pathway inhibitors in bone by high levels of PTH could contribute to maintaining bone health, but it is important to highlight that PTH-dependent reduction of the Wnt/ β -catenin pathway inhibitors in the vessels, could favor vascular calcification. Indeed, as mentioned earlier, recent studies in rats with CKD exposed to different concentrations of PTH suggest that elevated PTH favors vascular calcification. Instead, normal circulating PTH, levels appeared to be protective of aortic calcification despite high serum P (17).

It is important to highlight that even though the sclerostin inhibitors, such as Romosozumab, is one of the most promising therapeutic targets in the prevention and treatment of bone fragility fractures (147) its use in CKD patients is still a matter of controversy (148, 149). In fact, Romosozumab could have a negative action in the vascular system where the "natural" inhibition of the Wnt/ β -catenin pathway observed when severe vascular calcification is present, can play an important role protecting the vascular wall from further vascular mineralization.

Studies in animals with CKD and severe aortic calcification showed an increased aortic gene expression of some members of the sFRPs family (sFRPs 1, 2 and 4), suggesting the inactivation of Wnt/ β -catenin pathway in the vessels wall may constitute a "natural" protective mechanism against the progression of vascular calcification (109, 124). Thus, according to the present knowledge from experimental models, we can hypothesize that the increase in PTH, -a potent sclerostin suppressor-, progressively reduces the expression of sclerostin and the increment in other inhibitors, such as Dkk1 and/or sFRPs, of the Wnt/ β -catenin pathway could compensate the sclerostin reduction, helping to protect from further vascular calcification (109, 124, 130). However, other recent study has reported the hypothesis that the increased levels of serum sclerostin probably originating from excessive local production in calcified vessels, may contribute to the linkage between vascular disorders and impaired bone mineralization (150).

6. CARDIAC IMPACT

Among the cardiovascular disorders associated to abnormal Wnt/ β -catenin pathway activation, the abnormalities in left ventricular (LV) structure and function are also important. LVH is a well-recognized cardiovascular disorder, which occurs early in the course of CKD (151, 152).

Cardiomyocytes and fibroblasts are the cells implicated in the abnormal remodeling process leading to LVH, the cardiomyocytes increase their size, and the fibroblasts increase collagen synthesis prompting the onset of fibrosis. These changes progressively lead to cardiomyocyte apoptosis or necrosis, and the cardiomyocytes are replaced by fibroblasts and extracellular collagen (153). In addition, PTH promotes apoptosis of the cardiomyocytes (154), which in the long-term, either causes or exacerbates myocardial fibrosis.

Several clinical studies have shown that high PTH levels are associated with LVH (155, 156). Recently, it was demonstrated that myocardial specific R-spo3 acts mainly through the LGR4 receptor to promote coronary stem cell proliferation in the developing heart (157), demonstrating that this receptor and its ligand have an important role in heart development. Moreover, abnormalities in the canonical Wnt/ β -catenin pathway are fundamental in the establishment of cardiac lesions. Indeed, activation of β -catenin induces cardiomyocytes hypertrophy and myofibroblastic transformation of cardiac fibroblasts, increasing their ability to produce and secrete interstitial matrix components such as fibronectin and collagen I (158). Furthermore, a high OPG/RANKL ratio has been independently associated with LVH and abnormal LV structural remodeling in male overweight/obese children and adolescents (159). A better knowledge of the mechanisms that modulate the appropriate function of the discussed pathways may provide relevant information on novel therapeutic targets to attenuate LVH and myocardial fibrosis in CKD.

FGF23 is also elevated in CKD patients and it has been also related as a cause of LVH (160). Some authors have speculated that FGF23 could develop LVH through the Wnt signaling activation. In fact, the Wnt signaling inhibition improves cardiac function and could attenuate LV changes (114, 161).

7. FINAL COMMENTS

In summary, a better understanding of the intricate regulation of the RANK/RANKL/OPG/LGR4 and the Wnt/ β -catenin pathways in bone and vessels is a highly needed step to improve the diagnosis and treatment of these CKD complications.

The design of therapeutic strategies to prevent the deterioration of the bone-vessel axis in the progression of CKD requires a much better understanding of the interaction between classic

factors such as Ca, P, calcitriol, PTH and FGF23 with the activation and inactivation of the RANK/RANKL/OPG system and the Wnt/ β -catenin pathway. Unfortunately, still it is not possible to translate into the clinical practice a great part of the new important and challenging information discussed in this review because still in many of them, the serum levels of the components of the two main pathways are not able to predict their changes at bone and vascular level.

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AUTHOR'S CONTRIBUTION

N.C.L. and S.P. had the idea for the article, L.M.A. and S.F.V. performed the literature search and data analysis, S.P., M.N.D. and J.C.A. drafted the article and N.C.L., M.P.R.T., A.D.; J.C.A., M.N.D. and S.P. critically revised the article.

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FIGURE CAPTIONS

Figure 1. RANK/RANKL/OPG/LGR4 signaling in bone. A) Osteoblasts synthesize and secrete RANKL which binds RANK allowing their activation, maturation and prolonging the survival of osteoclasts. B) Osteoblasts also secrete OPG, a soluble RANKL decoy receptor, which prevents RANKL binding to RANK, inhibiting osteoclastogenesis. C) RANKL can also binds the LGR4 receptor on the surface of the osteoblasts, triggering signals of mineralization and bone formation.

Figure 2. Wnt/ β -catenin signaling pathway. A) In the absence of Wnt ligand, β -catenin is phosphorylated by GSK3 and destroyed avoiding its translocation to the nucleus to trigger the mechanisms of bone formation. B) If Wnt ligands binds to its LRP5/6 and Frizzled co-receptors, GSK-3 is inactivated, β -catenin is stabilized in the cytoplasm and translocate into the nucleus where activates target genes promoting osteoblast differentiation and osteocyte formation. C) In presence of Wnt inhibitors: Dkk1 or sclerostin or the sFRPs bind to LRP5/6 receptor or Frizzled respectively, thus the Wnt/ β -catenin pathway is inhibited. (Modified from Gordon MD et al. J Biol Chem. 2006; 281(32): 22429-22433 with permission from The American Society for biochemistry and Molecular biology.

**Title: Role of the RANK/RANKL/OPG and Wnt/ β -catenin systems
in CKD Bone and Cardiovascular Disorders**

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ABSTRACT

In the course of chronic kidney disease (CKD), alterations in the bone-vascular axis augment the risk of bone loss, fractures, vascular and soft tissue calcification, left ventricular hypertrophy, renal and myocardial fibrosis, which markedly increase morbidity and mortality rates.

A major challenge to improve skeletal and cardiovascular outcomes in CKD patients requires a better understanding of the increasing complex interactions among the main modulators of the bone-vascular axis. Serum Parathyroid hormone (PTH), phosphorus (P), calcium (Ca), fibroblast growth factor 23 (FGF23), calcidiol, calcitriol and Klotho are involved in this axis interact with RANK/RANKL/OPG system and the Wnt/ β -catenin pathway.

The RANK/RANKL/OPG system controls bone remodelling by inducing osteoblast synthesis of RANKL and downregulating OPG production and it is also implicated in vascular calcification. The complexity of this system has recently increased due the discovery of LGR4, a novel RANKL receptor involved in bone formation, but possibly also in vascular calcification.

The Wnt/ β -catenin pathway plays a key role in bone formation, when this pathway is activated bone is formed, when it is inhibited, bone formation is stopped. In the progression of CKD, a downregulation of the Wnt/ β -catenin pathway has been described which occurs mainly through the not coincident elevations of sclerostin, Dickkopf1 (Dkk1) and the secreted Frizzled Related Proteins (sFRPs).

This review analyses the interactions of PTH, P, Ca, FGF23, calcidiol, calcitriol and Klotho with the RANKL/RANKL/OPG system and the Wnt/ β -catenin, pathway and their implications in bone and cardiovascular disorders in CKD.

KEYWORDS: RANK/RANKL/OPG system, LGR4, Wnt/ β -catenin pathway, PTH, klotho, phosphorus.

RUNNING TITTLE: RANK/RANKL/OPG and Wnt/ β catenin systems in CKD

1. GENERAL ASPECTS: ROLE OF PARATHORMONE, PHOSPHORUS AND OTHER BIOMARKERS

The aging in the general population, which is markedly accelerated in chronic kidney disease (CKD) (1-8), is characterized by severe alterations in the bone-vascular axis that favour osteoporosis, fractures, vascular and soft tissues calcification as well as left ventricular hypertrophy (LVH), myocardial fibrosis and progression of renal damage.

In CKD patients, these abnormalities are associated to elevation in serum parathormone (PTH), phosphorus (P), fibroblast growth factor 23 (FGF23) and decrease in serum calcidiol, calcitriol and calcium (Ca), as well as a progressive reduction in renal Klotho and increase in the degree of systemic inflammation.

In the context of bone and mineral disorders, high serum P stimulates not only PTH synthesis and secretion but also parathyroid gland hyperplasia (9-12). These P/PTH interactions create a vicious bone-parathyroid gland circle, high PTH increases bone resorption releasing more P into the circulation and there is a trend to increase serum P levels because CKD patients cannot eliminate P efficiently, not only due to the reduced renal function, but also to the decrease in renal Klotho content that impair the phosphaturic response to FGF23.

High PTH induces high bone turnover and bone loss (13), but its effect in the vascular system is still controversial. While PTH 1-34 fragments inhibited vascular calcification in an atherosclerotic murine model (14), PTH 7-84 fragments increased vascular calcification in others (7, 13, 15). PTH seems to be not sufficient to directly induce vascular calcification (16). In fact, PTH can have synergistic effects with P, in addition to the well know capacity of PTH to increase osteoclast activity and bone turnover (16). A recent study has demonstrated in uremic rats and in cultures of vascular smooth muscle cells (VSMC), that high concentration of PTH increase the calcification induced by high serum P (17). Furthermore, the silencing of [PTH 1 receptor \(PTH1R\)](#), the most abundant PTH receptor in VSMC, partially abolished the pro-calcifying effect of high PTH, demonstrating an important PTH/PTH1R-driven induction of Ca deposition in the medial artery layer (17).

An additional critical consideration is that in advanced CKD, the serum levels of soluble Klotho cannot reflect the real reduction in renal Klotho content. In fact, soluble Klotho, due to its molecular weight cannot be filtered, so, its appearance in the urine to exert FGF23-independent

phosphaturic actions involves a process of transcytosis of soluble Klotho from the blood into the urine through renal tubular cells (18), a process that is impaired in a damaged kidney.

The decrease in urinary soluble Klotho could also partly explain the defects in renal tubular Ca reabsorption and its adverse impact on the skeleton. In fact, urinary soluble Klotho favors the anchoring of the Transient Receptor Potential Cation Channel Subfamily V Member 5 (TRPV5) Ca channel to the cell membrane through its sialidase/glucosidase activity, an action that attenuates the urinary excretion of Ca, preventing a negative Ca balance and, consequently, it could attenuate bone demineralization (19, 20).

This review will analyze the effect and interactions of the above mentioned factors with the two main bone pathways, Receptor Activator of Nuclear Factor (NF)- κ B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) ~~RANKL/RANKL/OPG~~ and Wnt/ β -catenin, describing their implications in bone and cardiovascular disorders in CKD.

2. THE RANK/RANKL/OPG/ (LGR4) SYSTEM

PTH is the main regulator of the ~~Receptor Activator of NF- κ B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG)~~ system that controls bone remodelling by inducing osteoblast synthesis of RANKL and downregulating OPG production. Both mechanisms favour osteoclastogenesis and bone resorption through a Protein Kinase A (PKA)-driven mechanism (21-23). PKA agonists mimic the PTH regulation of RANKL and OPG genes expression (22, 24).

The OPG/RANK/RANKL system was described in the mid_1990s as an essential regulator of bone modeling and remodeling (25). Its role in bone maintenance is well known, but some papers attributes to it an important role in vascular calcification (26, 27).

In bone, osteoblasts and osteocytes synthesize and secrete RANKL, which binds to its transmembrane receptor RANK in bone marrow-derived osteoclast progenitors, allowing the maturation, activation and survival of osteoclasts to initiate resorption. In summary, the RANKL action promotes and increases the osteoclastogenesis and bone loss. In addition, osteoblasts secrete OPG, a soluble decoy receptor for RANKL, which prevents the binding of RANKL to RANK, thus attenuating osteoclastogenesis (28) (Figure 1).

RANK, RANKL and OPG: The classical components of the pathway

RANK is fundamental for osteoclasts development. Also called Tumor necrosis factor (TNF)-related activation-induced cytokine (TRANCE) receptor, is a member of ~~the tumor necrosis factor (TNF)~~ receptor superfamily. It is a transmembrane receptor that consists in 616 aminoacids protein with 4 extracellular cysteine rich domains linked to a long C-terminal intracellular region (25, 28-30).

RANK is constitutively expressed in multiple organs and cells such us osteoclasts' precursors and mature osteoclasts, dendritic cells, mammary glands and vascular cells, among others. Its functions are associated with bone resorption, immune response, lymph node and mammary gland development and thermal regulation (31-35). RANK acts binding to the cytokine RANKL (31), however the RANK overexpression has shown to be enough to activate NF- κ B (36).

RANKL (also called OPG~~L~~ ligand) is the main stimulator of its specific receptor RANK (36-40). RANKL is a 316 amino acid protein with a molecular weight of 38 kDa. Its expression is also modulated by several cytokines, glucocorticoids, and PTH (41). RANKL is produced by osteoblasts, osteocytes and activated T cells. It promotes the formation, fusion, differentiation, activation and survival of osteoclasts, allowing increased bone resorption and mineral loss (42, 43) (Figure 1).

Activation of RANK by RANKL initiates the intracellular signaling cascade of NF- κ B (~~nuclear factor kappa B~~, a protein complex that functions as a transcription factor modulating many cellular processes) (44, 45). Indeed, the final step in RANK activation is the translocation of NF- κ B to the nucleus, which can take place through the classical or the alternative route initiated by their respective kinases, named inhibitor of nuclear factor- κ B (I κ B) kinase (IKK) ~~IKK~~ β and IKK α . The translocation of NF- κ B to the nucleus modulates the expression of different genes, such as c-Fos, Nuclear Factor of Activated T cells 1 (NFATc1) and some Bone morphogenetic proteins (BMPs) (25, 46).

OPG (also known as osteoclastogenesis inhibitory factor -OCIF-) is a decoy receptor for RANKL that regulates osteoclastogenesis disrupting the interaction between RANKL and its receptor RANK (28) (Figure 1). OPG is a 60 KDa glycoprotein, member of the TNF superfamily

that is normally secreted by the osteoblasts, even though it has also been detected in association with the cell membrane in lymphoid cells (47). OPG consists of 7 structural domains. Domains 1 to 4 give OPG its osteoclastogenesis inhibitory activity, domains 5 and 6 are considered death domains and involved in apoptosis. Domain 7 harbors the heparin binding region, a common trait of growth factors and signaling molecules (48).

OPG is produced in a wide variety of tissues including the cardiovascular system (heart, arteries and veins), lungs, kidneys, intestine and bone, as well as in hematopoietic and immune cells (30, 47, 49-52). Its expression and production are regulated by several cytokines, peptides, hormones, and drugs. Cytokines, including TNF α , interleukins 1 α and 18, transforming growth factor β (TGF β), bone morphogenic proteins (BMPs), and steroid hormones, such as 17 β estradiol, regulate OPG mRNA levels (53-55). Conversely, glucocorticoids (known to promote bone resorption), immunosuppressant cyclosporin A (which causes osteoporosis and vascular disease), PTH, prostaglandin E2, and fibroblastic growth factor (FGF) decrease OPG expression (53, 56-60).

It is well established that decreases in OPG favor, not only increases in osteoclastogenesis and bone resorbing activity, but also the increases in vascular Ca deposition. Indeed, OPG reduction was identified as an independent variable for coronary artery calcification (61). Furthermore, a recent elegant work has linked the anti-calcifying effects of higher vascular Pit2 expression to increases in OPG levels as suggested by the low levels of OPG in the Pit2 deficient mice (62).

In healthy subjects over 70 years old, RANKL and OPG plasma levels were gender-independent (63, 64), but different serum OPG levels were found in men and premenopausal women (65). There is a discrepancy between OPG concentration and ageing, some studies did not observe variations with age (64, 66), while others have shown a positive correlation (63, 65, 67) mainly in subjects over 60 years old (63). In two age related disorders, such as rheumatic polymyalgia and osteoarthritis, circulating OPG levels did not differ from the age matched controls, but soluble RANKL levels were higher in both diseases (63).

The RANK/RANKL/OPG system is of high clinical relevance in osteoporosis treatment. OPG is not used in clinical practice but its discovery and the studies of its actions were the base of the development of Denosumab, a human monoclonal antibody against RANKL that mimics the actions of OPG (reducing osteoclastogenesis and bone resorption). Denosumab has been worldwide long-term used to prevent the reduction of bone mass and to decrease the risk of bone fractures (68).

LGR4: A New RANKL receptor

The recent discovery of a new RANKL receptor, the leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4) (69), also called G-protein-coupled receptor (GPR) GPR48, provided a novel member of this system that regulates bone formation. This receptor counteracts RANKL-driven osteoclastogenesis and also activates the Wnt/ β -catenin pathway (70), it acts on bone formation but may also adversely promote vascular calcification.

LGR4 is essential to increase bone formation by increasing osteoblast maturation and mineralization (69). In addition, LGR4 inhibits osteoclast differentiation and maturation by the competition with RANK to the binding to RANKL. In this sense, LGR4 knock-out mice developed abnormalities in bone during embryonic and postnatal stages with delay in osteoblast differentiation and mineralization, reductions in osteoid formation and increases in osteoclast activity (71) ~~(Figure 4)~~. Also in humans, a nonsense mutation in LGR4 has been strongly associated with low bone mineral density and osteoporotic fractures (72).

However, the effect of PTH on LGR4 expression and the likely mechanisms involved are incompletely understood. A recent study has demonstrated that in uremic rats fed high P diet, LGR4 aortic expression markedly increased in response to high PTH. *In vitro*, the silencing of the LGR4 gene in VSMCs was capable to prevent PTH-induced vascular calcification without changes in RANKL and OPG expression (73) (Figure 1). Due to its recent discovery, is still early to envision if LGR4 will play a future role in the clinical management of bone and vascular disorders.

3. THE RANK/RANKL/OPG/(LGR4) SYSTEM. ROLE IN OSTEOPOROSIS AND VASCULAR CALCIFICATION

PTH indirectly regulates osteoclast differentiation and activity by increasing the production of RANKL and decreasing OPG synthesis in osteoblasts (28).

The biological effects of OPG are opposite to those mediated by RANKL, since OPG acts as a soluble inhibitor that prevents the interaction of RANKL with its receptor RANK and subsequently, its stimulation of osteoclastogenesis (74). The first evidence that this system was involved in vascular calcification derived from the study of the OPG knockout mouse, which presents osteoporosis and calcification of the aorta and renal arteries (51, 75).

OPG expression can be found in the media of large arteries (51) and in different cells types of the vessel wall such as VSMCs and endothelial cells (58, 76). In endothelial cells, OPG acts as

an autocrine survival factor (76). In contrast, RANKL and RANK have only been found in the calcified areas of aortas of transgenic mice prone to calcification, but not in the arteries of wild type mice (77).

The hypothesis that the RANK/RANKL/OPG system could establish a link between osteoporosis and vascular calcification is clinically based on the ~~presence~~ increased risk of arterial calcifications and cardiovascular disease in postmenopausal women and elderly people with osteoporosis (78-80). Other studies have shown that OPG inhibits the extensive calcifications of the aortic, carotid, femoral, mesenteric, hepatic, renal arteries induced by treatment with warfarin or toxic doses of vitamin D (81). Moreover, VSMCs calcification induced by β -glycerophosphate or RANKL was inhibited by OPG addition to the culture media (26).

The discovery that the OPG knockout mouse develops osteoporosis and severe arterial calcification (75) and the fact that RANKL expression increases in calcified arterial tissue (82), and also induces calcification of VSMCs through its binding to RANK and increases in BMP 4 expression (26), suggests that the OPG/RANK/RANKL axis could be an important autocrine/paracrine system involved in vascular calcification.

In the vasculature, increases in RANKL and decreases in OPG favor vascular calcification (26, 83). As it has been previously mentioned, LGR4 seems to promote vascular calcification in experimental models. In addition, LGR4 has also shown to potentiate Wnt/ β -catenin pathway through two mechanisms: the increase in Wnt receptors that involve a direct inhibition of the ubiquitinases Ring Finger Protein (RNF) 43 and Zinc And Ring Finger (ZNR) 3 that mediate their degradation, and also through the recruitment of the guanosine triphosphate (GTP)ase-activating protein Ras GTPase-activating-like protein (IQGAP1) to the Wnt/ β -catenin pathway complex, which results in a potentiation of both, the canonical and noncanonical Wnt/ β -catenin pathways (84). Recently, Apelin was identified as an important down-regulator of the activation of LGR4/ β -catenin signaling, sufficient to ameliorate aortic remodeling and fibrosis in models of transverse aortic constriction (85). As for most Wnt inhibitors, beneficial anti-calcifying actions in the vasculature could adversely impact adequate bone formation.

The role of RANKL and OPG as biomarkers of skeletal health has also been studied. In fact, the RANKL/OPG ratio is a useful tool to indirectly determining the degree of bone remodeling (86), however, its relationship with the degree of vascular calcification (87, 88) is still a controversial issue.

Denosumab, ~~which mimics the OPG action~~, has not shown any effect on vascular calcification. In the FREEDOM study (from Fracture Reduction Evaluation of Denosumab in Osteoporosis Every 6 Months), performed in osteoporotic women, the abdominal X-rays showed no differences in the progression of aortic calcification and the reported adverse cardiovascular events were found in the placebo and Denosumab groups (89).

4. WNT/ B-CATENIN PATHWAY

The Wnt/ β -catenin is an intracellular signaling pathway that plays a key role in bone formation, regulating the osteoblast activity (90-93). When the Wnt/ β -catenin pathway is activated bone is formed, when this pathway is inhibited, bone formation is stopped.

The activation of the Wnt/ β -catenin pathway start when the Wnt ligand bind to their receptors, Frizzled and Low-density lipoprotein receptor-related protein (LRP)5/6 inactivating the Glycogen synthase kinase 3 (GSK3) stabilizing β -catenin in the cytoplasm making possible the translocation of β -catenin into the nucleus ~~where replaces Groucho from T-cell factor (TCF)~~, initiating the transcription of bone forming genes, regulating the preosteoblast differentiation through Runt-related transcription factor 2 (Runx2) or/and Osterix induction, among others (94, 95). In the absence of the Wnt ligand, β -catenin is phosphorylated by GSK3, leading to its destruction avoiding its translocation to the nucleus and the osteoblast differentiation and osteocyte formation (Figure 2).

The Wnt/ β -catenin pathway has inhibitors such us the ~~Low-density lipoprotein receptor-related protein (LRP)~~ inhibitors Dickkopf1 (Dkk1) and sclerostin (also called Sost), that bind to LRP5/6 receptor allowing its internalization into the cytoplasm; and the frizzled inhibitors called secreted Frizzled Related Proteins (sFRPs), which are able to block the Wnt/ β -catenin pathway, inhibiting an decreasing the osteoblast differentiation and survival, respectively (96-98) (Figure 2).

The Wnt/ β -catenin pathway interacts with several hormones and factors such as PTH, FGF23, calcitriol, Klotho, and LGR4. The latter it is not only a RANKL receptor, but also a key receptor for R-Spondin (R-spoR-spo2) 2, which is a Wnt/ β -catenin pathway activator (99).

PTH is one of the main regulators of Wnt/ β -catenin pathway in bone. It is well known that PTH is an inhibitor of sclerostin, and this action is fundamental to increase bone formation (100-103); but PTH also affects the differential regulation of LRP5/LRP6 and the antagonist Dkk1 (100).

The use of anti-sclerostin monoclonal antibodies has shown to be effective in preventing bone loss in normal rats and rats with chronic renal failure (CRF) and low serum PTH (104), but not with elevated serum PTH (105); which suggested that serum sclerostin values, could be even a more sensitive and precise remodeling marker than circulating PTH. Both continuous and intermittent PTH administration decrease sclerostin levels (102, 103). Also, continuous PTH, increase the signaling receptor Frizzled 1 (103, 106) and co-receptors LRP5 and 6. However, there is not consensus in the effect of PTH over the agonist Dkk1. Several studies have shown that PTH decrease Dkk1 levels (100, 107-109) but others have found the opposite effect and PTH treatment was associated with increases in serum Dkk1 levels (110, 111).

Although there is not much evidence about the direct interaction of FGF23/Klotho with Wnt elements, it has been shown that the extracellular domain of Klotho binds to multiple Wnt ligands, inhibiting their ability to activate Wnt signaling (112, 113). It is interesting to note that in CKD, in parallel to the decrease of Klotho, and the increase of FGF23, there are also changes in the levels of Wnt inhibitors, such as sclerostin or Dkk1 (109, 114). FGF23 directly inhibits the osteoblastic Wnt/ β -catenin pathway through a soluble Klotho/mitogen-activated protein kinase (MAPK)-mediated process that requires Dkk1 induction (109). However, the relationship between FGF23/Klotho and Wnt / β -catenin pathway has not been sufficiently explored.

The soluble Klotho acts as an antagonist of Wnt/ β -catenin pathway activation through protein-protein interactions between soluble Klotho and extracellular activators of the Wnt/ β -catenin pathway (113). In CKD, the loss of kidney function is the most important cause of reduction in renal Klotho gene expression. As Klotho downregulates renal calcitriol production, its reduction could influence bone remodeling in CKD patients, acting through the complex PTH-calcitriol-FGF23 axis modulating through a direct protein-protein mechanism the interaction between the vitamin D receptor (VDR) and β -catenin (115, 116).

Since the activation of the Wnt/ β -catenin pathway is also involved in the progression of kidney damage (117), part of the renal and vascular anti-aging effect of blood-soluble Klotho could be explained by its ability to inhibit the Wnt/ β -catenin pathway (118, 119). The interactions between soluble Klotho and the extracellular activators of the Wnt/ β -catenin pathway may have negative effects in bone and positive effects in vessels, a matter of current research.

As mentioned earlier, LGR4 (and also LGR5 and LGR6) have been recently identified as second class receptors for the R-spondins (R-spos) family and it is another regulator of Wnt/ β -catenin pathway (70, 84) through the formation of complexes with recognized Wnt/ β -catenin

1 pathway modulators action such us Frizzled/LRP (120). R-spos activates the Wnt/ β -catenin
2 pathway through increasing phosphorylation of the Wnt co-receptors LRP5/6. They cannot directly
3 activate the Wnt/ β -catenin pathway, but they can do it indirectly acting as a key receptor for R-
4 spo2, which activates the canonical Wnt/ β -catenin pathway promoting osteoblast differentiation
5 and maturation (121-123). A similar effect may occur in osteoblast-like cells derived from VSMCs
6 to initiate vascular calcification. Other member of the family, R-spo1, may also play a role in bone
7 formation by synergizing with the Wnt ligand Wnt3A to induce osteoblast differentiation and OPG
8 expression (99).
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18 5. THE WNT/ β -CATENIN PATHWAY INHIBITORS AND VASCULAR 19 CALCIFICATION 20 21

22 As it has been discussed, the Wnt/ β -catenin pathway is fundamental for bone formation, but
23 it is also implicated in vascular calcification (17, 124-126). The pathophysiology of vascular
24 calcification involves a transition of the ~~vascular smooth muscle cells~~ (VSMCs) to a “osteoblast
25 like” phenotype, afterwards, a process of mineralization takes place in the vessel (127-129). In this
26 transformation, several hormones and proteins are involved as a promoters or inhibitors of the
27 vascular calcification. It is beyond the scope of this review to list and discuss the role of these factors
28 which have been analyses in detail in other publications (130-132) and also in other review of this
29 special ~~CTI~~ Calcified Tissue International supplement (133). Among them, the
30 RANK/RANKL/OPG pathway, -already discussed in this review-, and the Wnt/ β -catenin pathway,
31 play an important role in bone and vascular metabolism mainly through the inhibitory action of the
32 Wnt/ β -catenin pathway through Dkk1, sclerostin and the sFRPs. As a result of the action of these
33 Wnt/ β -catenin pathway inhibitors there is a decrease in the osteoblast differentiation and survival,
34 reducing bone formation (96-98) (Figure 2).
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47 During several decades, the excessive PTH suppression, mainly due to aluminum and Ca
48 load and overdosing of active vitamin D, with the consequent abnormally low serum PTH values,
49 have been considered the main responsible of the pathogenesis of low bone turnover, and low bone
50 formation observed in CKD. However, in recent years, this paradigm has been challenged, as a
51 result, the pathogenesis of low bone turnover is under reconstruction and the inhibitors of the Wnt/ β -
52 catenin pathway have been blamed as responsible of the early low bone turnover observed in CKD
53 patients. In fact, clinical and experimental data, both supported by bone biopsy, have shown that
54 bone sclerostin is increased and bone formation decreased in early stages of CKD (17, 109, 134,
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135). Unfortunately, these findings are difficult to translate in the clinical practice to guide therapeutic decisions, due to the fact there is a poor or a lack of correlation between the bone and serum values of the inhibitors of the Wnt/ β -catenin pathway, mainly in sclerostin, the most studied molecule (136, 137).

As vascular calcification and bone loss are age-dependent, the association between serum sclerostin levels and age has been investigated. A study performed in healthy pre and postmenopausal women (aged 20 to 79 years), showed the serum sclerostin significantly increased in postmenopausal women after the menopause (138). Similarly, other study showed that serum sclerostin levels were 46% higher in old women (mean age, 72.9 years) compared to young women (mean age, 30.0 years), but in contrast, sclerostin mRNA levels measured in bone biopsies were no different in the two groups (139), suggesting the age-dependent decrease in glomerular filtration rate may play a role and it should be considered in the interpretation of serum sclerostin levels.

In experimental models of ~~chronic renal failure~~ (CRF), sclerostin increase at very early stages, before the increase in P, PTH, and FGF23 (109, 134, 140). Furthermore, studies in humans (134), in a mouse model of slow developing polycystic disease (134, 140) and in a model of CKD with hyperphosphatemia (109), have also shown that the increase in sclerostin in bone precedes the increase in serum P, PTH and FGF223. In fact, the increments of these three factors are a later event which coincides with the decrease in bone sclerostin and with the increments in other inhibitors of the Wnt/ β -catenin pathway (109, 134, 140). In fact, bone biopsies from CKD patients have shown the signals of inhibition in the Wnt/ β -catenin pathway were associated with low levels of sclerostin in osteocytes (134), suggesting there may be a contribution of the other inhibitors of the Wnt/ β -catenin pathway in the pathogenesis of low bone turnover (141).

In fact, *in vitro* studies have shown that although the decrease in one Wnt/ β -catenin pathway inhibitors, is associated with greater vascular calcification, the increase in other Wnt/ β -catenin pathway inhibitors may play a role counteracting the decrease and attenuating the effect on vascular calcification (142-144). A good example is a study in diabetic rats with CRF, in which the neutralization of Dkk1 with monoclonal antibodies, was sufficient to prevent both bone and vascular damage (145).

Recent studies analyzing the direct effect of PTH and FGF23 on osteoblasts have revealed that elevated PTH inhibits not only the increases in sclerostin, but also the increment of other Wnt/ β -catenin pathway inhibitors and that FGF23 may have a direct inhibitory effect on the Wnt/ β -catenin pathway in osteoblasts through the induction of Dkk1 (109). On the contrary, the action of FGF23

would be opposite to that of PTH, since high FGF23, through the induction of increases in Dkk1, would inhibit Wnt/ β -catenin pathway in bone contributing to the bone loss, whereas in vessel could attenuate vascular calcification. In addition to these effects, sclerostin can influences serum concentration of calcitriol and FGF23, both implicated in the mineralization process (146).

The inhibition of sclerostin and other Wnt/ β -catenin pathway inhibitors in bone by high levels of PTH could contribute to maintaining bone health, but it is important to highlight that PTH-dependent reduction of the Wnt/ β -catenin pathway inhibitors in the vessels, could favor vascular calcification. Indeed, as mentioned earlier, recent studies in rats with CKD exposed to different concentrations of PTH suggest that elevated PTH favors vascular calcification. Instead, normal circulating PTH, levels appeared to be protective of aortic calcification despite high serum P (17).

It is important to highlight that even though the sclerostin inhibitors, such as Romosozumab, is one of the most promising therapeutic targets in the prevention and treatment of bone fragility fractures (147) its use in CKD patients is still a matter of controversy (148, 149). In fact, Romosozumab could have a negative action in the vascular system where the "natural" inhibition of the Wnt/ β -catenin pathway observed when severe vascular calcification is present, can play an important role protecting the vascular wall from further vascular mineralization.

Studies in animals with CKD and severe aortic calcification showed an increased aortic gene expression of some members of the sFRPs family (sFRPs 1, 2 and 4), suggesting the inactivation of Wnt/ β -catenin pathway in the vessels wall may constitute a "natural" protective mechanism against the progression of vascular calcification (109, 124). Thus, according to the present knowledge from experimental models, we can hypothesize that the increase in PTH, -a potent sclerostin suppressor-, progressively reduces the expression of sclerostin and the increment in other inhibitors, such as Dkk1 and/or sFRPs, of the Wnt/ β -catenin pathway could compensate the sclerostin reduction, helping to protect from further vascular calcification (109, 124, 130). However, other recent study has reported the hypothesis that the increased levels of serum sclerostin probably originating from excessive local production in calcified vessels, may contribute to the linkage between vascular disorders and impaired bone mineralization (150).

6. CARDIAC IMPACT

Among the cardiovascular disorders associated to abnormal Wnt/ β -catenin pathway activation, the abnormalities in left ventricular (LV) structure and function are also important. Left

~~ventricular hypertrophy (LVH)~~ is a well-recognized cardiovascular disorder, which occurs early in the course of CKD (151, 152). Cardiomyocytes and fibroblasts are the cells implicated in the abnormal remodeling process leading to LVH, the cardiomyocytes increase their size, and the fibroblasts increase collagen synthesis prompting the onset of fibrosis. These changes progressively lead to cardiomyocyte apoptosis or necrosis, and the cardiomyocytes are replaced by fibroblasts and extracellular collagen (153). In addition, PTH promotes apoptosis of the cardiomyocytes (154), which in the long-term, either causes or exacerbates myocardial fibrosis.

Several clinical studies have shown that high PTH levels are associated with LVH (155, 156). Recently, it was demonstrated that myocardial specific R-spo3 acts mainly through the LGR4 receptor to promote coronary stem cell proliferation in the developing heart (157), demonstrating that this receptor and its ligand have an important role in heart development. Moreover, abnormalities in the canonical Wnt/ β -catenin pathway are fundamental in the establishment of cardiac lesions. Indeed, activation of β -catenin induces cardiomyocytes hypertrophy and myofibroblastic transformation of cardiac fibroblasts, increasing their ability to produce and secrete interstitial matrix components such as fibronectin and collagen I (158). Furthermore, a high OPG/RANKL ratio has been independently associated with LVH and abnormal LV structural remodeling in male overweight/obese children and adolescents (159). A better knowledge of the mechanisms that modulate the appropriate function of the discussed pathways may provide relevant information on novel therapeutic targets to attenuate LVH and myocardial fibrosis in CKD.

FGF23 is also elevated in CKD patients and it has been also related as a cause of LVH (160). Some authors have speculated that FGF23 could develop LVH through the Wnt signaling activation. In fact, the Wnt signaling inhibition improves cardiac function and could attenuate LV changes (114, 161).

7. FINAL COMMENTS

In summary, a better understanding of the intricate regulation of the RANK/RANKL/OPG/LGR4 and the Wnt/ β -catenin pathways in bone and vessels is a highly needed step to improve the diagnosis and treatment of these CKD complications.

The design of therapeutic strategies to prevent the deterioration of the bone-vessel axis in the progression of CKD requires a much better understanding of the interaction between classic factors such as Ca, P, calcitriol, PTH and FGF23 with the activation and inactivation of the RANK/RANKL/OPG system and the Wnt/ β -catenin pathway. Unfortunately, still it is not possible to translate into the clinical practice a great part of the new important and challenging information discussed in this review because still in many of them, the serum levels of the components of the two main pathways are not able to predict their changes at bone and vascular level.

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AUTHOR'S CONTRIBUTION

N.C.L. and S.P. had the idea for the article, L.M.A. and S.F.V. performed the literature search and data analysis, S.P., M.N.D. and J.C.A. drafted the article and N.C.L., M.P.R.T., A.D.; J.C.A., M.N.D. and S.P. critically revised the article.

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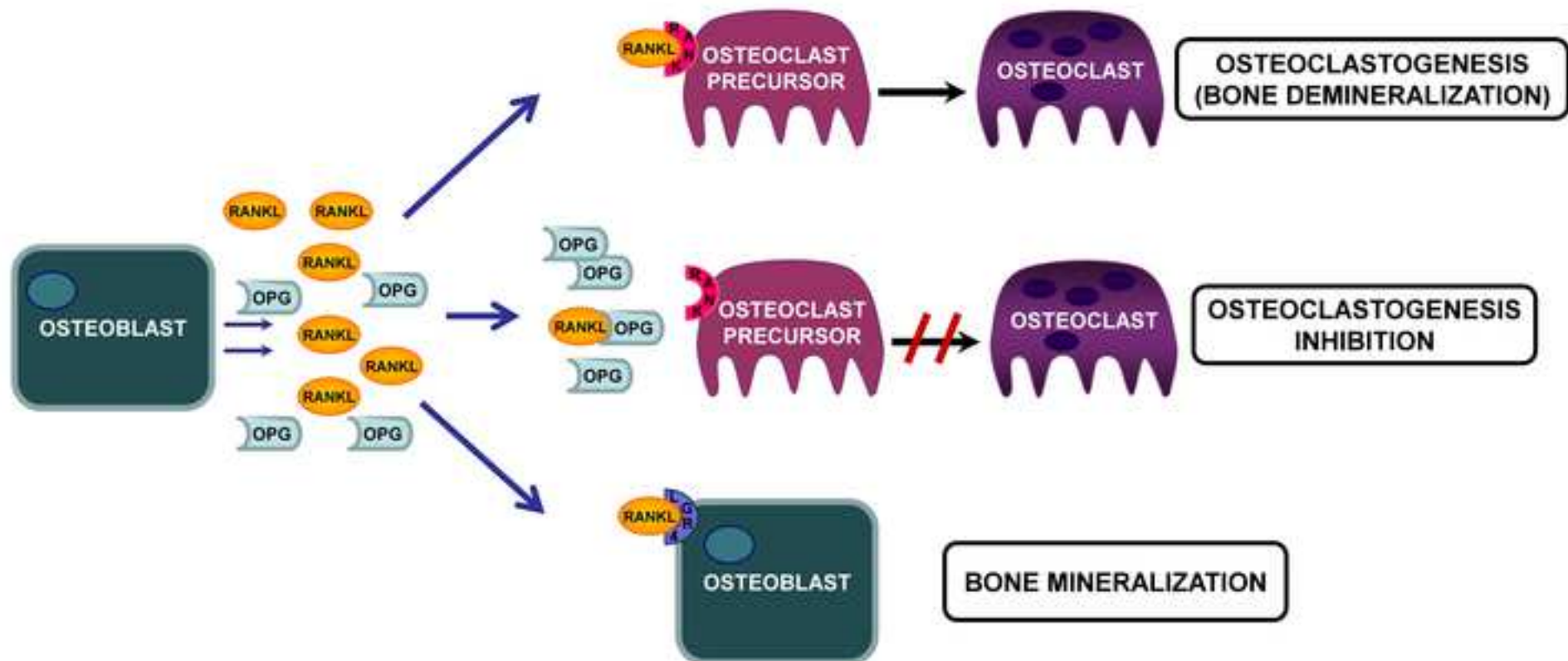
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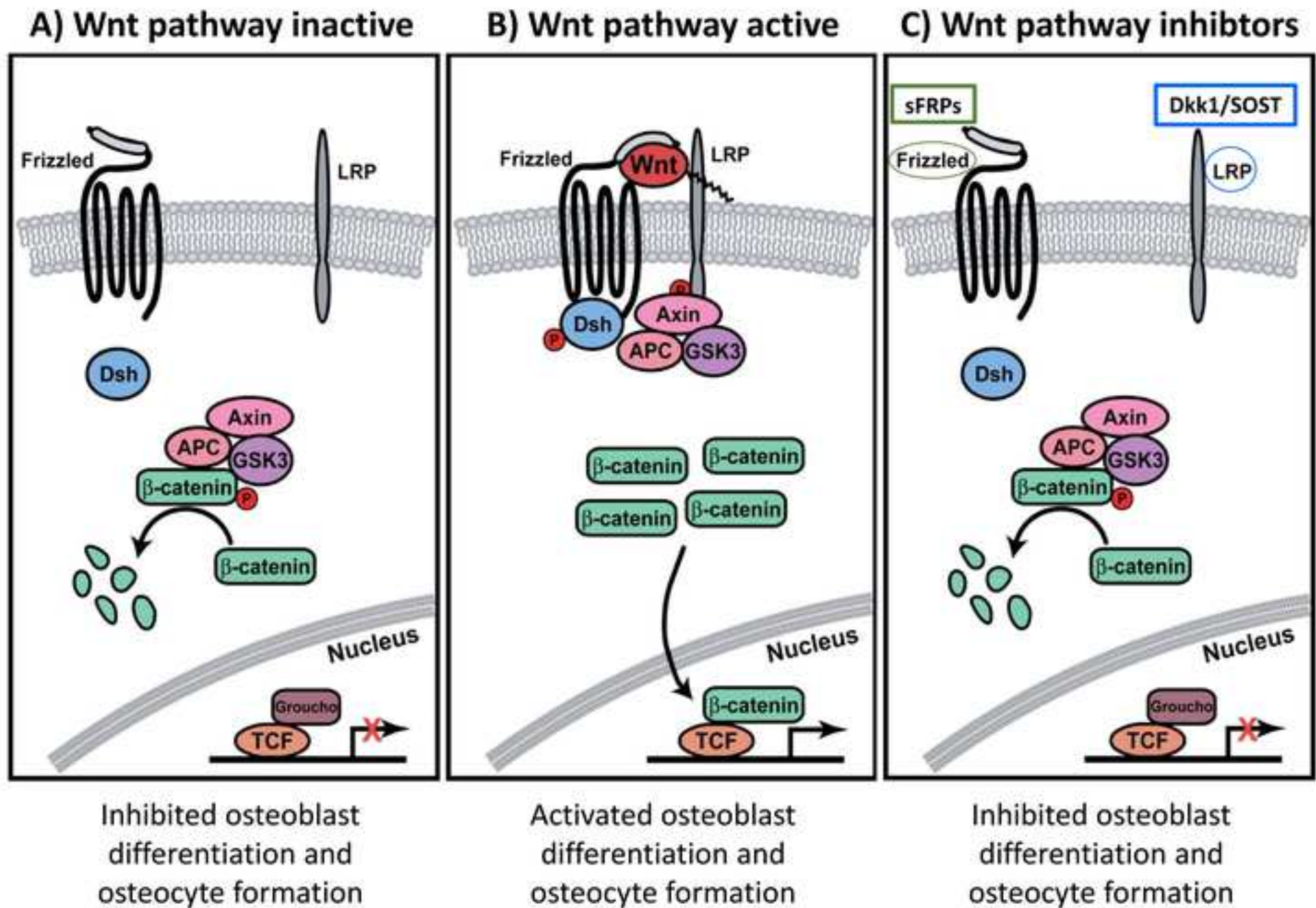
FIGURE CAPTIONS

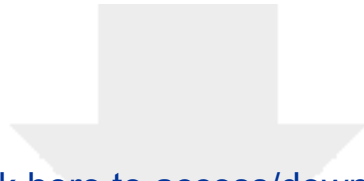
Figure 1. RANK/RANKL/OPG/LGR4 signaling in bone. A) Osteoblasts synthesize and secrete RANKL which binds RANK allowing their activation, maturation and prolonging the survival of osteoclasts. B) Osteoblasts also secrete OPG, a soluble RANKL decoy receptor, which prevents RANKL binding to RANK, inhibiting osteoclastogenesis. C) RANKL can also binds the LGR4 receptor on the surface of the osteoblasts, triggering signals of mineralization and bone formation.

Figure 2. Wnt/ β -catenin signaling pathway. A) In the absence of Wnt ligand, β -catenin is phosphorylated by GSK3 and destroyed avoiding its translocation to the nucleus to trigger the mechanisms of bone formation. B) If Wnt ligands binds to its LRP5/6 and Frizzled co-receptors, GSK-3 is inactivated, β -catenin is stabilized in the cytoplasm and translocate into the nucleus where activates target genes promoting osteoblast differentiation and osteocyte formation. C) In presence of Wnt inhibitors: Dkk1 or sclerostin or the sFRPs bind to LRP5/6 receptor or Frizzled respectively, thus the Wnt/ β -catenin pathway is inhibited. (Modified from Gordon MD et al. J Biol Chem. 2006; 281(32): 22429-22433 with permission from The American Society for biochemistry and Molecular biology.

Figure 1

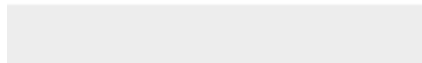


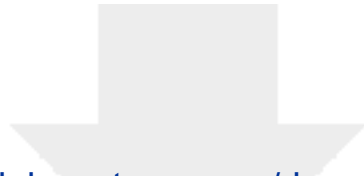




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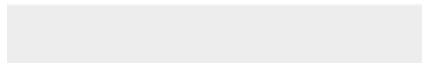
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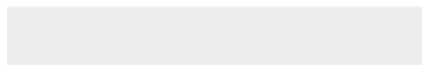
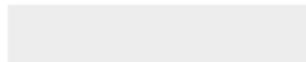
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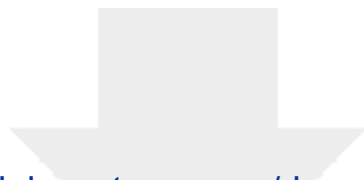




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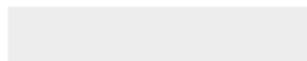
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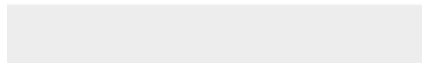
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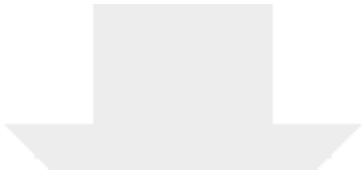
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