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REVIEW

Gut Microbiota, Immune System, and Bone

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Abstract The gut microbiota (GM) is the whole of commensal, symbiotic, and pathogenic microorganisms living in our intestine. The GM–host interactions contribute to the maturation of the host immune system, modulating its systemic response. It is well documented that GM can interact with non-enteral cells such as immune cells, dendritic cells, and hepatocytes, producing molecules such as short-chain fatty acids, indole derivatives, polyamines, and secondary bile acid. The receptors for some of these molecules are expressed on immune cells, and modulate the differentiation of T effector and regulatory cells: this is the reason why dysbiosis is correlated with several autoimmune, metabolic, and neurodegenerative diseases. Due to the close interplay between immune and bone cells, GM has a central role in maintaining bone health and influences bone turnover and density. GM can improve bone health also increasing calcium absorption and modulating the production of gut serotonin, a molecule that interacts with bone cells and has been suggested to act as a bone mass regulator. Thus, GM manipulation by consumption of antibiotics, changes in dietary habits, and the use of pre- and probiotics may affect bone health. This review summarizes evidences on the influence of GM on immune system and on bone turnover and density and how GM manipulation may influence bone health.

Keywords Osteoporosis · Gut microbiota · Bone · Immune system · Probiotics · Inflammation

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Introduction

The whole of the commensal, symbiotic, and pathogenic microorganisms living in our intestine has been defined as gut microbiota (GM). It is acquired at birth and derives almost entirely from the mother and changes accordingly to environmental factors such as diet, diseases, and use of drugs. The GM comprises about 1200 bacterial species, the main phyla represented are *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria,* and *Verrucomicrobia* [1]. Some of the identified species and of the common bacterial phyla varies between individuals [2]. Low microbial diversity has been identified as a risk factor for different chronic diseases such as intestinal inflammatory diseases, obesity, and insulin resistance [3–6]. Arumugam and colleagues suggested that individuals can be clustered according to the prevalence of different GM phyla and introduced the concept of "enterotypes." According to this definition, humans can be stratified on the basis of their microbial patterns dominated by *Bacteroides*, *Prevotella*, or by *Ruminococcus* [7].

In physiological condition, GM relationship with host is complex and comprehends various forms of symbiotic relationship such as parasitic, commensal, and mutualistic. GM helps in food digestion, in fighting pathogens and, during the first years of post-natal life, contributes to the maturation of the host immune system. During the whole life, GM interacts with the host and contributes to the modulation of gut and systemic immunity. Immune homeostasis disruption is the causal mechanism of several chronic non-communicable human diseases (NCDs) such as allergy, asthma, some autoimmune, cardiovascular and metabolic diseases, and neurodegenerative disorders. These disorders are characterized by a low grade of inflammation. Although inflammation and the pathways to disease are

multifactorial, the altered gut colonization patterns, associated with decreasing microbial diversity, are a central theme and are increasingly implicated in the physiologic, immunologic, and metabolic deregulation seen in many NCDs. Altered GM–host interaction has been indicated as a possible cause of immune deregulation and increased inflammation associated with several NCDs [8].

This review summarizes the evidences on the influence of GM on immune system and on bone turnover and density and how GM manipulation may influence bone health.

GM Influences Immune System

The interaction between immune system and GM has a central role in the maturation of immune system during the early post-natal period [9] and a role in the modulation of immune system and response to self-antigens during the whole life [9, 10]; thus it has been suggested that dysbiosis may play a role in the development of diseases characterized by immune deregulation such as allergies, autoimmune, and inflammatory disorders.

The role of GM in the development and maturation of host immune system in the early post-natal life has been demonstrated in germ-free (GF) mice, i.e., animal raised in sterile cages that maintains sterile gut. The use of this experimental model has shown that the absence of GM negatively influences the formation of lymphoid organs, in particular, GF mice have defective formation of the spleen and mesenteric lymph nodes, the intestinal Peyer's patches are smaller, and display a reduced number of CD4+ T cells and reduced production of IgA [11–16]. Also isolated lymphoid follicle and cryptopatches are reduced in GF mice [17, 18]. As regards, immune cells of different GM phyla were associated with the development of different T helper (Th) phenotypes: in animal model of rheumatoid arthritis (RA), the disease is reduced in GF mice thanks to a reduction of Th 17 [19]. Arthritic phenotypes are restored when GF animals are colonized with segmented filamentous bacteria, which enhance the differentiation and function of Th17 cells. In RA patients, a relationship between the disease and Prevotellaceae has been suggested, in particular *Prevotella copri* has been associated with increased risk of RA [20, 21], whereas *Prevotella histicola* seems to inhibit the development of arthritis [22]. Colonization of GF animals with *Bacteroides fragilis* restores a correct balance between Th1 and Th2 cells and redirects lymphoid organogenesis [14]. Resident bacteria, such as segmented filamentous bacteria and in particular some Clostridia-related species, have been associated to Th cells development and to Tregs cells induction [23, 24].

GM modulates immune system through the production of molecules with immunomodulatory and anti-inflammatory function that are capable to influence immune cells [25, 26]. In particular, GM produces several metabolites from digested food, by modifying host products and by the novo synthesis, among these molecules short-chain fatty acids (SCFAs) are the most widely investigated in the regulation of inflammation and immune system. It has been demonstrated that SCFAs have anti-inflammatory effects on intestinal mucosa, thus protecting the bowel from the development of inflammatory bowel disease [27–29] (Fig. 1).

Short-chain fatty acids signal to several non-enteral cell types through G-protein-coupled receptors also named free fatty acid receptors (FFAR) [30–32]. One of these receptors GPR109A/HCA2 is activated in immune system by butyrate [33], and the signal between GM and immune system is fundamental to regulate the homeostasis and to maintain the balance between immune tolerance to commensals bacteria and immunity to pathogens. The interaction of butyrate and GPR109A/HCA2 cooperates in the generation of immune tolerance and, in particular, mediates Tregs development [28, 29, 34, 35].

Butyrate regulates gene expression by inhibiting histone deacetylases (HDAC) [36], in particular butyrate inhibits HDAC1 and HDAC3 [37]. Also propionate acts as a less potent HDAC inhibitor [38]. Recently it has been suggested that inhibition of HDAC may increase Tregs development and function, hence this could be one of the mechanisms by which GM enhances Treg generation in the gut [39]. It has also been suggested that, depending on the cytokines milieu, interaction between SCFA and FFAR influences T cells differentiation not only toward Tregs, but also toward effector T cells. Park and colleagues suggested that, in certain conditions, SCFAs may induce T helper differentiation into Th1 and Th17 thus increasing the host defenses against pathogens [40]. SCFAs such as butyrate and propionate also modulate antigens presentation inhibiting the development of dendritic cells by HDAC inhibition [41–44] and by interaction with FFAR [34, 45].

Beyond SCFAs, GM produces other metabolites, such as indole derivatives and polyamines, from digested food that have important immunomodulatory function. These metabolites derive from dietary tryptophan and arginine, respectively, and have an indirect immune function. Indole derivatives favor the integrity of the enteral mucosa and the barrier defense toward pathogens by stimulating the production of anti-microbial peptides, mucins, and proliferation of intestinal goblet cells. Polyamines such as putrescine, spermidine, and spermine fulfill important roles in gene expression and proliferation. They enhance the development and maintenance of the intestinal mucosa and resident immune cells (Fig. 1). An immunomodulating role

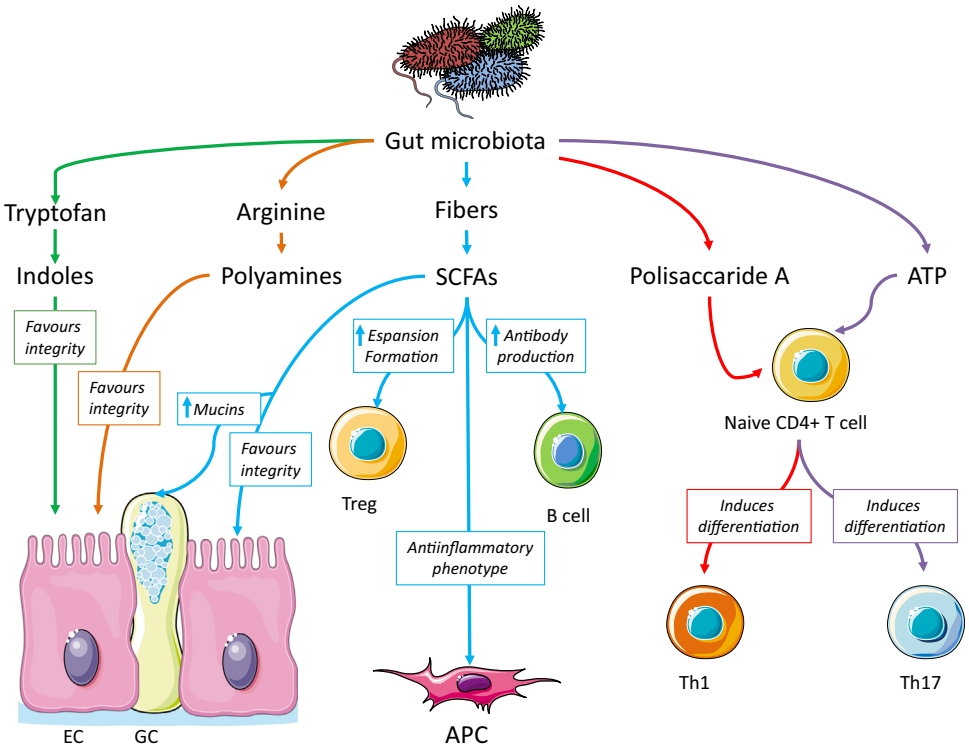

Fig. 1 The influence of gut microbiota on enteral barrier integrity and immune system through the production of several metabolites. *EC* enteral cells, *GC* goblet cells, *APC* antigen-presenting cells, *Treg* T regulatory cells, *Th1* T helper-1, *Th17* T helper-17 cells, *SCFAs* short-chain fatty acid

has also been postulated for other GM products such as metabolized bile acids; however, physiological role of these metabolites in health and disease is still an open question [46].

Germ-free mice have imbalance in T helper cells: reduced Treg, absence of Th17 cells, and altered ratio between Th1 and Th2 with increased Th2 response [26]. In these animals, gut colonization with *Bacteroides fragilis* induces the development of Th1 cells, thanks to the production of polysaccharide A [14]. Polysaccharide A is a bacterial product that influences T cells fate through its interaction with the toll-like receptor 2. Interacting with T cells, it favors immune tolerance by inhibiting Th17 differentiation and favoring Tregs activity [47]. Other bacteria, such as segmented filamentous bacteria and *Clostridium* spp., were shown to influence Th phenotype. The first stimulates Th17 immune response, through ATP or serum amyloid A production by innate immunity cells, whereas the latter promotes Treg cell response through SCFAs production [23, 48] (Fig. 1).

A recent study by Kim and colleagues suggests that GM may also affect B cells antibody production through SCFAs inhibition of HDAC and modulation of gene expression [49]; however, further studies are needed to clarify the underlying mechanism.

Taken together, these evidences suggest that GM influences T cells differentiation through the production of bacterial metabolites such as SCFAs and polysaccharide A at least at the intestinal mucosa level and T cells differentiation through cognate bacterial antigens [50] (Fig. 1).

The majority of the evidences thus suggest that GM metabolites and antigens may influence immune regulation and hence dysbiosis may be the environmental factor responsible for some immune and inflammatory disorders, both at gut level such as inflammatory bowel disease [51] and outside the gut such as Rheumatoid Arthritis [52], type 1 diabetes [53], and asthma [54]. However organs distant from gut, skin, and lung are not in direct contact with GM. This implies that GM has the ability to communicate with the host immune system in distant organs as well as in the gut. These signals have been identified in GM-derived products such as lipopolysaccharide, SCFAs, and bile acid and also in circulating antibodies or immune cells [2].

Relationship Between GM, Immune System Activation, and Bone Loss

Osteoporosis increases dramatically the risk of fractures: major osteoporotic fractures are a social and economic burden. In developed countries, the lifetime risk for osteoporotic fractures at the wrist, hip, or spine is 30–40%, very close to that for coronary heart disease. The number of new fractures in 2010 in the EU was estimated at 3,5 million, comprising approximately 620,000 hip fractures; 520,000 vertebral fractures; 560,000 forearm fractures; and

1,800,000 other fractures [55]. Osteoporotic fractures impair patients' quality of life and increase mortality: 20% of elderly patients suffering from femoral fractures will die within a year, and 50% of the survivors will loose independence. The most frequent cause of bone loss is postmenopausal osteoporosis (PMO) that is driven by estrogen deficiency at menopause. In PMO, there is an imbalance in bone turnover with increased bone resorption and reduced bone formation. It has been demonstrated both in experimental models and in humans that estrogen deficiency affects bone cells number and activation and bone turnover partially through its effect on immune system [56]. During estrogen deficiency, T cells increase their production of pro-inflammatory and pro-osteoclastogenic cytokines, such as TNF alpha and RANKL [57]; however, the reasons of this increased activity in osteoporotic women and not in non-osteoporotic subject are unknown. GM may be involved in the mechanism of PMO.

Some papers suggest that the absence of GM influences bone mass. The majority of the findings demonstrate that GF mice have increased bone mass, whereas a single study by Schwarzer and colleagues [58] demonstrated that GF mice have a growth retardation due to reduced level of IGF-1 and, consequently, reduced bone mass. These authors argued that the difference in the results may be due to the different genetic backgrounds used in the studies. Similarly, a study by Yan and colleagues reported an effect of GM on IGF-1 and consequently on bone growth. The study demonstrated an acute effect of GF colonization with GM obtained from conventionally raised mice on reduction of bone mass due to increased bone resorption, whereas the long-term colonization resulted in a net skeletal growth in young animals [59].

Even the studies on mice treated with broad spectrum antibiotics to alter GM bring to different conclusions regarding the effect on bone density. These discrepancies are possibly due to differences in animal age, sex, and protocols applied for antibiotic treatment [59–63].

The majority of the reports suggest that antibiotic-treated mice have increased bone density [60, 63, 64] and also best bone mechanical properties [64] than conventionally raised mice.

GF mice showed a reduced number of osteoclast and lower level of IL-6, RANKL, and TNFα in bone. These cytokines have a well-known pro-inflammatory and pro-osteoclastogenic effect [65, 66]. GF mice also displayed alteration of immune system with lower number of CD4+ T cells and no difference of CD8+ T cells. These features are normalized by colonization with GM from conventionally raised mice [65].

Recently, elegant studies demonstrated the role of innate immunity in mediating the effect of GM on inflammation and on bone metabolism. In particular, the role of toll-like receptor 5 (TLR5) [64, 66], Myd88, Nod1, and Nod2 has been studied.

TLR5 is the innate immune receptor for flagellin [67], and mice knock-out (KO) for this receptor develop an altered GM due to deficits in the immune system. TLR5KO mice have an altered host–microbe interaction, increased inflammation, and metabolic syndrome [68]. It has been demonstrated that metabolic phenotype in these mice depends on GM alteration as TLR5KO mice raised in GF conditions do not develop the metabolic phenotype [69]. Bone phenotype is significantly different in TLR5KO mice with respect to WT. These animals have larger cross-sectional area and moment of inertia with a reduction in whole-bone strength. The effect of antibiotic treatment and disruption of the GM on bone tissue material properties was different between WT and TLR5KO mice. In particular, TLR5KO mice display a greater reduction of the whole-bone femoral bending stiffness with respect to WT [64]. These differences may be due to several characteristics of TLR5KO mice: these mice are mildly obese and it is known that obesity influences bone mechanical competence [70]. Moreover GM is altered in TLRKO mice that display low microbial diversity, which might, *per se*, influence bone phenotype. Finally immune system is altered in these animals, and this could affect GM-immune system–bone interaction.

In order to study the role of innate immunity in mediating the effect of GM on bone health, Ohlsson and colleagues [66] evaluated the role of Myd88, NOD1, and NOD2. Myd88 is the main mediator of TLR activity on inflammatory response [71]; however, Myd88KO mice behave like WT mice when raised in GF environment and display a significant increase in cortical bone mass, this observation demonstrates that the effect of the GM on bone mass is independent of Myd88.

NOD1 and NOD2 bind bacterial peptidoglycan and cooperate to inflammatory response after bacterial recognition in the cytoplasm activating the NFκB pathway. NOD1 detects diaminopimelic acid-type peptidoglycan that is mainly expressed by Gram-negative bacteria [72]. Nod2 detects all types of peptidoglycans found in Gram-positive and Gram-negative bacteria [73].GF mice with deletion of NOD1 or NOD2 do not have increased cortical thickness nor increased expression of TNFα and RANKL; thus the effect of GM on the production of these cytokines and, hence, on bone mass is dependent on these molecules.

To investigate the role of GM in bone loss induced by sex steroid deficiency, this condition was induced pharmacologically in GF mice with the GnRH agonists leuprolide by Li and colleagues [74].These authors demonstrated that GM plays an important role in sex steroid deficiency-induced osteoporosis: GF mice are protected against osteoporosis and the increase in bone

turnover induced by sex steroid deprivation thanks to the lack of increase in TNF, RANKL, and IL-17. The authors also demonstrated that sex steroid depletion augments inflammation in the intestine by increasing gut permeability to bacterial antigens, namely, by decreasing the expression of claudin 2, 3, and 15, and of Jam3, which are modulators of intestinal barrier integrity [75, 76].

In humans, scarce data support results obtained in mice. Recently Wang and colleagues [77] in a very limited cohort suggest that GM component structure and diversity are altered in osteoporosis and osteopenia patients as compared with normal controls; however, they do not correlate with different GM components with inflammation and immune system, nor with bone turnover.

Relationships between immune system, estrogen deficiency, bone loss, and GM are summarized in Fig. 2.

GM and Bone Health Beyond Immune System

It has been suggested that GM composition and manipulation may affect bone health beyond immune system by influencing calcium absorption and the production of gut-derived serotonin.

A post hoc analyses on the use of *Lactobacillus reuteri* demonstrated that the use of this probiotic in healthy subject increases the level of serum 25OH vitamin D that influences calcium absorption and benefits bone health. The mechanism through which this probiotic influences vitamin D level is not clear; however, the authors argued that this may be due to a modification in the gut environment that specifically favors vitamin D absorption or to indirect effect on increased hepatic 25-hydroxylase activity or 7-dehydrocholesterol concentration due to reduced absorption of dietary and biliary cholesterol [78]. On the other hand, the relation between GM and vitamin D may also be inverse as it has been proposed that decreased vitamin D intake is associated with different GM profiles [79, 80].

Another possible mechanism through which GM benefits bone health is the increase in calcium absorption. It is well known that maintaining a positive calcium balance is important in achieving a good peak of bone mass that protects from the development of osteoporosis in older age [81, 82]. Dietary intake of fibers influences calcium absorption: after being fermented by GM, fibers improve calcium absorption by reduction of gut pH, thus reducing the formation of calcium phosphates and increasing the calcium absorption and by increasing the production of

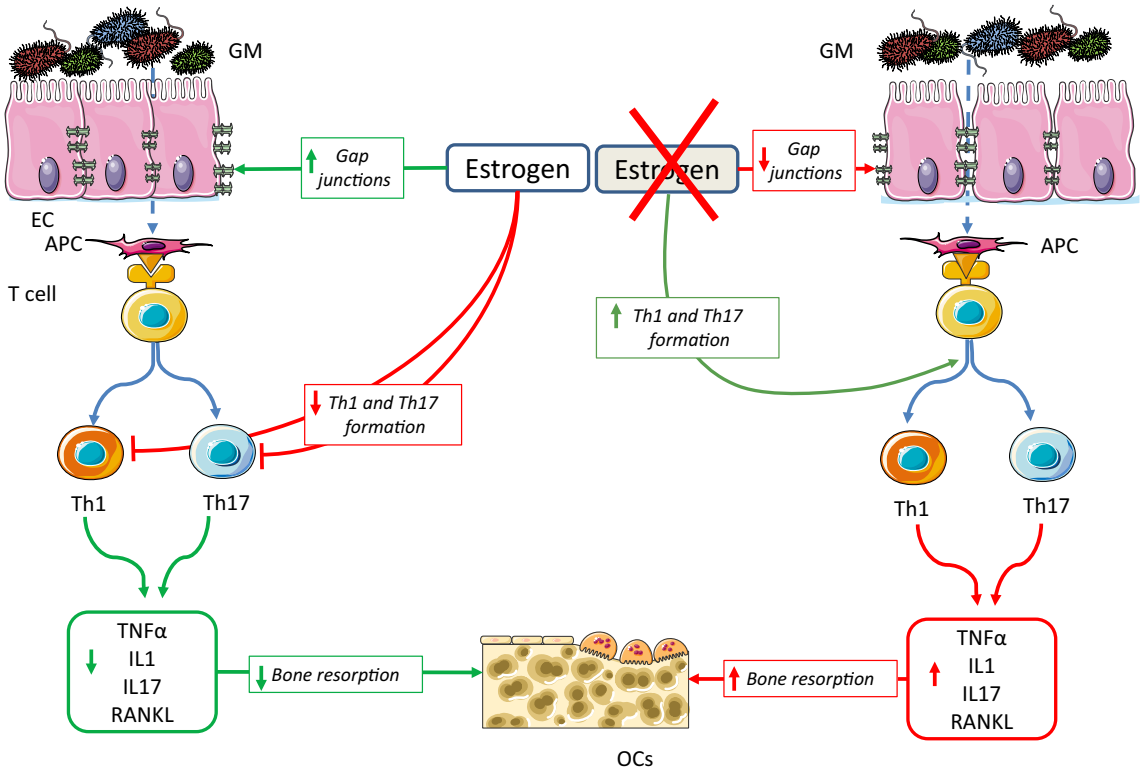

Fig. 2 The complex relationships between immune system, estrogen deficiency-bone loss, and gut microbiota: enteral barrier integrity, cytokine production, immune, and bone cells are involved. *GM* gut microbiota, *EC* enteral cells, *APC* antigen-presenting cells, *Treg* T regulatory cells, *Th1* T helper-1, *Th17* T helper-17 cells, *OBs* osteoblasts, *OCs* osteoclasts

SCFAs such as butyrate [83]. The effect of SCFAs may be more complex than the effect on gut pH, and in fact it has been demonstrated that SCFAs increase calcium transport through signaling pathway modulation [84]. As previously said SCFAs influence bone health also through immune system modulation; hence dietary fiber intake may be responsible for a healthier immune system and reduced inflammation. In fact, there is a general consensus recognizing that an adequate dietary fiber intake is associated with lower risk of chronic diseases such as cardiovascular disease [85].

Another possible mechanism through which GM influences bone health is mediated by its effect on the production of gut serotonin (5HT). In recent past, a dual effect of serotonin in the regulation of bone mass has been described depending on the site of production of this molecule [86]. In this review, we are interested in the role of gut-derived 5HT (g5HT), which is influenced by GM, as a bone mass regulator. Enterochromaffin cells of the duodenum are responsible for the synthesis of g5HT that is partially modulated by GM as SCFAs increase the synthesis of g5HT [87, 88]. It has been shown that 5HT interacts with bone cells and, in particular, decreases osteoblast proliferation via activation of 5-HT1B receptors on pre-osteoblasts [89, 90].These observations suggest that regulation of g5HT by GM may be a potential therapeutic strategy to improve bone health. Indeed, in animal models of ovariectomy-induced bone loss, pharmacological inhibition of g5HT synthesis results in the prevention of osteoporosis mediated by increased bone formation [91].

However, data on the effect of 5HT on bone health are quite controversial. Cui and colleagues [92] showed that mice KO for 5HT receptor 1 have no bone phenotype and that inhibition of this receptor with LP923941, an enantiomer of LP533401 used in a previous study with opposite results [91], decreases circulating 5-HT, but has no effect on bone density. Different results obtained may be explained by different techniques used [93].

Relationships between GM and bone turnover beyond immune system are summarized in Fig. 3.

GM Manipulation and Bone Health

GM composition may be manipulated in several ways such as the use of broad spectrum antibiotics, change in dietary habits and, more easily, by the use of prebiotics and probiotics, change in GM composition may affect bone health. The majority of experimental data produced in mice demonstrated that modulation of GM by the use of probiotics is able to increase bone mass and reduce sex steroid-associated bone loss [74, 94–96]. Probiotics used were different in different studies, both a single strain or a mixture of strains. The most used were *Lactobacilli spp.* that were demonstrated to have the higher anti-inflammatory and bone protective effect. McCabe and colleagues

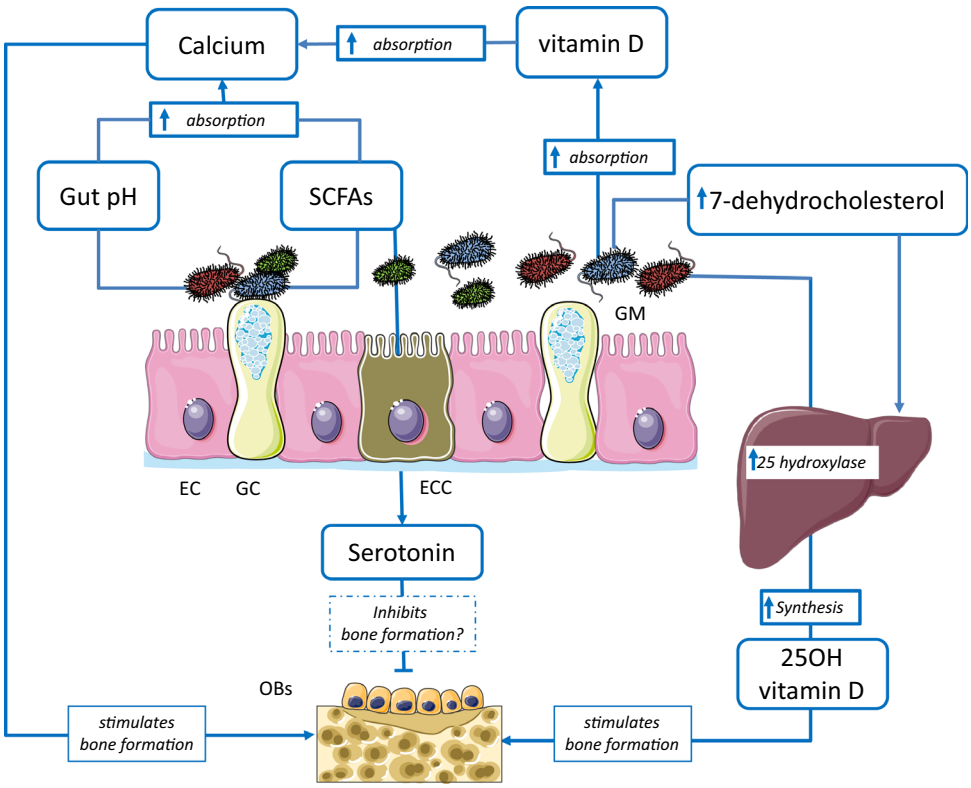

Fig. 3 The link between gut microbiota and bone turnover beyond immune system. *GM* gut microbiota, *EC* enteral cells, *ECC* enterochromaffin cells, *OBs* osteoblasts

suggested that short-term oral administration of the *Lactobacillus reuteri* enhanced bone density in male, but not in female mice [97]; however, in estrogen-deficient female mice the administration of this probiotic prevented bone loss [95]. In a further study, the authors suggested that *L. reuteri* is active on bone health also in intact females providing the presence of an inflammatory status. The authors speculated that estrogen deficiency is comparable to a mild inflammatory status, thus explaining their previous findings on intact female [98].

Also some data on the use of yogurt that contains different probiotics, but is also a source of calcium and proteins that are fundamental for bone health, have been produced [99]. All these studies showed a protective effect of probiotic yogurt on bone health. Moreover, it has been demonstrated that dairy products consumption in early life led to a higher peak bone mass [100]. Also in adults older than 60 years, consumption of dairy products was associated to increased bone density and lower risk of osteoporosis [101–104]. The use of probiotics has been proposed also as an adjuvant treatment in focal bone loss such as alveolar erosion in periodontitis. The ability of different *Lactobacilli* strains in reducing osteoclast number, alveolar erosions, and tooth movement in rat and mice has been demonstrated [105–107]. In humans, a recent meta-analysis concludes that current evidences suggest a possible use of probiotics as an adjuvant therapy in gingivitis and periodontitis [108].

In a geriatric population, the administration of *Lactobacillus helveticus* increases serum calcium [109]. In a prospective double-blind, placebo-controlled randomized clinical trial, the administration of *Lactobacillus casei Shirota* in 417 elderly patients with a distal radius fracture accelerates the healing process [110]. Also in osteopenic women, the administration of a multispecies probiotic (6 different species) increases markers of bone formation, decreases TNF alpha level, but has no effect on bone density during a 6-month period [111].

Another method to influence GM is the administration of prebiotics. Prebiotics are complex carbohydrates and fibers that influence composition and/or activity of GM in a way that favors host health. To generate beneficial metabolic products, GM needs substrate availability. Prebiotics partially provide these substrates, and can be used to modify the GM components and their metabolites. To be classified as a prebiotic, a substance should meet these criteria: be resistant to low gastric pH, hydrolyzed by mammalian digestive enzymes, and not be absorbable by humans, be fermented by GM, and stimulate the growth and activity of gastro intestinal tract [112]. Prebiotic supplementation in animal models favors the proliferation of *Bifidobacteria* and increases SCFAs production. As regards the effect of prebiotics on bone health, some experimental studies showed that they improved calcium absorption and bone density in animal models [113, 114]. In humans, the supplementation with different probiotics such as galactooligosaccharide and a mixture of short- and long-chain inulin-type fructans in adolescent girls improved calcium absorption and bone density [115, 116]. Recently, the corn-derived non-digestible carbohydrate, soluble corn fiber (SCF), has been evaluated for its ability to increase calcium absorption and improve bone health in humans. In particular, SCF administration enhances calcium absorption and its consumption is associated with a favorable change in GM, namely, increased presence of Bacteroidetes and Firmicutes known to ferment starch and fiber [117, 118]. In the study by Whisner and colleagues [117], increase in calcium absorption was positively correlated with bone formation marker, also the changes observed in GM phyla proportion was associated with calcium absorption. Parabacteroides significantly increase with larger SCF doses and are negatively correlated with calcium absorption. Firmicutes are positively correlated with calcium absorption. The results of this elegant study suggest that the role of GM in calcium absorption is complex due to different species.

Prebiotic fiber may influence bone metabolism both by the change in the composition of GM-favoring microbes with higher anti-inflammatory potential and by increasing SCFAs production thus increasing calcium absorption. It has also been suggested that prebiotics could have direct effect on immune system modulation and an anti-pathogen effect regardless to their effect on GM [119]. However, until now, in human studies on prebiotics only calcium absorption, markers of bone metabolism, and bone density have been investigated, whereas immune phenotype and inflammation have not been.

Conclusions

Gut microbiota is becoming one of the new players in the regulation of bone turnover by modulating immune system and controlling inflammation and also by influencing calcium absorption and vitamin D level.

Dysbiosis may favor bone loss in aged people and after menopause. Manipulation of GM may become a future adjuvant treatment in preventing osteoporosis, osteopenia, and other diseases characterized by focal bone loss such as periodontitis.

In the last years, several data obtained in animal models strongly supported the role of GM in the control of bone turnover; whereas less data have been published in humans, field in which confirmatory studies are needed. In particular, large clinical trials are needed to clarify the efficacy of

prebiotics and probiotics in favoring bone health during growth, aging, and post-menopausal bone loss.

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