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CONTRIBUTIONS OF THE INTESTINAL MICROBIOTA TO BONE

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ABSTRACT

Introduction: Overall bone metabolism is highly regulated by the intestine, mainly mediated by parathyroid hormone (PTH) and vitamin D. However, emerging pieces of evidence have shown this regulation is also strongly associated with intestinal microbiota by releasing metabolites, which affects directly or indirectly the bone. **Objective:** To review the contributions of the intestinal microbiota to bone. **Methods:** Thus, a narrative review from articles on contributions of intestinal microbiota to bone was conducted using database PubMed covering a period from 1997 until July 2023. Articles published in the form of original articles, systematic review or meta-analysis using the descriptors: bone and microbiota, bone and bacteria, gut microbiota and bone and probiotics were read and summarized throughout text. **Results:** Initially, we searched for understanding, which bacteria are resident or transitory and which are the factors released that either indirectly or directly act on bone, thus modifying its quantity or quality. Therein, we briefly explored how intestine, and bone are interconnected and finally about how the type of intestinal microbiota is associated with bone metabolism, quality and quantity. In particular, we reported some main studies on probiotics and bone health. **Conclusion:** This review brought together information from the literature on the role of intestinal bacteria in bone, revealing possibilities for directing the microbiota to maintain or gain bone in quantity and quality and thus prevent bone fractures in a close future, especially for osteoporotic individuals.

Key words: Microbiota, gut microbiota, bone, bacteria, probiotics.

INTRODUCTION

The human intestinal microbiota comprises bacteria, archaea, fungi, protists, and viruses that live together and interact with each other and with host cells. In particular, the human intestine is colonized by trillions of bacteria, important to digestion process and absorption, in addition to forming the most volume feces. Increasing pieces of evidence have shown a source of metabolites derived from microbiota has implications on different organs. The population of bacteria present along the gastrointestinal tract is widely diverse and determined strongly by microbial habitat (mouth, throat or intestine) (1). From this moment, the intestinal microbiota is simply defined as microbiota.

The main bacteria found in intestine are *Bacteroidetes* and *Firmicutes* account for approximately 90% and a lower extent *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (1, 2).

A consensus of the European society for clinical and economic aspects of osteoporosis, osteoarthritis and musculoskeletal

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diseases pointed out that intestinal microbiota may influence the response to osteoarthritis medications by regulating drug metabolism and bioavailability (3). Osteoporosis, characterized by low bone mass density and microarchitecture deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture (4). Bone is an organ with direct contact to muscles, tendons, cartilage, bone marrow by which it can regulate it. Although the intestine is not intimately attached to the bones, the hormones and metabolites derived from it, targets directly or indirectly bone cells (5).

Several studies show that bone maintenance, gain or loss (6) influence the bone. Specifically, metabolites from bacteria digestion as butyrate, acetate, and propionate are known energetic source for epithelium (7, 8), in addition to trimethylamine N-oxide *(9)*, tryptophan (10) are more recently, described as mediators of bone metabolism (CHEVALIER; KIESER; ÇOLAKOĞLU; HADADI (11). The immune system also regulates bone mass through mechanisms involving CD4+ T cells or through innate immunity, mediating the effects of microbiota on inflammation and bone metabolism (12). Thus, it seems to have a direct or indirect link among bacterial metabolites, the immune system, and the musculoskeletal system.

Probiotics are the main bacteria-containing food products used to influence the intestinal microbiota-bone; however, in general, studies showed a beneficial action of taking probiotics whereas others showed opposite effects, evidencing controversial role on bone health (13). Thus, this review brings to the light the importance of the intestinal bacteria to bone, which creates possibilities of targeting microbiota to maintain or gain bone quantity and quality.

METHODS

This narrative review was performed through a search of scientific articles on microbiota and its relationship with bone in the PubMed database, covering the period from 1979 to July 2023. Publications were selected in the form of original articles, integrative reviews, or systematic reviews, using the descriptors: bone and microbiota, bones and bacteria, intestinal microbiota.

FINDINGS

The resident human intestinal microbiota

Humans are colonized by bacteria before birth, and the microbiota eventually reaching maturity at about 3 years of age (14) and been important in every stage until extreme longevity (15, 16). Different parts of body contain its own bacteria population due a plethora of factors such as pH, diet, geography, age, disease, drugs, genetics and early exposure (lactation, natural birth or Cesarean section) as mapped by the Human Microbiome Project Consortium in 2012 (1).

The intestine represents the main source of bacteria, totalizing almost 2 kilograms or 95% of total amount of bacteria in adults (1, 17). The bacterial phyla representative of the human intestinal microbiota is *Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria*, and *Verrucomicrobia* with predominance of *Firmicutes* and *Bacteroidetes* whereas *Actinobacteria* (Bifidobacteriaceae family) and *Proteobacteria* (Enterobacteriaceae family) are found in a lower number. In the *Bacterioidetes phylum*, the most studied genera are *Bacteroides*, *Prevotella* and the *Firmicutes* phylum (including species of *Lactobacillus*) are related to intestinal health maintenance. The main bacterial taxonomic classification found in the intestines were recently summarized by Checa-Ros at al. in 2021 (18)

The outstanding importance of intestinal bacteria is to digest substances from food intake, synthesis of vitamins B and K and the metabolism of bile acids, other sterols and xenobiotics (19). Many of these groups are also responsible for body decomposition after death, including bone (20).

Association between intestine and bone

The digestive system is formed by an extensive gastrointestinal tract and its main associated organs such as tongue, teeth, salivary glands, pancreas, liver, and gallbladder. Regarding gastrointestinal tract, the part responsible for nutrients absorption (vitamins, minerals, carbohydrates, fats, proteins) is the small intestine, an organ of 5-6 meters divided in duodenum, jejunum and ileum continuously connected to large intestine, specialized in absorbing water and electrolytes and divided in colon, rectum and anus, producing and absorbing vitamins, and forming and moving feces toward the rectum for elimination (21).

Bone is a dynamic organ that undergoes significant turnover mediated by osteoclasts, osteoblasts, and osteocytes. In short, bone-resorbing osteoclasts are responsible for bone degradation releasing inorganic and organic products from bone matrix to serum in response to parathyroid hormone (PTH) whereas bone-forming osteoblasts synthesize bone matrix and proceed to its mineralization. In addition, osteocytes are responsible for orchestrating the bone acquisition during growth and the maintenance in a healthy skeleton (22).

Calcium is one of the abundant minerals available in the inorganic bone matrix, which provides strength to bone, preventing fractures. Calcium homeostasis is also controlled by an indirect PTH effect, but not direct, since PTH increases intestinal calcium absorption, via its effects on vitamin D metabolism. In the osteoclasts, PTH stimulates bone resorption, releasing a plethora of organic and inorganic products in the bloodstream, which calcium is collected by osteoblasts to build and mineralize the matrix. Thus, in combination, vitamin D and PTH are two important molecules involved in calcium metabolism for bone (23).

The intestine is the target of vitamins such as vitamin D (23). Vitamin D is a lipid-soluble vitamin that is absorbed in the small intestine at low concentration due to insignificant amounts available in the dietary sources or supplements or from synthetized by skin under ultraviolet light in an inactive form. The vitamin D activation pathway is initiated in the liver and then finalized in the kidney and its activated product is called 1,25-dihydroxycholecalciferol

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(calcitriol, 1,25-$(OH)_2$ vitamin D_3). Next, activating vitamin D induces calcium resorption in the intestine (23, 24)

Microbiota and bone metabolism

From microbiota digestion of carbohydrates, amounts of short fatty acids are generate, including butyrate which is rapidly absorbed in intestinal epithelium for energy acquisition of the epithelium and its growth in addition to growth inhibitor and differentiation inducer of many cell lineages (19).

The central participation of the immune system on bone health is strongly attributed to lymphocytes, in particular, T and B lymphocytes. These cells regulate bone density through the secretion of cytokines or via direct cell-cell contact with osteoblasts or osteoclasts. Indeed, the role of immune system and bone health is strongly linked by lymphocytes, for example, lymphocyte deficient Rag2 knockout mice were protected by antibiotic-induced bone loss, suggesting that lymphocytes and microbiota are related (25).

SJÖGREN et al. (26) point out that bone mass is influenced by intestinal microbiota, since those germ-free mice have decreased frequency of $CD4^+$ T cells and osteoclast precursor cells, resulting in high bone mass and that is reversed when germ-free mice are colonized with intestinal bacteria. The intestinal microbiota is considered an important agent in the function of epithelium barrier, immune system, endocrine system, food digestion and energetic metabolism, as well as bone metabolism such as calcium absorption (27-29). Some species promote the release of inflammatory mediators such as tumoral growth factor (TNF), interleukin (IL)-1 and IL-6, which play an important role in the osteoclast and osteoblast formation (30) in addition to mRNA coding endothelial nitric oxide synthase (eNOS). In a dose-dependent manner, eNOS at low concentration promotes proliferation, differentiation, and osteoblast survival whereas at high concentrations inhibits resorption and bone formation (31, 32).

A meta-analysis study showed that bone mineral density (BMD) in spine and hip was not affected by probiotics. On the other hand, massive studies in experimental models showed an improvement in bone parameters related to bone health as found in *Bifidobacterium* and *Lactobacillus strains, including L. reuteri, L. casei*, *L. paracasei, L. bulgaricus* and *L. acidophilus* (13). Therefore, the direct association between probiotic and bone needs caution since that species and dosages are not completely understood.

Important to notice that enterochromaffin cells constitute the largest population of intestinal epithelial enteroendocrine cells and secrete approximately 95% of total serotonin found in the body (33). An interesting experiment showed that single-stranded RNA from intestinal microbiota is perceived by intestinal channel Piezo1 expressed in enterochromaffin cells signaling to inhibit osteoblasts proliferation. Indeed, deletion of Piezo1 in the intestinal epithelium led to increased bone formation whereas the infusion of RNase A increased bone mass due to lower quantity of fecal RNA and decreased serum serotonin levels. Thus, this study reveals that serotonin produced by enterochromaffin cells due to microbiota acts as a negative regulator of bone formation, promoting the microbial ssRNA and intestinal Piezo1 axis as a potential therapeutic target for treatment of bone (34).

Pacifici group reported that microbiota produces butyrate, a short-chain fatty acid that bind to GPR43 receptor on dendritic cells and on CD4+ T cells and induce CD4+ T cells into regulatory T cels (Treg), which stimulates CD8+ T cells to produce Wnt10b, demonstrating that butyrate coming from microbiota is necessary for PTH-mediated bone gain (7). Wnt10b is an osteogenic Wnt signaling activator that stimulates bone formation (35). Indeed, B-catenin/Wnt signaling is crucial for bone gain, since signaling activation in bone stabilizes β-catenin and its consequent translocation into the nucleus where it binds to TCF/LEF transcription factor in order to stimulate Wnt target genes, resulting in elevated levels of osteoprotegerin (OPG), an osteoclastogenesis inhibitory factor, in addition to changes in cellular survival and bone metabolism (36). Wnt10b suppression and osteoblast and osteocyte apoptosis were identified as pivotal processes in preventing bone loss after long-term glucocorticoid treatment (37).

Moreover, another association between vitamin D and butyrate has been observed. Analysis of older men with higher levels of 1,25(OH)2D and higher vitamin D activation ratios are more expected to have butyrate producing bacteria such as those specially from *Firmicutes* phylum, Clostridia class, and Clostridia order and recognized to be producers of butyrate (38).

Recent and increasing studies have shown that dietary supplementation in animal models implicates altered bone mass. As reviewed by Pacifici (39) and recently by de Sire and collaborators (40), several dietary supplements (proteins, peptides, amino acids, micronutrients, prebiotics and probiotics) contribute positively on bone mass. In addition to benefits on bone mass, some researchers reported that supplementation with *Bifidobacterium adolescentis,* one of main species hosted in intestine of healthy adults, attenuated the systemic inflammatory response after fracture, accelerated callus cartilage remodeling, and enhanced protection of the intact skeleton following fracture (41).

Metabolic stress caused by fat-enriched diet impairs the bone marrow environment, in special bone marrow stem cells by which differentiates in endosteal osteoblasts and adipocytes. Two populations of bacteria are decreased in mice receiving fat-enriched diet, *Bacteroidetes* and *Firmicutes,* whereas increased in *Verrucomicrobia*, *Actinobacteria*, and *Proteobacteria* in the cecum and ileum. Interestingly, when vancomycin antibiotic against gram-positive bacteria was administered in mice with a fat-enriched diet, the bone marrow stem cells fate was rescued (42).

Environmental temperature correlates with selection of bacteria; pointing out an existence of a signaling axis between warmth and the bone that is mediated by the microbiota as postulated by high hip fracture incidence in Northern Europe and low in the Mediterranean.

By using ovariectomized rats (OVX), a model of estrogen deficiency, the bacteria diversity was altered, with several bacteria genes upregulated and downregulated in the ovariectomized

rats and bacteria abundance showed a continuous increase of some bacterial species, mainly *Helicobacter rodentium* (43). In a recent study, Chevalier, and collaborators (11) prevented bone loss and improved bone strength in ovariectomized-mice after microbiota exposure at 34ºC. Furthermore, the transplantation of warm-adapted microbiota prevented bone loss in a mechanism involving polyamine synthesis by genera *Bacteroides*, *Alitsipes*, and lower polyamine degradation by genera *Muribaculaceae* or *Lachnospirae*, which resulted in increased osteoblast activity and decreased osteoclast differentiation. A direct influence of the heat on the bacteria is established; however, it is unclear whether the lower food intake and/or movement of animals impacted at any extent.

The insulin-like growth factor type 1 (IGF-1), a hormone with endocrine and paracrine/autocrine actions on bone promotes its longitudinal growth in addition to promote bone formation and resorption via osteoblasts (44). In addition, IGF-1 is increased in colonized mice when compared to germ-free mice (44, 45). Importantly, IGF-1 is mainly synthetized by the liver, thus indicating an existence of intestine-liver-bone axis, and bone and muscle cells can locally produce IGF-1 in order to stimulate autocrine and paracrine effects (46).

Several other metabolites have been postulated as good candidates in affecting bone metabolism (**figure 1**), such as serotonin; however, it has been rejected and other candidates are still under investigation (30).

Figure 1.

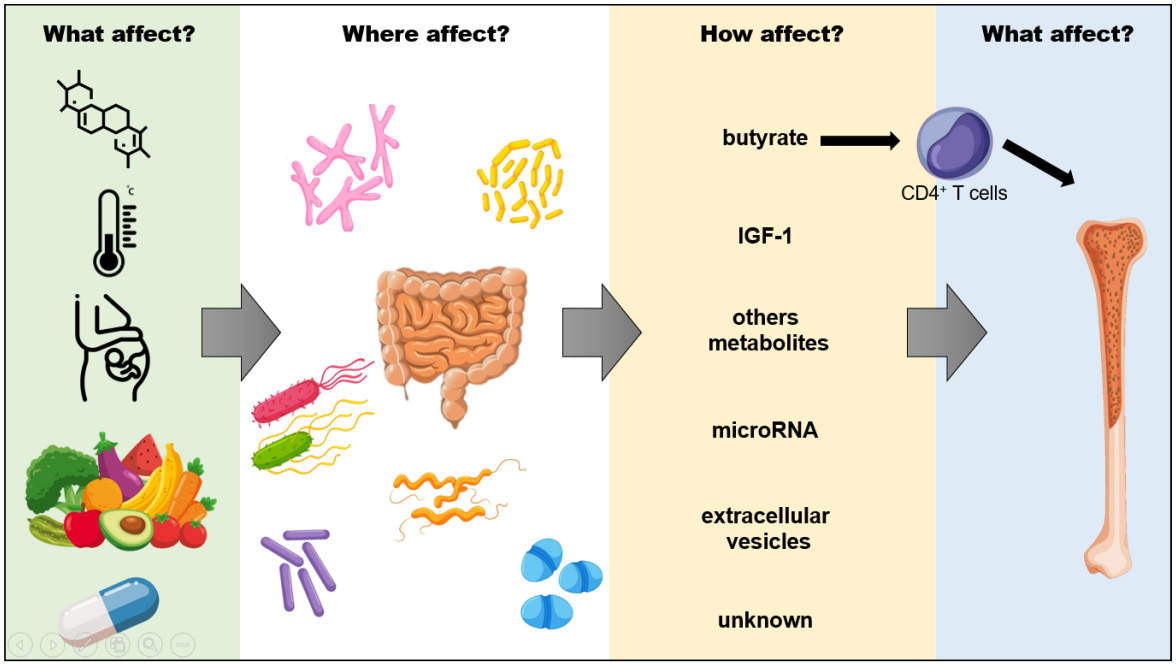

Figure 1. Intestinal microbiota communicate with several organs. The diagram shows that hormones, temperature, gestation, diet, and medications affect directly or indirectly the intestine and or its microbiota releasing butyrate and other metabolites, IGF-1, microRNA, and extracellular vesicles and still others unknown molecules that act on bone.

Microbiota and bone quantity and quality

Intriguing findings reported that mice treated with broad-spectrum antibiotic show increased bone mass (47) and even when they are treated with early antibiotic dose in mice mimicking pediatric patients (48). In this latter study, the early antibiotic dose reduced the ecological progression of the bacteria, with delays lasting several months with previous macrolide exposure whereas the control group rapidly recovered the microbiota with the diet.

A higher incidence risk of fractures by 5.6 times was observed in postmenopausal Japanese women with low numbers of bacteria from *Bacteroides* genus. It is known that vitamin K is a fat-soluble compound produced by *Bacteroides* genus and is pivotal in regulating bone matrix quality due to stimulates the osteocalcin and matrix Gla protein production (49). In the same study, *Rikenellaceae*, a family of Gram-negative bacteria, was more present in the low BMD group and with high circulating levels of bone resorbing osteoclasts, suggesting that this family may have a negative effect on bone resorption and bone density. In addition, individuals with low BMD had less abundance of the *Lachnospiraceae* family, culminating in higher fracture incidence.

Pieces of evidence showed altered bone tissue quality in aged mice (12-24 months of age) receiving low glycemic diet associated with antibiotics (6), indicating the importance of normal microbiota to bone. Furthermore, young mice (1-4 months of age) also receiving antibiotics had impaired bone quality (50). In humans, lower bone mineral density is associated with lower bacterial community composition and diversity (2). Specifically,

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in postmenopausal osteoporosis, the same evidence is noticed, with lower bacterial diversity and abundance (51). Taken together, these studies show a strong relationship between microbiota and bone quality.

If microbiota disrupts bone mass, germ-free mice might have increased bone mass. Indeed, researchers found increased microarchitectural parameters in male and females when compared to conventional caged mice; however, with differences sex-dependent (52). In addition, they also found increased tissue strength and matrix maturity. Another recent experimental report shows that depletion of intestinal microbiota using different antibiotics led to reduced bone strength without changes in bone quantity (53). Skeletal quality is transmitted by intestinal microbiota, as demonstrated in an elegant experiment that co-housed mice for 4 weeks normalized microbiota impacting positively in bone. Moreover, identified a segmented filamentous bacterium that negatively influence skeletal maturation by inducing intestinal Th17 cell expansion, an osteoclastogenic population of CD4+ T cells (54, 55).

Microbiota dysbiosis caused by a high fat diet decreased bone density due to changes in the *Firmicutes* to *Bacteriodetes* ratio. The abundance of *Actinobacteria phylum* (including Bifidobacteriaceae) positively correlates with bone volume (56).

How microbiota affect bone starts to be understood. Intrinsic microbiota have anti-anabolic effects in suppressing osteoblastogenesis and pro-catabolic effects, which leads to bone loss. These actions are immunomodulatory and mediated by the liver that release molecules targeting bone marrow (46). In addition, fecal transplantation containing segmented filamentous bacteria increased CTX (Type I Collagen Cross-Linked C-Telopeptide), a serum marker of bone resorption in recipient mice (55). Taken together, and with other researchers who claim that bone mass is strongly associated with microbiota and mainly with the type of bacteria colonizing the intestine.

Several process and molecules are involved, and microbiota might cause bone loss due to its action on hematopoietic progenitors in bone marrow as demonstrated that antibiotics decreased $CD4^+$ T cells (57). On the other hand, fecal microbiota transplantation from young mice to aged mice could rejuvenate hematopoietic stem cells (lymphoid/myeloid cell) (58).

Another important question to be answered is how the intestinal microbiota, which is located in a very specific niche that targets distant bones. Metabolites derived from bacterial digestion through intestinal epithelium toward connective tissue and deep inside blood flow (21). In addition, clarifying the pathway with a new mechanism about how intestinal bacteria affect distant bones, studies have shown extracellular vesicles from young but not senile people prevented bone loss and strength (59) and regulation and function of microRNAs by microbiota (60, 61).

Probiotics and bone

Probiotics are living bacteria-containing food products administered in proper quantity that provide beneficial effects to the intestinal microbiota of the host (62).

The majority of findings reported increased bone mass when the microbiota is absent (germ-free mice), depleted (antibiotics) or in dysbiosis (antibiotics or diseases) (63). However, supplementation with Lactobacillus strains (13), indicating that the certain bacteria present in the intestinal microbiota are crucial to maintenance of the bone mass and not all bacteria are negative regulators of bone mass.

The balance between Treg, an anti-inflammatory and Th17, a proinflammatory cell are part of the mechanism that indirectly regulates bone mass in postmenopausal mice model are demonstrated by studies from the same group using *Lactobacillus rhamnosus* or *Lactobacillus acidophilus* (64, 65).

The osteoblast regulators Runx2 and Bmp2 are upregulated when *Lactobacillus helveticus* are supplemented in ovariectomy-induced rats, which culminates in higher bone mineral density (66), evidencing that in addition to osteoclasts, the osteoblastic lineage is also affected, at least, in part, depending on the Lactobacillus strain used.

Several studies have shown that *Lactobacillus plantarum* is also able to attenuate or prevent bone loss with aging and estrogen depletion animal models (67-69).

Towards to this findings, a randomized, double-blind, placebo-controlled and multicentre trial study in early postmenopausal women treated with *Lactobacillus paracasei*, *Lactobacillus plantarum* DSM 15312, and *Lactobacillus plantarum* DSM 15313, once a day for 12 months revealed lower spinal bone loss compared to placebo group (70). Another study supplementing with *Lactobacillus reuteri* for one year neutralized the bone loss with the deleterious effects of the gut microbiota degradation in older women in a suggestive mechanism of reduced inflammation (71). In a study carried out on postmenopausal women with osteoporosis, the association of probiotics *Bifidobacterium animalis* subsp. lactis Probio-M8 (Probio-M8) with conventional treatments (calcium, calcitriol) was evaluated for three months. The results showed that co-administration of Probio-M8 improved bone metabolism, evidenced by increased levels of vitamin D3 and reduced levels of PTH and procalcitonin in the blood. Furthermore, co-administration of Probio-M8 increased genes related to carbohydrate metabolism pathways and other metabolic processes, suggesting that joint probiotic therapy with conventional treatments may be more effective in the management of postmenopausal osteoporosis (72).

However, the possible mechanism dissected in animals show that after antibiotic treatment, intestinal permeability is increased, although femoral bone mass was reduced by 30% whereas treatment with a mucus supplement prevented the intestinal barrier break as well as bone loss, thus indicating that antibiotics might have affect the intestinal permeability (73).

LIMITATIONS

Understanding the microbiota and its impact on bone is still evolving, with ongoing discoveries that may further refine our understanding of this complex interplay. Moreover, the specific

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mechanisms underlying how intestinal microbiota metabolites directly influence bone metabolism remain partially elucidated, posing a challenge for comprehensive conclusions. Lastly, while our review touched upon the potential of probiotics in enhancing bone health, more studies are warranted to establish definitive causal relationships and determine optimal probiotic strains and dosages.

CONCLUSION

Increasing pieces of evidence show that intestinal bacteria content is critical in regulating the metabolites and the immune system by diverse mechanisms. The studies report that intestinal microbiota regulate bone mass and that type of bacteria is determinant to increase or decrease bone mass; however, point out the importance of Lactobacillus genus supplementation to prevent or recover bone loss. This is a promising and fertile field to be further explored by researchers who claim to be elaborating strategies to prevent fractures or treat bone loss in elderly population.

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CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTION

Review design, data acquisition, discussion of the findings and drafting of manuscript were performed by RPC, CRO, and AG. All authors revised the manuscript and approved the final version.

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