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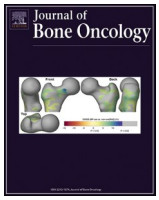

Bones and guts – Why the microbiome matters

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HIGHLIGHTS

- Bone metastasis is an emerging focus in the field of osteomicrobiology.
- Bone metastasis induces dysbiosis and alters microbiota composition in patients.
- Gut dysbiosis prevents immune cell egress to the bone *in vivo*.
- Lactobacillus supplementation enhances bone volume and reduces cancer-induced bone pain.
- Future studies are needed to further our understanding of bone metastasis and bugs.

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ABSTRACT

The importance of the gut microbiota in human health has become increasingly apparent in recent years, especially when the relationship between microbiota and host is no longer symbiotic. It has long been appreciated that gut dysbiosis can be detrimental to human health and is associated with numerous disease states. Only within the last decade, however, was the gut microbiota implicated in bone biology. Dubbed osteomicrobiology, this emerging field aims to understand the relationship between the gut microbiome and the bone microenvironment in both health and disease. Importantly, the key to one of the major clinical challenges facing both bone and cancer biologists: bone metastasis, may lie in the field of osteomicrobiology; however the link between gut bacteria and bone metastasis is only beginning to be explored. This review will discuss (i) osteomicrobiology as an emerging field, and (ii) the current understanding of osteomicrobiology in the context of cancer in bone.

1. Introduction

Despite the initial identification of animalcules, or bacteria, in human feces in the 1680s, it was not until surgeon John Goodsir's 1840s discovery of – what he believed to be – a pathogenic microbe in his patient's stomach fluid that the gut microbiome began to harbor clinical interest [1]. Advances in bacterial research have come a long way since the 1840s with modern techniques like 16S ribosomal RNA sequencing and shotgun sequencing shining a spotlight on the microbes contained within the human gut. The gut microbiota – comprised of bacteria, viruses, fungi, archaea, and protists – account for an estimated 38 trillion cells in the human body and play both beneficial and pathogenic roles [2]. In a healthy individual, bacteria present in the gut offer a mutualistic relationship. Gut bacteria are dependent on the host diet for nutrients essential to their survival [3]; in turn, the host depends on bacteria for the digestion and breakdown of otherwise indigestible compounds, promotion of angiogenesis and regulation of the enteric nervous system [4,5]. Importantly, commensal bacteria also support the chemical and immune barriers that separate the contents of the intestines from systemic circulation [4,6]. However, when the commensal and pathogenic bacteria become imbalanced and are in a state of dysbiosis, these protective barriers become compromised leaving the human host susceptible to disease [4]. Gut dysbiosis has been associated with the pathogenesis of numerous diseases including inflammatory bowel disease [7,8], cardiovascular disease [9,10], diabetes [11,12], and cancer [13–15]. Recently however, interest has emerged in a new field: osteomicrobiology, an area focused on understanding the role of the gut microbiota in bone health and disease [16].

The skeleton plays a quintessential role in the human body, providing a gateway for movement, protecting the vital organs, and serving as a reservoir of minerals [17]. Comprised predominately of bone-forming osteoblasts, bone-resorbing osteoclasts, and bone-regulatory osteocytes, the bone is defined by its constant remodeling essential for maintaining homeostasis [18,19]. The process of

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remodeling enables the skeleton to replace old or injured bone with new, preserving the skeleton's structural integrity and maintaining the body's balance of minerals [20]. However, this same constant overturn and abundance of minerals, chemokines, and vasculature that preserves the bone's health also makes it a prime target for cancer cell colonization [21]. In fact, it has been suggested that crosstalk between bone cells and bone metastatic cancer cells stimulates further bone metastatic progression, known as the "vicious cycle of bone metastasis" [22]. An incurable disease, bone metastasis results in pathological remodeling of the bone, resulting in debilitating pain and fractures among other side effects [23,24]. Pathological bone remodeling, a process mediated through multiple coordinated signaling mechanisms and defined by a dysregulation of osteoblast/osteoclast balance, is a hallmark of other disorders of the bone, such as osteoporosis and Paget's disease [25]. Despite an impressive body of research investigating effective therapeutics for bone disorders, the constant shifting of the landscape within the organ adds an element of difficulty when treating diseases of the bone. While anti-resorptive medications do exist and serve as the primary treatment for diseases of bone remodeling, their expensive cost and adverse side effects such as osteonecrosis of the jaw, adverse gastrointestinal effects, and atypical femur fractures can make treatment maintenance difficult [26]. Despite these adverse side effects often necessitating cessation of treatment, discontinuation of anti-resporptive therapies is associated with decreased bone density and elevates patients' risk of fractures [27]. As such, there is an apparent need to find effective ways to maintain bone health and safely target diseases of bone remodeling, and emerging evidence suggests that the key may lie in a new area of bone research, osteomicrobiology.

2. Osteomicrobiology

The field of osteomicrobiology began just over a decade ago when it was revealed that the gut microbiota regulated bone density in mice through immune mediated signaling mechanisms [28]. These researchers found that germ-free mice (i.e. mice without microorganisms) had elevated bone density, fewer osteoclast precursor cells, and decreased $CD4^{+}$T cells compared to conventionally raised mice [28]. Interestingly, when the guts of germ-free mice were recolonized, bone mass normalized, osteoclast precursor cell numbers increased, and $CD4^{+}$T cells were seen in higher frequency [28]. Since that discovery, researchers have worked diligently to understand the crosstalk between the gut microbiome and the bone in order to unveil potential therapies for a number of diseases of the bone. To date, the bulk of osteomicrobiology research pertains to osteoporosis, a disease defined by low bone mineral density (BMD) and increased fracture risk as a result of imbalanced bone remodeling that affects over 200 million people worldwide [29]. Interestingly, osteoporosis has been identified as a complication of several diseases of the gastrointestinal (GI) tract; however, treatments for osteoporosis targeting the gut microenvironment do not currently exist [30]. A decade of research focused on understanding gut-osteoporosis crosstalk has yielded some success alleviating osteoporosis by manipulating the gut. For example, *Lactobacillus reuteri ATCC PTA 675* supplementation was found to enrich short chain fatty acids (SCFAs) and reduce detrimental bacteria in the gut [31]. Importantly, gut metabolites such as SCFAs may be a key to understanding gut-bone crosstalk. The SCFA butyrate for example leads to bone formation by increasing intestinal and bone marrow regulatory T cells which stimulate Wnt secretion from $CD8^{+}$T cells, activating the Wnt pathway in osteoblast cells [32]. Further, osteoclast differentiation can be inhibited through the downstream activity of SCFAs propionic acid and butyric acid, which can reduce the expression of osteoclast associated genes through oxidative phosphorylation [33]. While this data suggests that gut microbiota and their metabolites may be important for osteoanabolic activity, very little research has been conducted exploring these interactions outside of osteoporosis. Only recently has research begun to carry the field of osteomicrobiology into other diseases of the bone, especially cancer bone metastasis.

3. The gut microbiome and cancer in the bone

Cancer in bone places enormous financial, emotional, and physical burdens on those who face the disease [34,35]. While surgery and chemotherapy have brought the 5-year survival rate for patients with primary bone cancers to approximately 66 % [35], unfortunately there is no curative therapy for cancer metastasis to the bone. As such, an effective, affordable treatment for cancer in bone – especially metastatic cancer in bone – is urgently needed, and the gut may hold the key to this therapy.

3.1. Microbiota profiling in patients with bone metastasis

Only recently has the gut microbiota come to the forefront of cancer research in the bone microenvironment. A recent profiling of the gut microbiota comparing patients without cancer (n = 25), patients with breast cancer (BCa) without metastases (n = 32), and patients with BCa bone metastasis (n = 22) revealed significantly reduced number of observed species in patients with bone metastases [36]. Reduced diversity of the gut microbiota has previously been found to be associated with a wide variety of human diseases [37]. Despite reduced gut microbiota diversity, an abundance of Proteobacteria, *Streptococcus, Campylobacter, and Moraxellaceae* was found in both cancer bearing groups [36]. Interestingly, Proteobacteria, *Streptococcus,* and *Campylobacter* have all been linked to colorectal cancer [38–40]. In the non-cancer controls, *Megamonas*, *Akkermansia, Clostridia, Gemmiger,* and *Paraprevotella* were significantly more abundant [36]. Akkermansia manipulation has previously been shown to have a protective effect against cancer and increase treatment responsiveness in mice [41–43]. *Megamonas* has been found to be reduced in older adults with intestinal permeability [44]. Interestingly, supplementation of *Clostridiales* strains have been found to improve immunotherapy treatment in murine melanoma and colorectal cancer by inducing CD8+ T-cell infiltration and activation at the tumor site [45]. Therefore, these bacteria may provide protective effects against BCa. When comparing BCa patients without metastases and BCa patients with bone metastases, higher abundances of *Pasteurellaceae, Haemophilus, Planococcaceae, Lysinibacillus,* and *Neisseria* were found in patients with bone metastases; whereas *Megamonas, Lactobacillales, Bacilli, Streptococcus, Akkermansia,* and *Oxalobacter* were elevated in BCa patients without metastases suggesting a different bacterial signature in patients with bone metastases [36]. While further investigation is warranted to determine the roles of these bacteria in BCa bone metastasis, predictive biological processes analyses attempted to evaluate the potential functions of these microbiota in BCa bone metastasis. Interestingly, it was revealed that BCa patients with bone metastasis have elevated metabolic pathway activity, steroid hormone synthesis, and bile acid metabolism [36]. This data supports the idea that the gut microbiome contributes to cancer progression by contributing to sex hormone activity [46]. Some members of the gut microbiota can metabolize estrogen and progesterone through B-glucuronidase activity, contributing to BCa occurrence [46].

3.2. Impact of Radium-223 on the gut microbiome

To date, no other studies have been conducted specifically profiling the gut microbiota in patients with cancer in the bone; In addition to gut bacteria alteration in BCa bone metastasis, evidence suggests that cancer type and treatment can also have effects on the gut microbial composition. One recent study sought to determine the effect of Radium-223 (Ra-223) on the gut microbiomes of prostate cancer (PCa) patients with bone metastases (n = 3) compared to healthy controls (n = 2). Ra-223 is the only treatment targeting PCa bone metastasis that significantly improves overall survival, but unfortunately only by a few months (mean = 3 months) [47]. Interestingly, the phyla relative

abundances of Proteobacteria and Actinobacteria were elevated in PCa patients with bone metastasis compared with controls before receiving Ra-223 treatment [48]. Increased Actinobacteria abundance has been found to be a risk factor for both breast and lung cancers [49]. Interestingly, Proteobacteria were also more abundant in patients with osteoporosis and osteopenia compared to those with normal BMD [50]. Indeed, increased abundance of Proteobacteria has been previously identified as a potential indicator of inflammation and disease [51]. In contrast, Lactobacillus, *Clostridium leptum, and Clostridium coccoides* were decreased in PCa patients [48]. Lactobacillus has been shown to have anti-cancer effects in colorectal cancer, and both Clostridium species contribute to SCFA production [52–54]. Moreover, *Lactobacillus animalis* supplementation in patients with osteonecrosis of the femoral head has been found to have protective effects on the bone microenvironment, inducing angiogenesis and bone growth [55]. After receiving Ra-223, levels of Bacteroidetes, *Prevotella, Lactobacillus, Bidifidobacterium, Clostridium coccoides, and Bacteroides fragilis* were all decreased [48]. Taken together, this data suggests that bone metastatic PCa patients have altered gut microbial signatures, and these signatures are further altered by Ra-223 treatment.

3.3. Preclinical studies

Importantly, research has begun to unveil the crosstalk between the gut microbiota and cancer in bone *invivo*. In an attempt to characterize the gut microbiome in osteosarcoma, Nu/J mice were injected subcutaneously with the 143B human osteosarcoma cell line (n = 6) or sham (n = 6) [56]. Stool analysis over 63 days revealed that *Roseburia* and *Akkermansia* genera were more abundant in the osteosarcoma group whereas Lachnospiraceae NK4A135 and *Muribaculaceae* were elevated in the control group [56]. Interestingly, literature predominately suggests a protective role of *Roseburia* and *Akkermansia* in cancer contrary to the results of this study [41–43,57,58]. It was hypothesized that the observed increases in butyrate synthesizing *Roseburia* and mucin degrading *Akkermansia* abundance may be a protective mechanism to restore bacterial diversity in the gut [56]. In line with the literature, the Lachnospiraceae family has been found to promote tumor immune surveillance in colorectal cancer [59], and *Muribaculaceae* can ameliorate DSS-induced colitis [60]. Further, time-course analysis revealed an increase in the firmicutes:bacteroidota ratio in the osteosarcoma group as the study progressed [56]. Importantly, an increase in the ratio of firmicutes:bacteroidota is associated with dysbiosis [61]. Despite these apparent changes in gut microbial compositions and dysbiosis in patients and animals with cancer in bone, very few studies exist exploring the underlying mechanisms. To date, there is just one study investigating the mechanisms through which the gut microbiome effects bone metastasis. To investigate the role of the gut microbiota in melanoma growth in bone, the gut bacteria were ablated to mimic dysbiosis using antibiotics and intracardiac or intratibial injections of B16-F10 melanoma cells were performed [62]. Findings revealed that ablation of the gut microbiota significantly enhanced tumor growth in the bone compared to mice with intact gut microbiomes [62]. Indeed, this is consistent with earlier literature demonstrating that antibiotic manipulation of the gut microbiota enhanced BCa growth in mice [63]. Further, the antibiotic ablation also prevented natural killer (NK) and type 1T helper (Th1) cell egress to the bone marrow [62]. After confirming that melanoma injection in bone does indeed promote NK and Th1 cell expansion to the bone marrow using C57BL/6 Kaede mice, which allow for tracking of photoconverted cells [64], this expansion did not occur in mice with antibiotic ablated guts [62]. Interestingly, it was found that blocking NK and Th1 egress from intestines or influx into the bone marrow both enhanced melanoma growth in bone [62]. This study suggests that gut microbiota crosstalk with cancer in bone may occur through immune mediated mechanisms.

3.4. The gut microbiota and Cancer-induced bone pain

While it appears that the gut microbiota play an important role in cancer progression, the gut microbiota have also emerged as a player in cancer-induced bone pain (CIBP). CIBP, a debilitating pain state characterized by spontaneous breakthrough pain, affects upwards of 60 % of patients with bone metastasis [65]. Unfortunately, management of CIBP often presents as a clinical challenge due to the severe side effects associated with current treatments [66]. Medications used to manage pain, such as non-steroidal anti-inflammatory drugs and opioids, are often associated with lethargy, nausea, vomiting, and other gastrointestinal issues [66]. Treatments targeting the bone, such as bisphosphonates, radiation therapy, and surgery, can result in hypercalcemia, renal complications, osteonecrosis of the jaw, bone marrow toxicity, leukopenia, and infection among other side effects [65,67]. As such, safer, more effective therapies are essential for treating CIBP, and the gut microbiome is beginning to emerge as a potential solution. A recent study, the only one to date exploring the gut microbiome and CIBP, found that Lactobacillus rhamnosus GG and butyrate supplementation in rats could reduce CIBP [68]. Interestingly, when rats received supplementation with Lactobacillus, expression of mu-opioid receptor was increased in the spinal cords [68]. Further, Lactobacillus supplemented rats experienced less pain following morphine injection [68]. Moreover, Lactobacillus supplementation was able to enhance Lachnospiraceae and reduce Clostridiaceae and Enterobacteriaceae to levels seen prior to tumor inoculation. Both Lactobacillus and Lachnospiraceae are known for the production of beneficial SCFAs and butyric acid [50]. Interestingly, when rats received supplementation with SCFA butyrate, increases in mu-opioid receptor expression and reduction in CIBP were also seen [68]. When Lactobacillus and butyrate supplementation were combined, mu-opioid receptor was increased, and histone deacetylase 2 (HDAC2) expression was inhibited [68]. HDAC2 levels have previously been found to increase after nerve injury; however, when HDAC2 was returned to basal levels, hyperalgesia was reduced [69]. Overall, this study highlights the analgesic effect of both Lactobacillus and butyrate in CIBP and further suggests that the gut may be a valuable analgesic target. Despite seeing reduction in CIBP following Lactobacillus rhamnosus GG and butyrate supplementation, the effect of this supplementation on tumor growth was not reported [68].

In summary, while the body of evidence suggesting that the gut microbiota plays a role in cancer progression in bone and CIBP is growing (Fig. 1), further studies are certainly necessary to understand the crosstalk between the gut and cancer in the bone microenvironment.

4. Conclusion

In this article, we have detailed the current understanding of the gut microbiota's involvement in cancer in bone, an emerging area under the larger umbrella of osteomicrobiology. While research regarding the effect of the gut microbiome on cancer in bone is only just emerging, the gut microbiota holds promise as a therapeutic target. Treating bone metastases remains a clinical challenge [23]. Anti-resorptive agents are associated with many side effects and ultimately do not increase survival [26]. Their osteoanabolic counterparts – medications designed to facilitate bone growth by either activating parathyroid receptors or inhibiting sclerositin, a Wnt signaling antagonist – are administered clinically; however these agents too have severe side effects including hypercalcemia, osteonecrosis of the jaw, and cardiovascular events [70]. Notably, supplementation with bacteria and SCFAs, such as Lactobacillus and its metabolite butyrate, may serve as a potential therapy for both cancer in bone and associated pain. Butyrate specifically is known to induce osteoanabolic activity through downstream activation of Wnt signaling by regulatory T cells [32]. As such, supplementation with butyrate holds promise as an osteoanabolic agent. The importance of the gut in immune activity has also become increasingly apparent, and the gut may be able to be targeted to induce immune activity in the bone

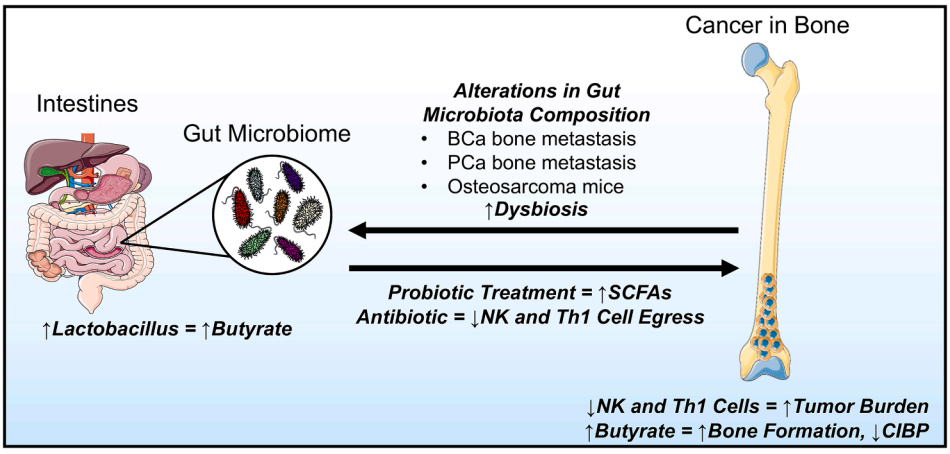

Fig. 1. Osteomicrobiology in the context of cancer in the bone. Osteomicrobiology research in the context of cancer in the bone has yielded important insight into the crosstalk between the gut microbiome and the bone-cancer microenvironment. Gut microbiota profiling in patients has revealed that the microbial composition is altered and species diversity is reduced in patients with breast cancer (BCa) bone metastasis compared with non-metastatic and healthy controls. These findings suggesting that the gut is in a state of dysbiosis as a result of cancer in bone were corroborated in both a cohort of prostate cancer (PCa) patients with bone metastasis and in an *in vivo* osteosarcoma model. This dysbiosis can have profound effects on both the intestinal tissue and the metabolites produced by bacteria within the gut. When mice were treated with antibiotics, a model of gut dysbiosis in which gut bacteria are ablated, a decrease in egress of intestinal immune cells – namely NK and Th1 cells – to the bone was seen. This reduction in intestinal immune egress to the bone resulted in an increase in bone tumor burden suggesting that gut dysbiosis may promote tumor growth in bone. The gut microbiome can also be manipulated to have a positive effect on the cancer bone microenvironment. When cancer bearing mice were treated with probiotic Lactobacillus, increases in butyrate were observed. Increased butyrate leads to increased bone formation and reduced cancer-induced bone pain (CIBP). Taken together, these studies suggest that the gut microbiome plays an important role in bone metastasis, and future studies are certainly warranted to expand upon the mechanisms through which these interactions occur. Graphics adapted from Smart Servier Medical Art (https://smart.servier.com/).

marrow. With this new evidence suggesting the importance of the gut microbiota in cancer in bone comes several new questions:

1. Can we identify a bacterial signature specific to cancer in bone?
2. What are the functions of the gut microbiota increased in patients with cancer in bone?
3. Can the gut microbiota be manipulated to reduce bone metastatic tumor growth or CIBP?
4. Do the gut microbiota have an effect on patient response to therapy?
5. What are the effects of gut microbial metabolites on cancer in bone?
6. Can intestinal immune cells be harnessed to target cancer in the bone marrow?

While further research elucidating the crosstalk between the gut microbiota and the bone metastatic microenvironment is certainly warranted, the gut may serve as a much needed therapeutic target for cancer in bone and CIBP.

CRediT authorship contribution statement

Kelly F. Contino: Writing – original draft, Writing – review & editing. **Katherine L. Cook:** Writing – review & editing. **Yusuke Shiozawa:** Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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