Gut Microbiota, Immune System, and Bone

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Running title: *Microbiota and Bone*

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 as immune cells, dendritic cells and hepatocytes, producing molecules 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

Introduction

The whole of the commensal, symbiotic and pathogenic microorganisms living in our intestine has been defined gut microbiota (GM), it is acquired at birth and derives almost entirely from the mother, it changes accordingly to environmental factors 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 have been identified as a risk factor for different chronic diseases as intestinal inflammatory diseases, obesity and insulin resistance [3,4,5,6]. 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 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) 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 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.d. animal raised in sterile cages that maintains sterile gut. The use of this experimental model have 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 patch are smaller and displays 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 different GM phyla where 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 is restored when GF animals are colonized with segmented filamentous bacteria, which enhances the differentiation and function of Th17 cells. In RA patients a relationship between the disease and Prevotellaceae has been suggested, in particular *Prevotellae 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 *Bacterioides fragilis* restores a correct balance between Th1 and Th2 cells and redirect lymphoid organogenesis [14]. Resident bacteria as segmented filamentous bacterium 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, amongst 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).
SCFAs signals 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], 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 HADAC may increase Tregs development and function, hence this could be one of the mechanism 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 towards Tregs, but also towards 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 as butyrate and propionate also modulates 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 from digested food that have important immunomodulatory function as indole derivatives and polyamines, these metabolites derive from dietary tryptophan and arginine respectively and have an indirect immune function. Indoles derivatives favor the integrity of the enteral mucosa and the barrier defense towards pathogens by stimulating the production of anti-microbial peptides, mucins, and proliferation of intestinal goblet cells. Polyamines as putrescine, spermidine, and spermine fulfill important roles in gene expression and proliferation; enhance the development and maintenance of the intestinal mucosa and resident immune cells (Fig. 1). An immunomodulating role have also been postulated for other GM products as metabolized bile acids, however physiological role for this metabolites in health and disease is still an open question [46].

GF 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 thought 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, 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 trough SCFAs production [23, 48] (Fig. 1).
A recent study by Kim and colleagues suggests that GM may affect also B cells antibody production through SCFAs inhibition of HDAC and modulation of gene expression [49] however further studies are needed to clarify the underling mechanism.

Taken together these evidences suggest that GM influences T cells differentiation through the production of bacterial metabolites as SCFAs and polysaccharide A at least at the intestinal mucosa level, and T cells differentiation trough cognate bacterial antigens [50] (Fig. 1).
The majority of the evidences thus suggested 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 as inflammatory bowel disease [51] and outside the gut as Rheumatoid Arthritis [52], type1 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 to the host immune system in distant organs as well as in the gut. These signals have been identified in GM derived products as lipopolysaccharide, SCFAs, and bile acid but also 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% to 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 lose independence. The most frequent cause of bone loss is post-menopausal 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 their 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 is 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 background 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 conventional 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 conventional raised mice.

GF mice showed a reduced number of osteoclast, 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 interactions, 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 as 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 as 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, that might, *per se*, influence bone phenotype; finally immune system is altered in these animals, these 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 this cytokines and, hence, on bone mass is dependent by 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 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 profile [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 SCFAs as butyrate [83]. The effect of SCFAs may be more complex that the effect on gut pH and in fact it has been demonstrated that SCFAs increase calcium transport trough signaling pathway modulation [84].As previously said SCFAs influence bone health also trough immune system modulation, hence dietary fibers intake may be responsible for an 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 as cardiovascular diseases [85].

Another possible mechanism through which GM influences bone health is mediated by its effect on the production of gut serotonin (5HT). In the 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), that 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 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 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 to 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 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 adjuvant treatment in focal bone loss 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 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 months period [111].
Another method to influence GM is the administration of prebiotics, prebiotics are complex carbohydrate and fibers, that influence composition and/or activity of GM in a way that favors host health. To generate beneficial metabolic products GM need substrate availability, prebiotics partially provides these substrates, and can be used to modify the GM components and their metabolites. To be classified as prebiotic a substance should meet these criteria: be resistant to low gastric pH, hydrolysis 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 as galacto-oligosaccharide and a mixture of short- and long-chain inulin-type fructans in adolescent girls improved calcium absorption and improved 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 increases with larger SCF doses and negatively correlated with calcium absorption. Firmicutes positively correlated with calcium absorption. The results of this elegant study suggests that the role of GM in calcium absorption is complex and due to different species.
Prebiotic fiber may influence bone metabolism both by the change in composition of GM favoring microbes with higher anti-inflammatory potential and by increasing SCFAs production thus increasing the calcium absorption. It has also been suggested that prebiotics could have direct effect on immune system modulation and an anti-pathogen effects 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 were investigated, whereas immune phenotype and inflammation were not.

Conclusions

GM 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 as periodontitis.
In the last years several data obtained in animal models strongly supported the role of GM in the control of bone turnover, 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.

Funding: F. Sassi is supported by a grant from MIUR PRIN 2015.

Compliance with Ethical Standards

Conflict of interest: the authors declare that they have no conflict of interest.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure legends

Fig. 1 The cartoon summarizes how gut microbiota influences enteral barrier integrity and immune system through the production of several metabolites
Abbreviations used: enteral cells (EC), goblet cells (GC), antigen presenting cells (APC); T regulatory cells (Treg); T helper-1 (Th1), Thelper-17 cells (Th17), Short Chain Fatty Acid (SCFAs)

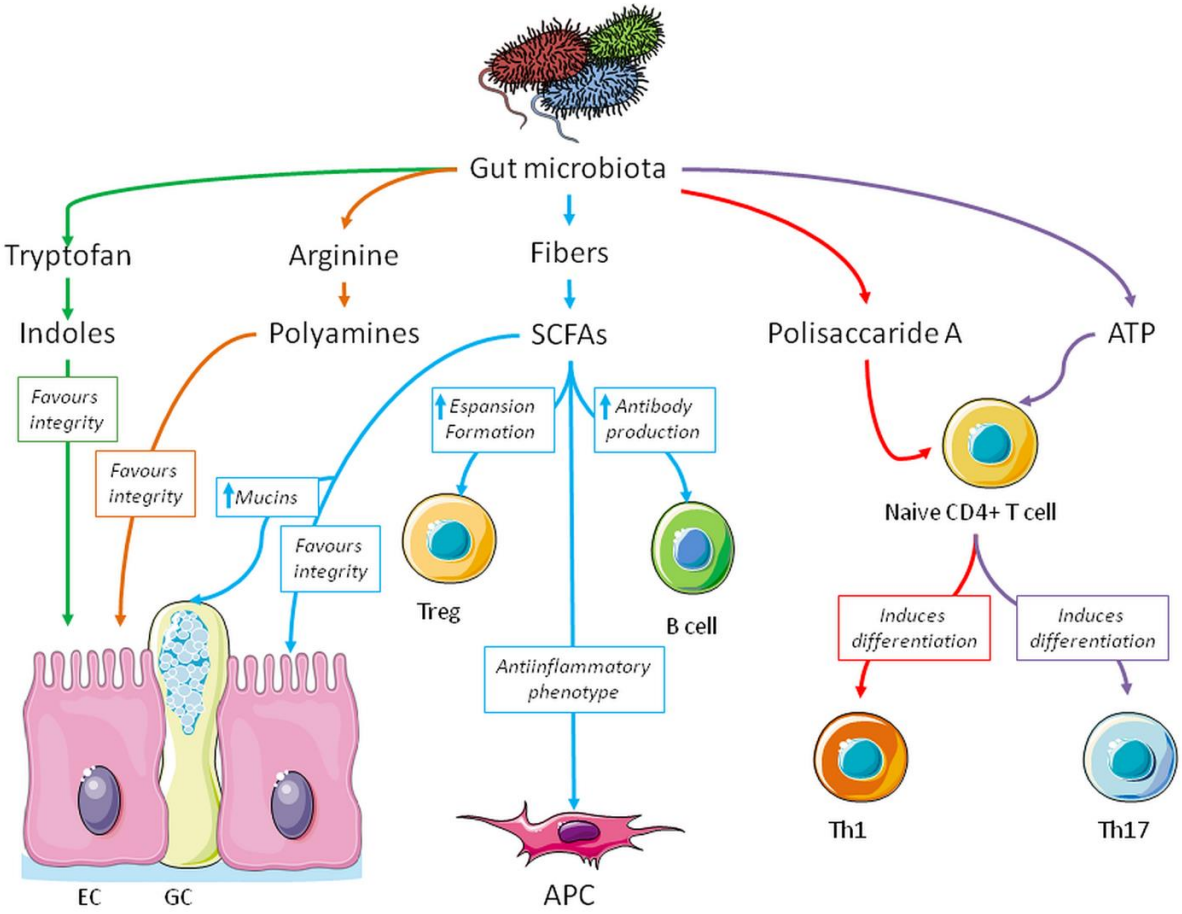

Fig. 2 The cartoon summarizes the complex relationships between immune system, estrogen deficiency-bone loss and gut microbiota: enteral barrier integrity, cytokine production, immune and bone cells are involved

Abbreviations used: gut microbiota (GM) enteral cells (EC), antigen presenting cells (APC); T regulatory cells (Treg); T helper-1 (Th1), Thelper-17 cells (Th17), osteoblasts (OBs), osteoclasts (OCs)

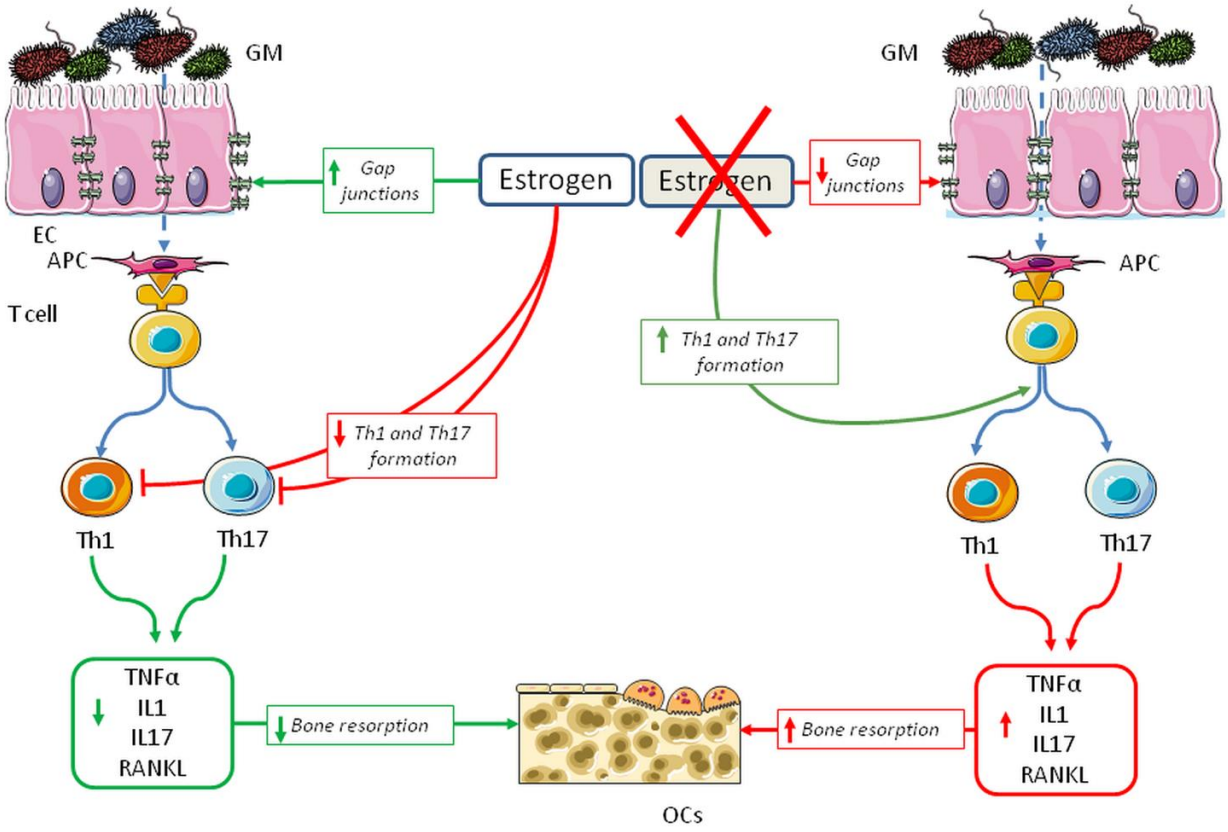

Fig. 3 The cartoon summarizes the link between gut microbiota and bone turnover beyond immune system

Abbreviations used: gut microbiota (GM), enteral cells (EC), enterochromaffin cells (ECC), osteoblasts (OBs)