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Epithelial barrier function in gut-bone signaling

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Abstract

The intestinal epithelial barrier plays an essential role in maintaining host homeostasis. The barrier regulates nutrient absorption as well as prevents the invasion of pathogenic bacteria in the host. It is composed of epithelial cells, tight junctions, and a mucus layer. Several factors, such as cytokines, diet, and diseases can affect this barrier. These factors have been shown to increase intestinal permeability, inflammation, and translocation of pathogenic bacteria. In addition, dysregulation of the epithelial barrier can result in inflammatory diseases such as inflammatory bowel disease. Our lab and others have also shown that barrier disruption can have systemic effects including bone loss. In this chapter, we will discuss the current literature to understand the link between intestinal barrier and bone. We will discuss how inflammation, aging, dysbiosis and metabolic diseases can affect intestinal barrier-bone link. In addition, we will highlight the current suggested mechanism between intestinal barrier and bone.

1. Introduction

The gastrointestinal (GI) epithelium plays an essential role in maintaining host health through its ability to digest and absorb nutrients. At the same time, it is essential for providing a selective barrier that prevents translocation of harmful substances as well as pathogens and their products from the external environment to the blood stream. The intestinal epithelium is composed of a continuous single layer of intestinal epithelial cells (IECs) that are sealed together by tight junctions (TJ) proteins. This epithelial layer allows the movement of materials from the mucosal side of the epithelium to the serosal side via transcellular and paracellular pathways. A mucus layer, secreted by specialized epithelial cells (goblet cells), is located on the surface of the epithelium and is important for limiting the ability of gut bacteria and pathogens to access host cells. The lumen of the GI tract also harbors a variety of commensal microorganisms referred to as the gut microbiota which

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accounts for 90% of the cells in the human body, approximately 10^{14} bacteria total. The intestine also secretes immunoglobulins, defensins, and other antimicrobial products that contribute to maintaining a healthy environment. Beneath the epithelial layer is the lamina propria which contains immune cells, fibroblasts and plasma cells.

Disruption of the epithelial barrier can 1) affect efficient nutrient absorption, 2) facilitate pathogen translocation into the bloodstream and cause systemic inflammation, and 3) alter gut microbiota composition [1]. As a consequence, barrier disruption can trigger the development of GI diseases such as inflammatory bowel disease (IBD), celiac disease, and colon cancer [2–5]. Other systemic and metabolic diseases such as type I diabetes can also be influenced by barrier changes [6, 7]. However, whether barrier dysfunction is causal or consequence of these systemic and metabolic diseases is controversial. Recent studies from our lab and others demonstrate that GI barrier dysregulation can critically affect bone health [8, 9]. In this chapter, we will review several important aspects of intestinal epithelial barrier function including: tight junction protein composition, the mucus layer, epithelial barrier integrity measurements, barrier alterations associated with disease processes, and barrier dysregulation-induced bone loss during aging, dysbiosis, and metabolic diseases.

1.1 Pathophysiology of tight junction proteins

Tight junction (TJ) proteins connect adjacent epithelial cells on their apical side and therefore are critical for controlling paracellular permeability by selectively regulating the flow of ions, solutes, and small molecules across the epithelium. TJ proteins respond to a variety of stimuli including changes in diet, dysbiosis, viruses, inflammation, antibiotic treatment, and/or humoral or neuronal signals [10][1][4]. Stimuli can have positive or adverse effects on paracellular permeability depending on the physiological status of the host [1, 11–13].

TJ protein complexes are composed of junctional adhesion molecules (JAM), occludins, desmosomes, claudins, and cytoskeletal linker proteins such as zonula occludens (ZO) (1–3) (Figure 1). The ZO is a family of proteins (ZO-1, ZO-2, ZO-3) that link the TJ proteins to the actin cytoskeleton. This interaction, between the TJ and the actin cytoskeleton, is essential to maintain TJ structure and cytoskeletal regulation of the epithelial barrier. Desmosomes do not directly connect adjacent epithelial cells. Instead, they provide the adhesive force to ensure the integrity of the epithelial layer [14][1]. Alterations of the TJ complexes can increase paracellular permeability and pathogen translocation that can induce sustained activation of the mucosal immune system and tissue damage.

Several cytokines can modulate TJ complexes and affect intestinal permeability. For example, the proinflammatory cytokine tumor necrosis factor alpha (TNFα) can directly increase intestinal permeability in cultured intestinal epithelial cells and mouse epithelium by dysregulating TJ proteins. *In vitro*, in a colon epithelial cell line (Caco-2), TNFα-induced increases in TJ permeability were associated with increased nuclear factor kappa beta (NF-κB) activation and nuclear translocation of NF-κB p65 [13]. TNFα has also been shown to increase the expression of the myosin light-chain kinase (MLCK), and inhibition of MLCK can prevent TNFα induced increases in permeability in intestinal epithelial cells [15, 16]. Similarly, interferon-γ (IFN-γ) increases paracellular permeability in T84 colonic epithelial

cells. IFN-γ decreases ZO-1 protein synthesis, increases internalization of the TJ proteins and rearranges the actin cytoskeleton [17]. Interleukin-1β (IL-1β) can also regulate transepithelial permeability *in vitro*. IL-1β caused a progressive time-dependent increase in transepithelial permeability in Caco-2 cells. This increase in permeability was attributed to the rapid activation of the NF-κB by IL-1β [18]. On the other hand, the role of the anti-inflammatory cytokine interleukin 10 (IL-10) in TJ regulation has been demonstrated in IL-10 knockout mice. IL-10 knockout mice present with an increase in ileal and colonic permeability at 2 weeks of age. However, this effect was ablated in IL-10 gene-deficient mice raised under germ-free conditions, suggesting a role of the microbiota in intestinal permeability in IL-10 knockout mice [19].

Studies in IBD patients indicate that TNFα levels are increased in the serum, stool, and intestinal mucosa [20] and, correspondingly, patients display increased intestinal paracellular permeability that is characterized by suppression and re-distribution of occludins, and claudins 5,8, whereas claudin-2 expression was upregulated [21]. Interestingly, people at high risk of developing Crohn's disease exhibit increases in small intestinal permeability [22]. Together, these studies demonstrate that TJ proteins are regulated by cytokines, modify intestinal permeability and are linked to disease pathogenesis.

1.2 GI Mucus Layer

The GI epithelium is covered by a layer of mucus that creates a physical barrier. This barrier prevents the interaction of luminal microorganism with the surface of the epithelium. It also serves as the first line of host defense and allows the exchange of water, nutrients and gases with the underlying epithelium. This layer is formed by high molecular weight glycoproteins called mucins (MUC) that are synthesized and secreted by goblet cells. Mucins are produced and stored in granules in the goblet cell and then are transported and secreted into the lumen. They can be secreted by continuous fusion (constitutive/basal secretion) or by exocytosis (exocytosis/regulated secretion). There are two groups of mucins: secreted mucins and transmembrane mucins. MUC2, MUC5AC, MUC5B and MUC6 constitute the secreted mucins and are responsible for the formation of the mucus layer. The transmembrane mucins (MUC1, MUC4, MUC13 and MUC16) don't play a role in mucus production. Under normal physiological conditions, goblet cells continually produce mucins, however, factors such as cytokines, toxins, microbes and microbial product can negative or positively regulate this process [23]. Disruption of this process has been associated with GI diseases.

Inflammatory cytokines such as TNFα, IL-1β, and IL-6 are known to be major regulators of mucin synthesis and exocytosis. *In vitro*, in colon cells, TNFα and IL-6 increased the expression of the secreted gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6) [24]. TNF-α enhanced MUC2 transcription through the activation of the NF-κB pathway in colon cells [25]. Similarly, the anti-inflammatory cytokine IL-10 enhances MUC2 expression in goblet cells [26].

The gut microbiota depends on the mucus layer which serves as an energy source for bacteria. The mucus layer also serves as a matrix for commensal bacteria attachment and colonization that ultimately prevents opportunistic pathogenic bacteria from binding/growing within the mucus. Cross talk between the intestinal microbiota and mucus layer

contributes to the regulated production of mucin by goblet cells [27–29]. Studies in germ-free mice demonstrate that microbiota-deficiency leads to fewer goblet cells and thinner mucus layer in comparison with conventionally-raised mice; supporting the role of the microbiota and/or microbial products in modulation of mucin synthesis and secretion [27]. Correspondingly, treatment of intestinal epithelial cells (HT-29) with the probiotic *Lactobacillus planetarium* enhanced MUC2 and MUC3 mRNA expression levels [30]. Similarly, commensal microbiota break down non-digestible carbohydrates into short-chain fatty acids (SCFAs) such as acetate, propionate, and especially butyrate, which at low concentration can increase mucus production and secretion [28, 29, 31]. Other microbial products, such as lipopolysaccharides and flagellin, also increase mucin synthesis [32]. If mucin production is chronically stimulated, goblet cells become depleted of mucin and the lack of mucus secretion can lead to increased permeability and disease [33]. Taken together, mucus secretion is highly regulated by a variety of conditions/factors and, along with tight junctions, contributes to intestinal barrier integrity.

2. Epithelial barrier integrity measurements

The intestinal epithelial barrier plays an essential role in host health as well as in GI diseases. Several tests have been developed to improve the diagnosis of GI diseases such as IBD. These tests (discussed below and in Table 1) include measures of serum, fecal, and urine biomarkers that are altered as a consequence of intestinal barrier dysfunction (i.e., inflammation) or that directly assess barrier permeability (i.e., endotoxin).

2.1. Serum

Serologic markers for epithelial barrier integrity in IBD patients includes C-reactive protein (CRP) measurements [34, 35]. CRP levels are elevated in the serum of patients with acute IBD, with almost 100% of patients with CD showing an increase of this protein in the serum [34][36]. Serum measurements of inflammatory markers such as cytokines and neutrophils can also be performed. However, plasma/serum levels of inflammatory markers, including CRP, are not specific for gut inflammation since they can also be increased in other inflammatory conditions.

Breakdown of the epithelial barrier can lead to the translocation of the microbiota or their toxic products. Markers of bacterial antibodies for *Escherichia coli* and *Pseudomonas fluorescens* have been used to identify children with IBD with 67% sensitivity and 76% specificity [37]. Bacterial metabolic products, such as D-lactate or cell wall components such as LPS/endotoxin, are also commonly measured. Baseline levels of these markers are low in healthy individuals, whereas increased circulating LPS/endotoxin levels are related to an impaired mucosal barrier and increased levels of D-lactate are correlated with intestinal injury [38].

To estimate enterocyte damage, measurement of the fatty acid binding proteins (FABP) can be performed in the urine or plasma. FABP is located on top of the villi and an increase in FABP in the blood or urine can be used as a marker of early stage intestinal diseases [39–41]. Levels of FABP rise rapidly after GI inflammation and have been correlated with the histological status of the epithelium after GI inflammation.

2.2 Feces

Invasive methods to test gut epithelial barrier inflammation in humans are not feasible. However, fecal proteins such as calprotectin and lactoferrin are specific markers for mucosal inflammation in intestinal diseases [42] and can identify patients with IBD, assess disease activity, and predict relapse [43, 44]. Calprotectin is a 36 kDa calcium- and zinc-binding protein. Approximately 60% of the cytosolic protein content in the neutrophils is made up of calprotectin. During intestinal inflammation, neutrophils migrate to the mucosa and any break in the mucosal barrier results in the leakage of neutrophils into the lumen. Hence the presence of calprotectin in the feces indicates the migration of neutrophils to the intestinal mucosa and potential leakage of these cells into the lumen. Calprotecin is stable in feces, and its concentration represents an indirect measure of neutrophil infiltration and barrier breaks. Lactoferrin is an iron-binding protein that is also found in neutrophils, specifically neutrophil granules. Lactoferrin is secreted during inflammation; when the intestine is inflamed and neutrophils are present, lactoferrin levels increase in the lumen and stool [42] [45].

2.3 Urine

Intestinal permeability can also be assessed by using small to large-sized probe molecules [46, 47]. This approach involves oral ingestion of sugars, such as mannitol and lactulose, and measuring their subsequent concentration in the urine over a period of time (usually 5 hours). Mannitol is a monosaccharide with a molecular weight (MW) of 182 Da and a molecular radius of ≤ 0.4 nm. Lactulose is a disaccharide with an MW of 342 Da and a molecular radius of 0.42 nm. The different sizes of the molecules allows for the measurement of transcellular and paracellular routes of permeability across the epithelia [45]. Large molecules, such as lactulose, are thought to traverse the epithelium by paracellular pathways. Small molecules, such as mannitol, cross the epithelium predominantly by the transcellular pathways. Neither sugar should be fermented by bacteria or metabolized in the body. Thus, the ratio of urinary excretion of the relatively large molecule is compared with that of the relatively small molecule and permeability is expressed as the ratio [46].

One concern with this approach is that many factors can influence the uptake of these sugars by epithelial cells, including (1) GI motility, (2) the use of medications such as nonsteroidal anti-inflammatory drugs, (3) intestinal transit time and surface area, (4) mucosal blood flow and (5) renal clearance; these effects can potentially yield false-positive results. However, when both the large and small molecules are combined in the test solution at a fixed concentration ratio, the effects of variables, such as gastric emptying, intestinal transit time, and renal clearance will apply equally to both. Thus, the urinary excretion ratio will be influenced only by the difference in gut permeability for each molecule.

Polyethylene glycols (PEG) have also been used to test intestinal barrier function. PEGs that have a molecular weight of 400–4000 Da can only cross the intestinal mucosa under conditions of barrier integrity loss. PEG can be used to measure both small and large intestinal permeability and are not degraded by bacteria. PEG have been used to test changes in permeability in IBD and Crohn’s patients [48, 49].

3. Barrier pathophysiology in development

3.1 Pediatrics

The intestine at birth is not fully developed and many factors, such as diet, stress and microbiota have been implicated in influencing its permeability during development [50][51][52]. Increased intestinal permeability in infancy may lead to diseases that persist throughout childhood as well as those that appear later in life, such as IBD. Immune system involvement, which is developed alongside microbiota and diet changes, is also a significant indicator of the health of the intestinal barrier. Because of the fundamental differences in development between children and adults, it is important to consider pediatric intestinal barrier physiology separately.

The intestinal mucosal barrier significantly matures after birth, coinciding with changes in microbial composition and diet changes. At birth, the child is introduced to microbes, traditionally through contact with the birth canal, that colonize the intestine. It has been shown that vaginally born infants have higher numbers of *Bifidobacteria* and *Bacteroides* when compared with infants born through cesarean section [53]. In addition, the presence of *Bifidobacteria* in breast-fed infants, corresponds with breast-fed infants having lower intestinal permeability than cow's milk formula-fed infants [54]. Other studies have shown that *B. infantis* promotes intestinal barrier function by regulating tight junctions. Infant mice treated with *B. infantis* exhibited decreased internalization of claudin 4 and occludin, which effectively decreased the incidence of necrotizing enterocolitis [55]. Mucin production also contributes to barrier integrity due to its importance in building the mucosal layer. In mice, the maturation and production of these glycoproteins occurs after weaning, signifying the role of diet as well as hormonal and other age-associated factors in barrier development [56].

Increased intestinal permeability is associated with a variety of intestinal as well as extra-intestinal diseases, many of which persist or manifest in adulthood. Since the intestinal microbiota takes approximately 2.5 years to become functionally mature, the clinical impact of any large shifts in microbial composition during this developmental period can significantly impact intestinal permeability [57]. It has been suggested that traumatic GI events in early infancy, during the period of barrier maturation, are more powerful indicators of eventual disease than events occurring outside this "critical window". As evidence for the possibility of an early life disturbance creating lasting effects on barrier function, the trauma of maternal separation during weaning has been shown to predispose adult rats to enhanced intestinal permeability in response to stress [51].

The intestinal immune system in infants develops upon antigen exposure, directing attention to the importance of gut microbial colonization in relation to successful barrier function. Immunoglobulin A (IgA) is a class of antibody first received in breast milk and is then produced by the gut mucosa. Interestingly, by 24 months, both mono- and dizygotic twins had IgA responses comparable to unrelated children although significant differences were observed at older ages, suggesting a level of maturation acquired by age two [58]. This roughly coincides with the stabilization of the makeup of the microbiome at 2.5 years. The interplay among microbial colonization, diet, immune system and the intestinal barrier is fundamental to the health of the gastrointestinal tract. Insults to the intestinal barrier at a

young age can induce diseases such as IBD that appear in childhood and possibly persist through adulthood.

Early life disturbances such as premature birth, which can increase intestinal permeability, can also affect bone density [59]. In fact, 16–40 % of very low birth weight (VLBW, <1.500 kg) and extremely low birth weight (ELBW, <1.000 kg) infants are estimated to develop bone metabolic diseases [60]. Examination of growth and bone mineralization among children born prematurely (birth weight less than 1.5 kg) indicates reduced lumbar bone mineral density and content compared to full-term children [60]. During this early period of life, the intestinal barrier is particulary permeable to allow antibodies in the mother's colostrum to cross into the infant's blood. This increased permeability in neonates can cause intestinal inflammation and can lead to necrotizing enterocolitis (NEC) [61]. While a direct link between early changes in bone density with reduced barrier function in neonates and children has yet to be proven, studies in inflammatory bowel patients and animal models support this link (see section on IBD below). For example, chemically increasing barrier permeability in young (5 week old) growing mice causes reduced bone density and stunted growth compared to control mice [62]. It is noteworthy, that when the inflammatory insult is removed the young mice are able to fully regain bone density and length 5 weeks later [62]. Taken together, the data support the need for more studies to understand the role of the gut epithelial barrier in early life disturbances in bone physiology.

3.2 Aging

Several studies have shown that aging can have profound effects on the GI tract. Approximately 35–40% of elderly patients report having at least one GI tract complication during a routine medical exam [63]. Effects of aging in the GI tract includes changes in permeability, motility, inflammation, and disruption of the gut microbiota. However, the mechanisms by which aging contribute to shifts in any of these effects and their influence on epithelial barrier and bone health are poorly understood. This is in part because it is difficult to discern if changes are due to normal aging, common age-related disorders, or result from disease treatments. This section will discuss the effects of aging on intestinal permeability, inflammation and microbiome and while no papers directly link barrier changes with age related bone loss, we will discuss potential connections.

3.2.1 Changes in permeability—Several studies have shown that aging can have detrimental effects on intestinal barrier permeability. A study looking at 34 vs. 133-week-old rats demonstrated that the younger rats excreted 34.3% of the administrated PEG 400 in the urine, while 43.6% was excreted by the older rats [64]. Similarly, another study showed that as rats age (12–112 weeks), intestinal permeability to PEG 400 and mannitol increased [65]. Colonic mucosal biopsies from young and old non-human primates (baboons) demonstrate significant differences in permeability and TJ proteins. The older baboons displayed a significant decrease in ZO-1, occludin, and JAM-A proteins, and an increase in claudin-2 expression, all of which correlated with increased permeability in the old aged group [66]. Another study in monkeys found that old monkeys have an increase in FITC dextran flux compared to young monkeys [67].

A cross-sectional study of non-smoking healthy adults, between 60 and 85 years old, showed no difference in the permeability index (PI= lactulose/mannitol) between young and older humans. However, the study had some limitations such as that the older age group consisted of predominantly males and sex difference may play a role in intestinal permeability [68]. In an *ex vivo* assay by Man et.al [69], the authors demonstrated that the transepithelial electric resistance (TEER) is affected in the aged humans. In their study, the effects of age on TEER was tested using ileal biopsies from healthy humans, young (7–12 years), adult (20–40 years), and aging (67–77 years). The TEER was significantly reduced in the aging biopsies whereas no difference was observed between the two younger groups. The increase in permeability in the aging group appeared to be restricted to solutes since the permeability to macromolecules was not affected by aging [69]. There were no changes in mRNA expression of ZO-1, occludin, and JAMA-1 in the aging group compared with adults and young individuals. On the other hand, they observed that levels of claudin-2 were significantly increased in the aging group and not in the adult group; suggesting that claudin-2 play an important role in intestinal permeability [69]. It has also been proposed that the changes seen in intestinal permeability in aging people can be due to an increase in inflammation and/or disruption of the gut microbiota.

3.2.2 Changes in mucosal immune system—Aging is associated with a decline in the immune response [70, 71]. About 50% of the older age group are affected by low-grade chronic inflammation known as “inflammageing” [72]. In a steady-state situation, the IECs communicate with the intestinal immune system to regulate intestinal homeostasis. IECs regulate the intestinal immune homeostasis through the secretion of cytokines that control dendritic cells and T-regulatory cells. This interaction helps to discriminate between invasive pathogenic organisms and harmless antigens. Several studies have reported a dysregulation in the intestinal immune homeostasis in different models of aging. Specifically, it has been shown that the function of the gut-associated immune system is impaired in elderly humans [67, 69, 73, 74].

Using monkeys as a model of human aging, researchers found that old monkeys have greater systemic inflammation as compared to young monkeys. This increase in inflammation was attributed to an increase in serum CRP [67]. In a similar study using baboons, it was found that aged baboons have a significant increase in IFN-γ, IL-6, and IL-β in colonic biopsies. In the same study, the old animals presented an increase in colonic permeability [75]. These results suggest that dysregulation of the immune system can alter intestinal permeability.

Several studies in humans have also confirmed the effects of aging on the intestinal immune response. Ileal biopsies from young (7–12 years), adult (20–40 years) and aging (67–77 years) individuals were assessed for inflammatory cytokines levels. They noticed an increase in the expression of IL-6, but not IFNγ, TNF-α, and IL-1β in the aging group. The increase in IL-6 was attributed to an increase in dendritic cells. They also demonstrated a correlation between IL-6, claudin 2 and permeability [69]. Many studies indicate that IL-6 expression is induced with aging. Animal studies showed that the decline in the production of IL-1 β, TNFα, and IL-12, in response to LPS in aging is restored in aging IL-6-deficient knock-out mice, suggesting that IL-6 is responsible for the changes in the mucosal immune system

during aging [76]. In conclusion, the pro-inflammatory state observed in aging populations may be related to dysfunction of the intestinal barrier.

3.2.3 Changes in intestinal microbiota—In a healthy intestinal tract, the microbiota and the gut immune system interact to maintain a homeostatic equilibrium. Perturbation of this homeostatic equilibrium has been strongly associated with many human diseases such as obesity and IBD [77, 78]. The intestinal microbiota supplies nutrients as well as protects the intestinal barrier against pathogens [79]. A variety of factors including the host, microbiological, dietary and environmental factors can disrupt the gut microbiota. Because of the crucial role of the intestinal microbiota in host homeostasis, it is important to study the age-related differences in microbiota and how it influences intestinal function.

It has been demonstrated that the human intestinal microbiota undergoes maturation from birth to adulthood and is further altered with aging. A study looking at age-related differences in the gut microbiota composition among young (average 31-years old), elderly (average 72-years old), and centenarians humans (average 100- years old) demonstrated that the composition and diversity of the gut microbiota do not differ between young adults and elderly groups. However, there was a significant difference between the elderly and centenaries [80]. These differences were attributed to an increase in facultative anaerobes, mostly belonging to Proteobacteria and Bacilli in the centenarians group. The Firmicutes/Bacteroidetes ratios did not differ between the young and centenarian groups. In the same study, measurements of inflammatory cytokines were performed. An increase in IL-6 and IL-8 was observed, but not TNFα. They also found a positive correlation between bacteria belonging to the phylum Proteobacteria with IL-6 and IL-8 [80]. Species diversity was found to change with age in bacteria isolated from fecal samples from healthy young and elderly adults. On the other hand, the overall numbers of organisms were similar at the genus level [81]. In a different study, the results showed a change in bacterial genera with age and a reduction in the numbers Bacteroides and Bifidobacteria in the elderly group. These reductions were accompanied by reduced species diversity [82]. The Firmicutes/Bacteroidetes ratio of the human microbiota increased with age [83]. This shift in microbiota composition might result in a greater susceptibility to diseases by altering intestinal permeability among other consequences.

3.2.4 Intestinal changes and bone health—While we do not know of any study directly linking the effect of aging on gut barrier-to-bone signaling, the intestinal changes that occur with aging are associated with bone loss in other conditions. Specifically, the strongest link between gut permeability and bone loss comes from colitis studies where barrier disruption in adult animal models leads to bone loss, even without weight loss [84][85][62]. Thus, as animals and humans age, intestinal permeability increases [64][65][66][67][62][69] and could contributes to age-related bone loss. In addition, increased permeability likely promotes the low-grade chronic inflammation, termed “inflammageing” [72][67][75], and many studies link low grade inflammation with bone loss [84][85][62]. In the future, studies are need to test if a direct link exists between barrier function and bone health in the elderly, since this could be a promising target for new therapeutics.

3.3 Menopause

Menopause is the natural cessation of menstruation and decline in reproductive hormones. One of the most significant reproductive hormones in females is estrogen, produced primarily in the ovaries. It is also produced at extra-gonadal sites including adipose tissue, skin, osteoblasts, osteoclasts, aorta, and the brain. After menopause, adipose tissue is the main source of estrogen. There are several forms of estrogen, 17β estradiol being the most prevalent circulating estrogen. Only a small amount in the plasma is free and active, most is bound to globulin or albumin. The two primary receptors for estrogen are estrogen receptor α (ERα) and estrogen receptor β (ERβ), both of which are nuclear receptors. ERα is typically associated with secondary sex characteristics and regulation of the menstrual cycle in females and sperm maturation in males [86]. ERβ has less of a role in the classical estrogen target tissues and has been found to be more dominant in the brain, cardiovascular system and the colon [87, 88]. A decrease in estrogen levels during menopause has been attributed to osteoporosis that occurs in post-menopausal women but the role of declining estrogen in intestinal permeability is only beginning to be understood. In ovariectomized Wistar rats, colonic paracellular permeability was increased significantly and this was reversed by estrogen treatment (oestradiol benzoate) [89]. Consistent with this, colonic paracellular permeability decreases during the oestrus phase (high levels of estrogen) of the rat when compared to the dioestrus phase (low levels of estrogen) [89]. Although estrogen treatment has been shown to predispose ovariectomized rats to development of ulcerative colitis-induced tumor development [90], most studies to-date have shown that estrogen treatment decreases colonic paracellular permeability and reduces IBD symptom severity [89, 91, 92]. ERβ is the predominant estrogen receptor in the intestinal tract. Whole body ERβ knockout mice display altered intestinal cell proliferation, decreased apoptosis and abnormal villus/crypt architecture throughout the intestine [93]. One potential mechanism that could account for estrogen effects on the intestine is through its alterations in TJ and adhesion molecules which would alter intestinal permeability [94]. In models of IBD (IL-10 deficient mice and HLA-B27 rats), ERβ mRNA levels were decreased and colonic permeability increased [92]. Similarly, treatment of cell culture models of intestinal epithelial layers (HT-29, T84, Caco-2) with estrogen receptor antagonists increases permeability while estrogen treatment prevents this outcome [89, 92]. Our lab demonstrated a significant increase in intestinal permeability 1 week post-surgery in the absence of estrogen (OVX model) in mice. Section specific changes in permeability were also measured *ex vivo* by Ussing chambers which demonstrated that the ileum had the most dynamic changes. This study indicates that estrogen deficiency induces region-specific effects on intestinal permeability [94]. Thus, estrogen appears to predominantly inhibit increases in intestinal permeability and therefore an increase in permeability during menopause could lead to increase in systemic and bone inflammation that could contribute to bone loss.

In addition to altering epithelial barrier function, estrogen has also been shown to impact calcium absorption in the intestine, which is important for bone maintenance. Several studies identified decreases in intestinal and renal calcium absorption following estrogen deficiency [95–100]. Though the exact mechanism is not well understood, it is thought that estrogen deficiency leads to down regulation of the expression of transcellular calcium transport proteins plasma membrane calcium pump 1b (PMCA1b), transient receptor potential cation

channel subfamily V member 5 (TRPV5) and calbindin-D 28K (CaBP28k) [95]. Furthermore, estrogen has been found to increase vitamin D receptor (VDR) gene and protein expression as well as $1,25(OH)_2D_3$ activity in the colon, which leads to increased intestinal calcium absorption [101, 102]. Taken together, these studies suggest that estrogen could modulate bone heath via multiple mechanisms that depend on intestinal barrier function (permeability and calcium/vitamin-D metabolism).

Given that one out of two postmenopausal women will fracture a bone [103], the potential for using the gut as a therapeutic target to treat osteoporosis has increased research in this area. Recent studies support a role for intestinal health in the prevention of bone loss in ovariectomy (Ovx) mice [104][8]. Decreasing intestinal inflammation or altering the gut microbiome leads to the prevention of bone loss [104][8]. Our lab has shown that treatment with the probiotic *Lactobacillus reuteri* significantly protected Ovx mice from bone loss. This prevention of bone loss by *Lactobacillus reuteri* was attributed to a decrease in osteoclastogenesis and an increase in bone marrow CD4+ T-lymphocytes. *Lactobacillus reuteri* also modifies microbial communities in the Ovx mouse gut [104]. In a different study, researchers found an increase in gut permeability and cytokines (TNFα and IL-17) in the small intestine of Ovx mice. Surprisingly, in the germ-free mice the effect of estrogen deficiency in gut permeability and cytokines dysregulation was ablated; suggesting a role of the gut microbiota in Ovx induce bone loss. Treatment with the probiotic *Lactobacillus rhamnosus* GG (LGG) or the probiotic supplement VSL#3 reduces gut permeability, intestinal inflammation, and completely protects against bone loss induced by estrogen deficiency [8]. Together, these data highlight the role that of the gut epithelial barrier and microbiota in bone loss induced by estrogen-deficiency.

4. Barrier pathophysiology in disease

4.1 Dysbiosis

The intestinal microbiota has been described as a virtual organ that exhibits a complex bidirectional crosstalk with the environment and other systems throughout the body [105, 106]. The intestinal barrier acts as a wall between the intestinal microbiota, and the host's immune system. Under normal conditions the intestinal epithelium has numerous adaptations such as anti-microbial peptides and mucins that keep the intestinal microbiota away from the gut epithelial layer [107–110]. The TJ also impede microbial invasion into the host tissue [111]. This intestinal epithelial barrier and its adaptations are not static, but can be regulated by a variety of external factors such as alteration to the gut microbiota (i.e. dysbiosis).

A number of factors can alter intestinal microbial composition. These include medications such as antibiotics, psychological and physical stress, radiation, altered peristalsis and dietary changes [112–116]. This can lead to alterations in bacterial metabolism as well as overgrowth of potential pathogenic bacteria [117]. Changes to the gut microbiota during dysbiosis have now been linked to a myriad of diseases such as IBD, irritable bowel syndrome (IBS), obesity and rheumatoid arthritis [118–121]. Importantly, dysbiosis can also lead to disruption of epithelial barrier leading to unwanted consequences [50] (Figure 2).

Altered gut microbiota can signal through pattern recognition receptors on gut epithelial cells, activating the NF-κB pathway and leading to changes in gut homeostasis [122]. Epithelial cell NF-κB activation increases pro-inflammatory cytokines such as TNFα, IL-1, and INFγ [123]. An increase in gut INFγ and TNFα protein levels have been shown to increase intestinal permeability [124, 125]. This altered protein composition decreases barrier properties and leads to leaky gut properties. The exact mechanism behind these effects is not well characterized (20). Dysbiosis has also been linked to increase in IL-1β, which has also been shown to increase permeability by decreasing TJ protein occludin expression [18].

Additionally, dysbiosis of the gut microbiota also influences other adaptations such as mucin production which in turn influences gut barrier function. Intestinal mucins can inhibit bacterial adhesion to the intestinal epithelial cells, limiting immune responses and maintaining barrier function. The commensal microbiota has been shown to regulate production of intestinal mucins [126]. The abundance of mucolytic bacterium, which has the ability to degrade mucins, has been shown to increase 100 fold during dysbiosis observed in IBD [127]. In addition, under dysbiosis pathogens can secrete proteases that have been shown to cleave MUC2, the main mucin-component, thereby decreasing mucin levels [128]. Decreases in the mucin layers have been shown to compromise the intestinal barrier function leading to increases in intestinal permeability and microbe penetration [129]. All of the effects of dysbiosis on the gut barrier function can also lead to systemic changes in the body including bone loss [130–132].

Intestinal dybiosis has also been shown to affect bone density [8, 104, 133–135]. The role of the microbiome in regulating bone remodeling was shown in germ free mice (in C57BL/6 background) which have increased femoral bone density (both trabecular and cortical) when compared to conventionally raised mice [132]. This increase in bone density was attributed to a decrease in osteoclast, as well as, inflammatory cytokines in the bone and bone marrow in the germ free mice vs conventionally raised mice [132]. However, the effects of microbiome on bone density, as determined by studies using germ free mice, are not consistent across mouse strains and/or sex [136][8][130][132]. In addition, the impact of the microbiota changes on the epithelial barrier were not been fully examined. It is possible that changes in the microbiome that promote greater barrier function could benefit bone density while a more pro-inflammatory microbiome could cause bone loss, thereby explaining the inconsistencies between studies.

Previous work has demonstrated that gut dysbiosis promotes inflammation in the bone marrow that correlates with bone loss [137]. It has been hypothesized, that dysbiosis disrupts barrier function leading to increases in inflammation and activates T-cells leading to enhanced expression of TNFα in bone marrow [138, 139]. The increase in TNFα stimulates osteoclastogenesis and/or enhances osteoblast apoptosis, thus disrupting normal bone homeostasis leading to bone loss [138, 139]. The mechanism by which activated T-cells are increased in the bone marrow in response to changes in the gut microbiota are not completely understood; T-cell activation could be due to gut antigens crossing the intestinal barrier consequent to dysbiosis [140].

The finding that dysbiosis can alter bone density led to studies investigating the role of both pre- and probiotics in bone health. Prebiotics are non-digestible fermentable nutrients which promote the growth of beneficial microorganisms [141]. *In vitro* studies indicate that prebiotics can enhance intestinal epithelial barrier function and increase tight junction protein expression [142]. Under healthy and estrogen-deficient conditions, prebiotics (such as fructo-oligosaccharides (FOS) and inulin) also increase bone health parameters [143, 144] [145, 146]. In addition, probiotics (live microorganisms which have a beneficial effect on the host) have been shown to increase barrier function and bone health. The probiotic *Lactobacillus reuteri* has anti-TNFα properties, reduces gut inflammation, and strengthens gut barrier function *in vitro* [104][134][147]. When given to mice, *L. reuteri* treatment was found to increase bone density in healthy male mice in addition to preventing bone loss in both female ovariectomized mice and type 1 diabetic male mice[134][104, 148][149]. Taken together, these data demonstrate the role of the microbiome and intestine in maintaining bone density.

4.2 Colitis/IBD

Inflammatory bowel disease is characterized by damage to the intestinal epithelial barrier resulting in increased permeability and the resultant dissemination of the commensal microbiota. This translocation of the luminal contents into the lamina propria persistently stimulates the immune system leading to its hyper-activation and eventual damage to the intestine. IBD can occur in two different forms, through either ulcerative colitis, which affects only the large intestine, or Crohn's Disease, which can occur anywhere in the gastrointestinal tract. This idea that IBD is caused by the improper localization of the microbiota and other luminal contents is largely supported through animal models of intestinal inflammation in that it is difficult to elicit these diseases in germ-free conditions [150]. In animal models, decreased epithelial resistance has been shown to precede microscopic inflammation [151]. This highlights the importance of maintaining a healthy epithelial barrier to protect and regulate the permeability and translocation of the microbiota.

An important element in maintaining this healthy barrier is the constant maintenance and restoration of the epithelial cells comprising this barrier as these cells age and eventually undergo apoptosis (approximately every week). To maintain a healthy barrier the epithelial cells are constantly in a balance of proliferation, migration, and differentiation, migrating from the base of the crypts to the crypt surface or villous tip. Once their journey is complete, these epithelial cells are removed through shedding/apoptosis that does not result in inflammation, normally associated with mass apoptosis. In a disease state, such as IBD, this apoptosis is greatly upregulated resulting in damage and increased permeability in the epithelial barrier and impairment of its basic functions.

As mentioned before, one of the key factors maintaining the integrity and permeability of the epithelial barrier are the TJ. Additionally, inflammatory conditions can influence this regulation resulting in alterations in the mucosal barrier. Increases in proinflammatory cytokine such as TNFα, IL-4, IL-6 and IL-13 have all been shown to increase epithelial permeability and have been tied to increased expression of claudin-2 in animal and human models as well as decreased expression of JAM-A and occludin [152, 153]. For example,

TNFα is responsible for the removal of claudin-1 from tight junctions. TNFα also induces occludin degradation while promoting MLCK phosphorylation thus resulting in augmented paracellular permeability [154, 155]. Not only can the expression of these TJ proteins be influenced but also their localization within the cell can be dysregulated. Claudins 3, 5 and 8 as well as occludin and JAM-A have all been observed to be internalized rather than expressed on the membrane in biopsies from patients with colitis [156]. These effects on the TJ proteins by inflammatory cytokines is in part mediated by myosin light-chain phosphorylation through myosin light-chain kinase (MLCK). This phosphorylation induces actomyosin contraction that can lead to openings in the junctional gap. In fact, mice continuously expressing MLCK are more susceptible to experimental colitis [157]. Furthermore, improper activation of protein kinase C and Rho can modulate the actin cytoskeleton and influence tight junction regulation and function [158].

In addition to causing a leaky barrier, IBD and ulcerative colitis negatively impact bone [62, 137, 159, 160]. Patients with IBD have a 40% higher risk of developing osteoporosis than the general population [161]. Inflammatory cytokines also increase in IBD and it is know that they can have negative effects in the bone [162]. Although it is not well known whether loss of intestinal barrier per se in IBD patients is causal to bone loss in these patients (see chapter on IBD and bone for further details), our lab has shown that intestinal inflammation without weight loss in an IBD model can lead to significant bone loss suggesting a link [85]. Despite these results, more studies need to be perform to further understand the role of intestinal disruption in IBD effects on bone.

4.3 Type 1 Diabetes

Type 1 diabetes (T1D) is characterized by hyperglycemia and hypoinsulinemia and requires treatment with exogenous insulin therapy. Intestinal health has been shown to play a key role in the development of T1D [163–165]. Additionally, T1D induced changes in intestinal health and function have been suggested to contribute to further T1D complications, such as osteoporosis [166]. Intestinal changes that have been reported to precede or be caused by T1D which can influence bone health include intestinal barrier function or permeability and the intestinal microbiota [167–174].

Intestinal barrier function has been implicated in the development of T1D [167, 171, 174–177]. In rodent models of T1D, intestinal permeability or the “leakiness” of the gut has been studied by measuring the amount of disaccharides and monosaccharides in the urine following their oral administration. The spontaneously diabetic biobreeding (BB) rat model of T1D shows an increased amount of permeability in the stomach, small intestine and the colon [169]. The increased permeability in both the stomach and small intestine appear prior to the development of overt diabetic symptoms [169]. During the pre-diabetes stage, BB rats that are diabetes prone have increased intestinal permeability, altered tight junction proteins (specifically claudin 7), increased gut infiltration by neutrophils and decreased numbers of gut natural killer cells in comparison to BB rats which were diabetes resistant [168, 178].

Examination of intestinal permeability in human patients with T1D has been limited and has shown diverse outcomes. An initial study examining the permeability of the monosaccharide mannitol in T1D patients showed an increase in intestinal permeability [172, 179]. However,

a subsequent study using pediatric T1D patients showed no difference in the permeability to lactulose or mannitol except in patients with a high-risk allele for celiac disease [172]. As of now, the role of gut permeability in T1D is not well understood and further research is needed to understand how hypoinsulinemia affects barrier function.

The gastrointestinal system has the largest immune population in the body and creates an interface between the external environment and the host and has been linked with numerous other autoimmune diseases [180] in addition to T1D. T1D is an autoimmune condition resulting from T-cell mediated destruction of insulin-secreting pancreatic β cells. As the gut houses the largest population of immune cells, it is not surprising that alterations in the intestine can predispose patients to the development of T1D. Furthermore, microbial communities within the intestine have also been shown to alter immune cell number and differentiation [181–186].

Development of T1D has been shown to be preceded by changes in the microbiome in both human and rodent studies. Studies examining the microbiome in T1D patients and control subjects found that T1D patients had less microbial diversity, less microbial population similarities between individual patients, as well as an increase in non-butyrate producing bacteria when compared with non-diabetic subjects [187]. In a study examining the microbiota in children prior to the development to T1D (children were negative for anti-islet antibodies, however, they possessed the predisposing HLA genotypes), several bacterial taxa correlated with the development of anti-islet antibodies found in T1D children; indicating that alterations in the microbiome may precede the development of T1D [188]. In rodent models of T1D (non-obese diabetic (NOD) mouse and biobreeding rat (BBR)), exposure to specific bacterial strains or metabolic products as well as vivarium hygiene can modulate T1D incidence [189–199]. Furthermore, comparison of the microbiome between NOD and the genetically related, but T1D-resistant mouse (NOR) demonstrated an increase in beneficial microbe populations in NOR mice [200]. Fecal transplant of NOD stool into NOR mice increased insulin resistance, however NOR stool transplant into NOD mice did not prevent the development of T1D [200]. As the microbiome is highly influential in the development and activity of the immune system within the gut, researchers have sought to determine the role of intestinal immune function in the development of T1D. In humans with T1D, duodenal samples had increased expression of pro-inflammatory cytokines, increased leukocytic infiltration, as well as alterations in microbial populations within the microbiome (increase in Firmicutes), which all contributed to a pro-inflammatory environment as compared to healthy controls [201].

As the microbiome was found to be altered in both human and rodents with T1D, studies have gone on to show that T1D prevention can be achieved by supplementation with both prebiotics and probiotics. Recently, in the non-obese diabetic (NOD) mouse model, T1D severity was shown to be inversely correlated with levels of acetate and butyrate, microbial metabolites. When these metabolites were replaced in the diet, NOD mice were protected from the development of T1D [202]. Furthermore, the authors found that acetate supplementation decreased the activation of autoreactive T cells while butyrate supplementation increased the number and function of regulatory T cells, thereby preventing autoimmune development of T1D [202]. Several studies examining different probiotics have

shown that altering the microbiome can influence both the inflammatory state of the intestine as well as prevent the progression of T1D [203].

In addition to intestinal changes, T1D is associated with many complications including bone loss [204][205][206] (Figure 3). Our lab demonstrated that treatment with the probiotic *Lactobacillus reuteri* 6475 prevents T1D-induced bone loss in mice; suggesting a role of the gut microbiota in T1D-induce bone loss. Interestingly, the probiotic treatment prevented several of the bone pathologies of T1D including marrow adiposity, suprpressed Wnt10b expression and suppressed osteoblast activity [149]. The prevention of bone loss occurred despite metabolic dysregulation as indicated by high blood glucose levels. It remains to be seen whether T1D-induced changes in gut permeability can directly influence T1D bone loss. Even if not causal, it would be of interest to examine if reversing increases in T1D-induced intestinal permeability can also reverse T1D bone loss.

4.4 Obesity

Obesity is typically associated with several metabolic disorders and is characterized by low-grade inflammation; the molecular origin of which remains unclear [207]. Several studies have reported that serum levels of bacterial LPS are modestly increased in a high-fat diet and that LPS is capable of inducing metabolic disease onset [208–211]. This increase in serum LPS suggests that in obesity the intestinal barrier is compromised. *In vivo*, animal models of obesity have demonstrated increased whole intestinal permeability via measurement of 4kD FITC-dextran transport to the serum [209, 210] (Figure 3). *Ex vivo*, studies utilizing the Ussing chamber have reported increased permeability in the small intestine [211]. Interestingly, while the large intestine has the highest bacterial density and highest levels of LPS, experimental models do not support a definitive causative role for colonic gut barrier dysfunction in obesity. However, a role for increased colonic permeability in obesity cannot be conclusively ruled out as further specific studies are required [212].

The effect of obesity on intestinal permeability in humans is inconclusive. Studies using lactulose (L) and mannitol (M), two sugar probes commonly used to evaluate small intestinal permeability in humans, have shown either no change or modestly increased permeability [212]. In a study investigating obese patients with nonalcoholic steatohepatitis (NASH) the ratio of L/M excreted was similar to healthy controls suggesting no change in intestinal permeability [213]. This is supported in a study by Brignardello et al [214] that looked at gut permeability in asymptomatic, non-smoking obese volunteers and observed no differences compared to healthy controls. In contrast, a study by Teixeira et al [215] reported obese females exhibited higher levels of lactulose excretion but not mannitol than the lean controls; suggesting that small intestinal paracellular permeability may be altered in obese individuals.

Investigations into the mechanisms behind the increased intestinal permeability in animal models have focused on expression of the TJ proteins. In a study by Brun et al [211] using *ob/ob* and *db/db* mice, distribution of occludin and zonula occludens-1 (ZO-1) were reported to be profoundly modified in the small intestine; suggesting disruption of TJ links with the cytoskeleton, a condition known to compromise the sealing properties of TJs [211]. Obesity-induced changes to TJ expression are further supported in studies by Cani et al [209, 210].

In these studies, small intestine gene expression of ZO-1 and occludin were reduced in mice fed a high-fat diet and distribution altered in *ob/ob* mice. Expression of these genes had a significant negative correlation with intestinal permeability [209, 210].

An increase in body weight due to obesity has been commonly considered to have a positive effect on bone. However, recent studies demonstrated that bone quality can be compromised in obesity [216, 217]. As mentioned before, obesity can have several effects on gut epithelium, but their effects on bone density are not well known. It has been suggested that a high fat diet may affect intestinal calcium absorption and therefore decrease bone formation. Free fatty acids can form unabsorbable insoluble calcium soaps and therefore decrease calcium absorption [218]. Several gut peptides whose levels are altered in obesity, such as ghrelin and incretins, may be involved in bone metabolism [219][220][221]. Future studies need to be perform to further understand the role of obesity and complex effects on the intestine and their subsequent impact on bone health.

Conclusions

In this chapter, we discussed the importance of the intestinal barrier in maintaining host homeostasis. We discussed different non-invasive methods such as serum, fecal, and urine biomarkers, to further understand intestinal health. We also present information in how disruption of this barrier can have detrimental effects in the host including effects on bone. Factors such as inflammation, changes in microbiota, aging, menopause and disease have been shown to dysregulate this barrier. In addition, there is now emerging evidence that dysregulation of the intestinal barrier can affect distant organs such as the bone.

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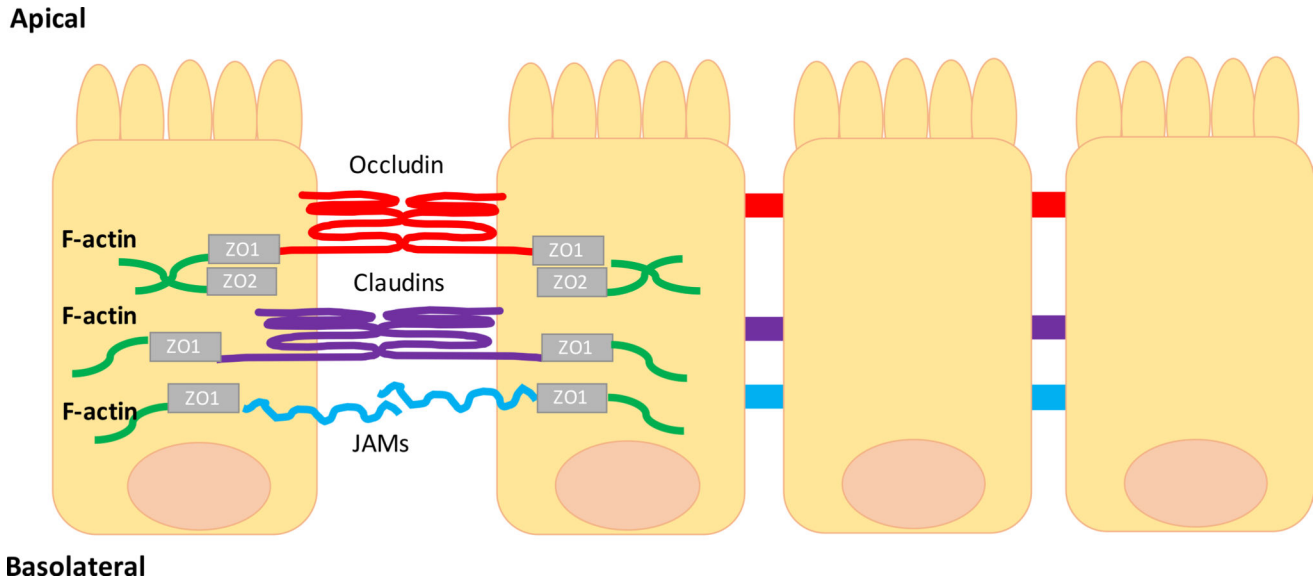

Figure 1. Schematic representation of the intestinal tight junctions proteins and their location

Tight junctions are protein complexes that span between epithelial cells to form a tight barrier. They are comprised of transmembrane proteins, such as occludin (red) and claudins (purple) and they are connected to the actin cytoskeleton via a zona occludens (ZO-1 and ZO-2 (grey)). The transmembrane receptor JAM (junctional adhesion molecule (blue)) is also found at tight junctions complexes. Abbreviations: JAM, junctional adhesion molecule; ZO, zona occludens.

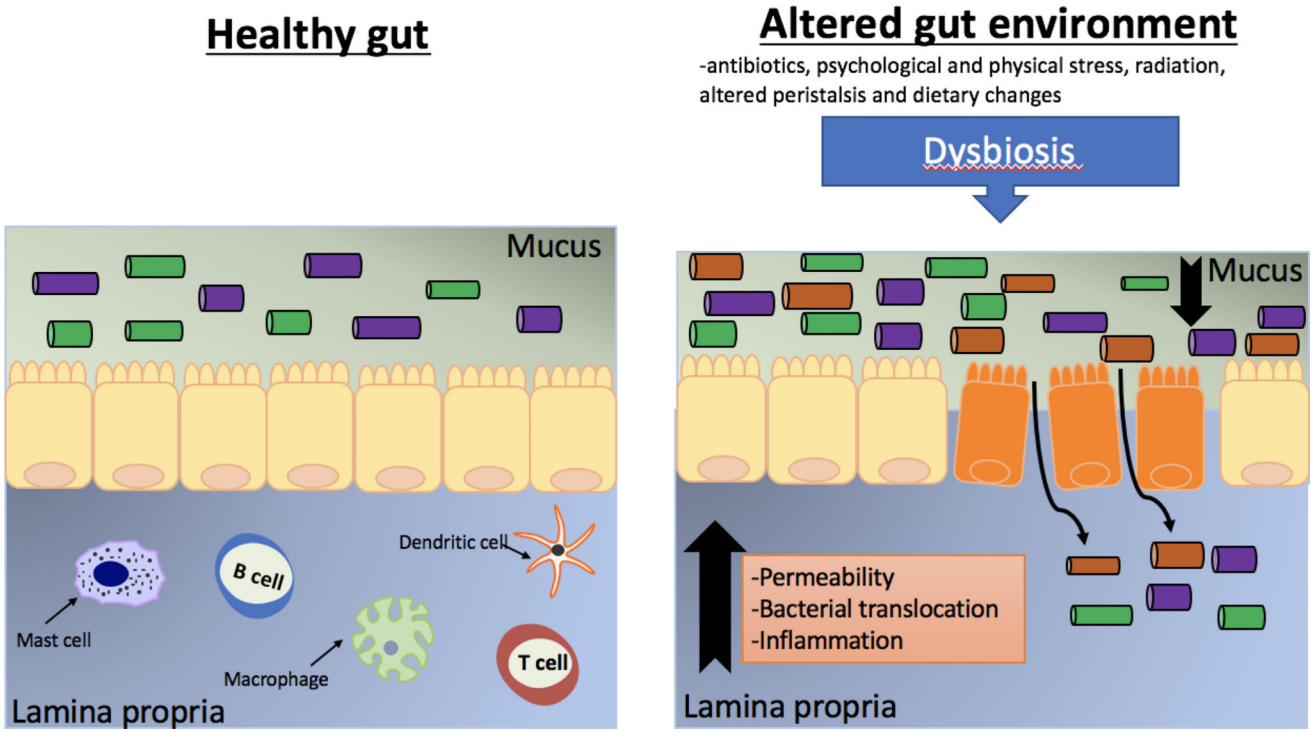

Figure 2. Schematic representation of the gut epithelial layer in healthy gut vs dysbiosis

In the normal state, the mucus layer prevents the interaction between the gut microbiota and the intestinal epithelial barrier. Underneath the epithelial layer is the lamina propria. The lamina propria is composed of connective tissue and cells of the innate and adaptive immune system: mast cells, macrophages, dendritic cells, and lymphocytes (T and B). The composition of the intestinal epithelial layer can be influenced by many factors including antibiotic treatment, psychological and physical stress, radiation, age, and diet. This can lead to alterations in bacterial metabolism as well as overgrowth of potential pathogenic bacteria. This dysbiosis is associated with increased levels of permeability, bacterial translocation and inflammation.

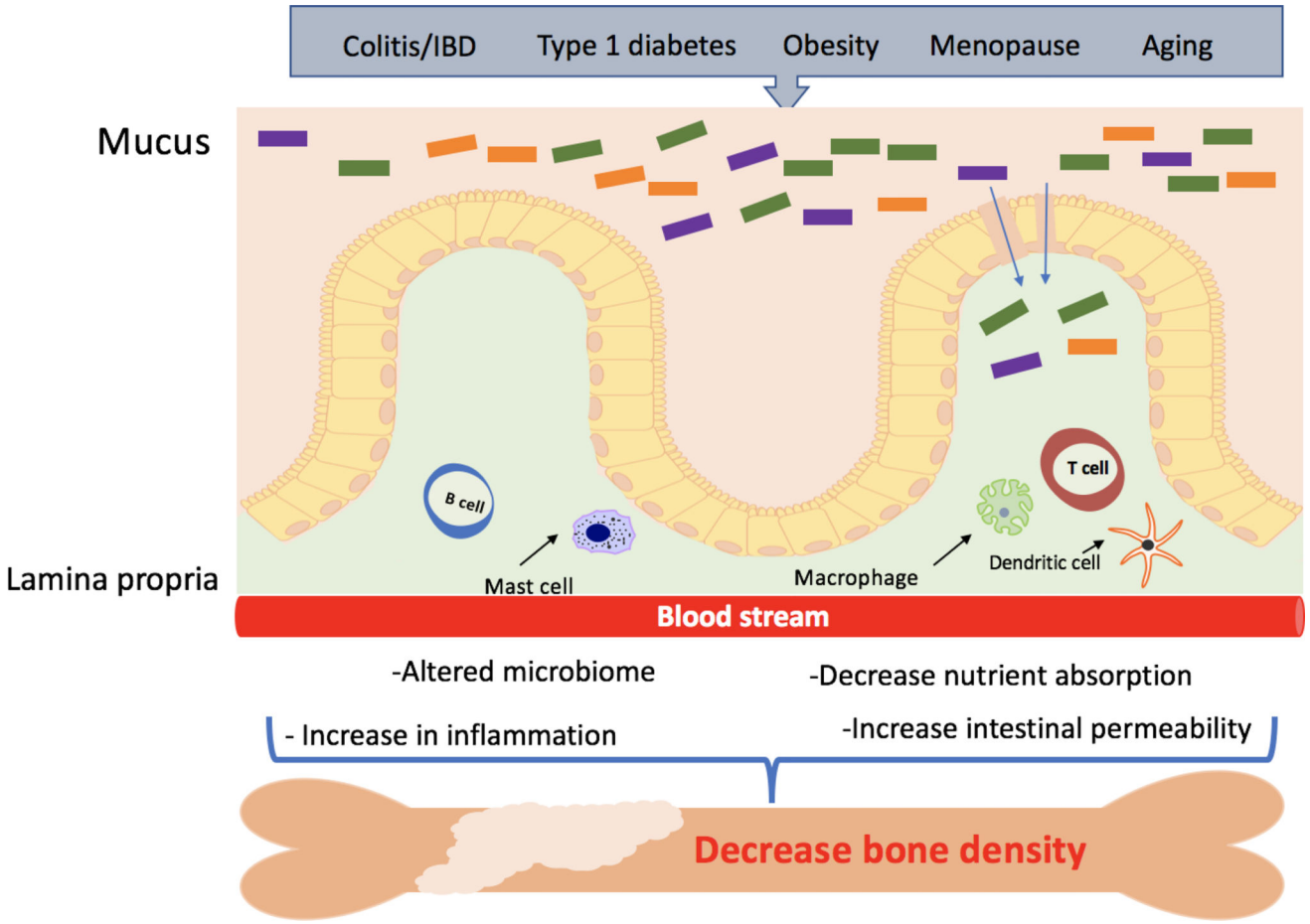

Figure 3. Model of intestinal epithelial disruption signals that can regulate bone density

Many factors such as aging, menopause and metabolic diseases are known to disrupt the intestinal epithelial layer. They can modulate gut microbiota composition and activity, increase intestinal permeability, inflammation and decrease nutrient absorption. These changes can result in local and systemic responses that can affect bone density.

Table 1

Methods for the assessment of intestinal epithelial barrier integrity.

Test	Measured in	Advantages	Disadvantages
Ex vivo			
Ussing chamber	Small and large intestine	Site specific	Invasive
In vivo			
C-reactive protein (CRP)	Serum	Non-invasive	Low sensitivity
Inflammatory markers (e.g. cytokines)	Serum	Non-invasive	Non- specific
Bacteria metabolic products (e.g. LPS)	Serum	Non-invasive	High sensitivity
Fatty acid binding proteins (FABP)	Seum /urine	Non-invasive	
Calprotectin and lactoferrin	Feces	Non-invasive	Non-specific
Dual sugar test (e.g. mannitol, lactulose)	Urine	Non-invasive	Time consuming
Polyethylene glycols (PEG)	Urine	Non-invasive Small and large intestine	Time consuming