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Effect of fungal microbiota on RANKL and sclerostin in patients with Crohn's disease

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ABSTRACT

The etiology of Crohn's disease (CD) is still unknown. However, many factors, including a dysregulated immune system, altered microbiota, inheritance, and environmental factors, have been implicated. This work was conducted to estimate the effect of fungal microbiota on two bone mineral density markers, RANKL and sclerostin, in addition to the correlation between these markers and vitamin B12, D3, and zinc in CD patients, along with their potential effect on fungal microbiota and vice versa. Peripheral blood and carry-Blair Stool samples were collected from 88 participants (60 newly diagnosed with CD without treatment and 28 healthy controls) to detect serum levels of RANKL and sclerostin, and culture media were used to grow, isolate, and identify fungi attendant to CD and its effect on RANKL and sclerostin levels. Sociodemographic data (vitamin B12, D3, and zinc levels) were collected from patients' medical records. The results showed significant differences in RANKL and sclerostin levels in various types of fungal microbiota in CD patients along with a significant increase in RANKL and sclerostin levels in these patients. Moreover, RANKL levels were negatively significantly correlated with Zinc, while sclerostin levels correlated negatively with vit D3. The findings of this study suggest that fungal microbiota may play a role in the inflammatory process and interactions with bone density by affecting levels of RANKL and sclerostin, vitamin D3, and zinc, suggesting that the use of the fungal microbiota in the monitoring and treatment of CD patients.

INTRODUCTION

The intestinal mucosa is the site of origin and manifestation for the two primary phenotypes of inflammatory bowel disease (IBD), Crohn's disease (CD), and ulcerative colitis (UC) [1]. CD inflammation can affect any part of the digestive system, but the terminal ileum, colon, and perianal areas are the most commonly implicated in a discontinuous pattern. The CD is distinguished from ulcerative colitis by submucosal thickening, transmural inflammation, and chronic granulomas [2]. CD inflammation can affect any part of the digestive tract, from the mouth to the anus, and is accompanied by discontinuous transmural lesions of the gut wall [3]. Compared to the general population, patients with CD are more likely to develop osteoporosis and sustain osteoporotic fractures [4]. Chronic inflammation, diminished vitamin and mineral absorption, severe small-bowel disease or resection, corticosteroid use, advanced age, inactivity, smoking, and nutritional inadequacies are all factors that contribute to bone loss in CD patients [5]. The relation between CD and lower bone density is still unclear. However, it seems to be directly related to inflammation or other osteoporosis risk factors, such as vitamin deficiency, that are frequently observed in CD. However, corticosteroids have been found to increase the risk of poor bone mineral density and osteoporosis [6]. The etiology of CD is complex, involving environmental variables, genetic

predisposition, and the host's impaired immunological interaction with the gut microbiota [7].

Most research on host-associated microbiota in health and disease has concentrated on bacterial microbiota even though fungal illnesses cause a significant infectious disease burden. As a result, little has been realized about the relevance and function of the fungal microbiota [8]. Fungi are considered an important component of the human flora because they represent a dynamic and ecologically diverse microbial population. In a healthy gastrointestinal tract, the diversity of bacterial and fungal microbiota is inversely correlated, and the two are dynamically balanced [9]. Aside from bacterial dysbiosis, earlier research has identified a distinct fungal microbiome dysbiosis in CD, which is characterized by changes in biodiversity and composition [10]. Shifts in fungal microbiota modify immune response and disease status [11]. The development of skeletal disorders is significantly influenced by gut microbiota, which includes bacteria, fungi, and viruses, and may be a target for treatment [12]. A dynamic equilibrium known as physiological bone remodeling is the outcome of several biological processes that are tightly orchestrated and controlled by the complex interactions between the various cell types that comprise bone, principally osteoclasts and osteoblast lineage cells [13]. RANKL (receptor activator of nuclear factor kappa-b ligand) is a transmembrane protein generated by stromal and osteoblast cells. It belongs to the tumor necrosis factor receptor family. It is mostly found on chromosome 18q22.1. It is made up of 616 amino acids and is found in osteoclast precursors and mature osteoclasts [14]. RANKL regulates osteoclast development and function via binding to the RANK on osteoclast progenitor cells [15].

Moreover, osteocytes, the most prevalent type of bone cell, play a critical role in the formation of bones and resorption. They also serve as endocrine cells, secreting proteins that regulate skeletal metabolism as well as global mineral and nutrient balance [16]. Sclerostin SOST is a bone tissue protein that is encoded by the SOST gene [17]. It is a glycoprotein released by osteocytes that prevents osteoblasts from forming new bone, which results in bone loss [18]. Several research on bone health have found that vitamin (Vit) B12, (vit) D3, and zinc all contribute to the quality of human bone growth, homeostasis, and bone diseases [19-21]. A few studies focused on the effect of microbiota on Iraqi patients with CD, so that, the current study aims to assess the impact of fungal microbiota on bone mineral density, with an emphasis on its potential effect on RANKL and sclerostin levels, as well as their correlation to vitamin B12, D3, and zinc levels in Iraqi CD patients.

MATERIALS AND METHODS

Study design

A total of 88 Iraqi individuals (male and female) were enrolled in this study. Sixty patients were clinically early diagnosed with CD without treatment during their attendance at Al-Kindy Teaching Hospital in Baghdad, Iraq. The study extended from January 2022 to November 2022. The age of CD patients ranges from (15–65) years. In addition, 28 healthy individuals; their age matched the patient group, and the participants had no CD or other gastrointestinal diseases. Sociodemographic information (fungal infection kinds, vitamin B12, D3, and zinc levels), as well as patients' height, weight, age, sex, blood groups, and smoking habits, were obtained from their medical records.

Ethical considerations

This work has been approved by the ethical committee of the biology department at the University of Bagdad, College of Education for Pure Sciences/ Ibn Al-Haitham in Baghdad, Iraq. On 2/1/2022, the authorization was acquired under reference number EC-45.

Collection of blood samples

The venous blood sample was drawn from CD patients and controls. Each participant had a vein punctured to draw 5 mL of blood, which was then carefully pumped into disposable serum tubes filled with separating gel. After allowing the blood in the gel tubes to coagulate for 15 minutes at room temperature, it was centrifuged for another 15 minutes at 3000 rpm. The serum is kept for later use at -20°C.

Determination of RANKL and sclerostin levels by using ELISA

Commercial enzyme-linked immunosorbent assay (ELISA) kits from Cloud Clone Crop (USA) were used to measure the levels of RANKL and sclerostin in serum samples, employing the sandwich enzyme immunoassay concept. A microplate has been pre-coated with antibodies unique to each parameter. A biotin-conjugated antibody that is specific to the parameters is then pipetted into the wells along with the samples and standards. Each well was then filled with Avidin coupled with Horseradish Peroxidase (HRP) and incubated. After adding tetramethylbenzidine (TMB) substrate solution, only the wells containing the investigated parameter, biotin-conjugated antibody, and enzyme-conjugated Avidin showed a color shift. A sulfuric acid solution was added to halt the enzyme-substrate reaction, and the color shift was detected using spectrophotometry at 450 nm ± 10 nm. The concentration of the parameter studied in the samples was ascertained by comparing the optical density (OD) of the samples to the standard curve.

Quantitative measurements of vitamin D3, vitamin B12, and Zinc in serum samples

The quantitative measurements of vitamin D3, vitamin B12, and zinc in the sample of human serum were performed using an ELISA kit (BioSource, USA) following the manufacturer's instructions. The human kit used in this study was a sandwich enzyme-linked immunosorbent assay. A microplate was pre-coated with the antibody unique to each parameter. A biotin-conjugated antibody specific to the parameters was pipetted into the wells along with the samples and standards. Next, HRP conjugate was then added. Following incubation, the unbound avidin-HRP conjugate was eliminated during a wash step. A substrate solution reactive with HRP was added to the wells. A colored product was formed in proportion to the sample's parameters. The color development was monitored with an ELISA plate reader, and absorbance was measured at a wavelength of 405 nm.

Stool sampling and analysis

In order to identify the fungus linked to CD, 120 Carry-Blair stool samples were obtained from patients and controls. A variety of culture media were made in accordance with guidelines for sterilizing culture media and were used to grow, isolate, and identify fungi. 15 minutes at 121° C and 15 Psi of pressure.

Isolates and culture samples

After homogenizing the sample in a tube with sterile saline and leaving it to rest for 30 minutes, 100 µl of the supernatant was transferred to potato and sabouraud dextrose agar (Oxoid, UK) and incubated for a week at 25–37° C. The features of mold colonies were based on color constancy and the reverse; direct microscope examination was used to diagnose fungi [22].

Preparation of sabouraud dextrose agar

Sabouraud dextrose agar (SDA) medium was prepared according to the manufacturer's instructions (Oxoid, UK) by dissolving 51 g of powder medium in 5 liters of distilled water and putting it on the electric heater for a minute. After the powder was mixed with water and chloramphenicol to prevent bacterial growth, it was autoclaved at 121 °C and 15 psi of pressure for 15 minutes, then left to cool to 41 °C before being poured into 250 mg/L dishes. The solution is placed in dishes until solidified and becomes ready to culture the samples.

Preparation of potato dextrose agar

Potato dextrose agar (PDA) medium was prepared according to the manufacturer's specifications (Oxoid, UK) by dissolving 39 g of it in 1 liter of distilled water. The pH was set at 6.8. After that, the medium was sterilized in an autoclave at 121°C and a pressure of 15 psi for 15 minutes. After cooling the medium, the antibiotic chloramphenicol was added to prevent bacterial growth at a concentration of 250 mg/L.

Determination of body mass index

The formula of Body Mass Index (BMI) = weight (kg) / height^2 (m)^2 is used to calculate the body mass index (BMI). Underweight is defined as having a BMI beneath 18.5, normal weight as being between 18.5 and 24.9, overweight as being between 25 and 29.9, and obese as being over 30 [23].

Statistical analysis

SPSS version 23 was used for statistical analysis of the acquired data. A difference was considered statistically significant if its P value was less than 0.05. The information was presented as Mean ± Standard Error (SE). The t-test and analysis of variance (ANOVA) were used to compare the groups statistically. Utilizing the Pearson correlation coefficient, the relationship among RANKL, sclerostin, zinc, vitamin D3, and vitamin B12 was investigated.

RESULTS

Serum levels of RANKL and sclerostin in CD patients

The present study revealed a highly significant increase (P≤0.001) in RANKL levels in CD patients compared to control as illustrated in Figure 1A. Sclerostin level showed a slightly significant (p≤0.05) increase in patients with CD compared to the control group (Figure 1B).

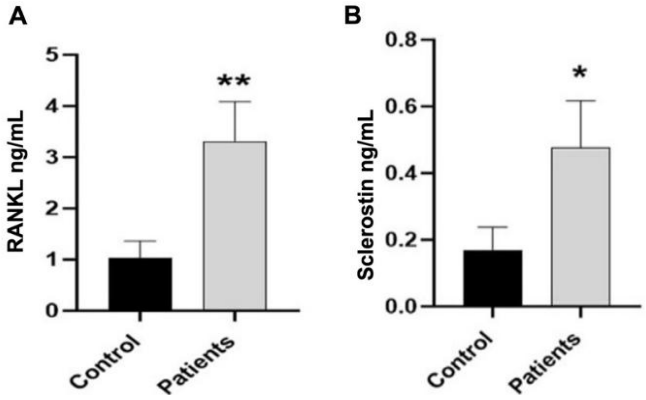

Figure 1. A) Serum levels of RANKL. B) Serum levels of sclerostin. Mean ± standard error (SE). ** High significant increase in RANKL levels in CD patients compared to control at probability level p≤0.001. *A slightly significant increase in patients with CD compared to controls at probability at probability (P≤0.05).

Serum levels of RANKL and sclerostin among different fungal microbiomes in CD patients

A highly significant (p≤0.001) variation in RANKL levels among the several fungal microbiome types, is shown in Table 1. Additionally, the sclerostin level was slightly significant (p ≤ 0.05) in the different types of fungal infection as shown in Table 2.

Table 1. RANKL levels with different types of fungi in CD patients.

Fungus types	Count of patients	RANKL levels
Candida albicans	1	0
Candida tropicalis	1	0
Candida glabrata	2	0.07
Pencillium	1	0
Candida tropicalls, Asprgillus	1	0
Candida albicans, Asprgillus, Fussarium	4	1.364
Candida albicans, Asprgillus, Pencillium	3	5.817
Candida glabrata, Pencillium, Mucor	4	3.61
Candida glabrata, Aspirgillus	6	2.586
Candida glabrata, Mucor, Rhizopus	4	2.568
Candida albicans, Asprgillus	5	4.497
Candida albicans, Pencillium, Saccharomyces	4	8.141
Candida glabrata, Asprgillus, Cryptococcus	4	7.317
Aspirgillus, Trichophyton	2	0.736
Candida gulliermondi, Mucor	5	9.952
Candida glabrata, Pencillium	4	3.946
Mucor, Saccharomyces	4	5.196
Saccharomyces	5	10.371
Mean±S.E.		6.83±0.35
T test		19.63
P-value		0.001*

The data represented serum RANKL levels in different fungal species compared to one another in CD patients. Significant at probability level p≤0.001

Table 2. Sclerostin levels with different types of fungi in CD patients.

Fungus types	Count of patients	Sclerostin levels
Candida albicans	1	1.641
Candida tropicalis	1	0
Candida glabrata	2	0
Pencillium	1	0
Candida tropicalls, Asprgillus	1	0.11
Candida albicans, Asprgillus, Fussarium	4	0.136
Candida albicans, Asprgillus, Pencillium	3	0.128
Candida glabrata, Pencillium, Mucor	4	0.019
Candida glabrata, Aspirgillus	6	1.206
Candida glabrata, Mucor, Rhizopus	4	0.188
Candida albicans, Asprgillus	5	0
Candida albicans, Pencillium, Saccharomyces	4	0.244
Candida glabrata, Asprgillus, Cryptococcus	4	0.283
Aspirgillus, Trichophyton	2	0
Candida gulliermondi, Mucor	5	1.140
Candida glabrata, Pencillium	4	1.320
Mucor, Saccharomyces	4	0.195
Saccharomyces	5	0
Mean±S.E.		1.14±0.34
T test		3.326
P-value		0.05*

The data represented serum sclerostin levels in different fungal species compared to one another in CD patients. *Significant at probability level p≤0.05

Serum levels of RANKL and sclerostin among different fungal microbiomes in controls

The results also showed a non-significant difference p>0.05 in RANKL and sclerostin among control in different Fungi as shown in Tables 3 and 4.

Table 3. Levels of RANKL in control with different types of fungi.

Fungus types	Count of individuals	RANKL control (Mean±SE)
Candida albicans	16	0.2414±0.11
Candida glabrata	4	0.3090±0.18
Candida tropicalis	8	0.2798±0.16
p-value		0.945*

The data represented serum RANKL levels in different fungal species compared to one another in the control group. *Non-significant at probability p>0.05.

Table 4. Levels of sclerostin in control with different types of fungi.

Fungus types	Count of individuals	Sclerostin control (Mean±SE)
Candida albicans	16	0.02±0.0338
Candida glabrata	4	0.12±0.1553
Candida tropicalis	8	0.05±0.0878
p-value		0.204*

The data represented serum sclerostin levels in different fungal species compared to one another in the control group. *Non-significant at probability p>0.05

Comparison of RANKL and sclerostin levels between patient and control groups with different fungal infections

In addition, our results showed a slightly significant difference ($p \leq 0.05$) in RANKL levels between patients and the control group with *Candida* spp., as shown in Table 5. Moreover, the sclerostin level was slightly significantly higher ($p \leq 0.05$) in patients with *Candida albicans*, but not significantly different ($p > 0.05$) in patients with other *Candida* spp. compared to the control group with *Candida* spp. as shown in Table 6.

Table 5. Comparison of RANKL with different fungal infections in CD patients.

Fungus types	Control group (Mean ±SE)	Patients group (Mean ±SE)	P value
Candida albicans	0.241 ±0.11	0.00 ±0.00	*0.048
Candida glabrata	0.309 ±0.18	0.0175 ±0.01	* 0.0491
Candida tropicalis	0.279 ±0.16	0.00 ±0.00	* 0.0431

*Significant at probability level p≤0.05

Table 6. Comparison in sclerostin with different fungal infections in CD patients.

Fungus types	Control group (Mean ±SE)	Patients group (Mean ±SE)	P value
Candida albicans	0.00 ±0.00	0.274 ±0.18	*0.044
Candida glabrata	0.0175 ±0.01	0.00 ±0.00	0.252 N.S
Candida tropicalis	0.00 ±0.00	0.00 ±0.00	0 N.S

*Significant at probability level p≤0.05. N.S Non-significant at probability p>0.05

Serum levels of RANKL and sclerostin are based on blood groups

There were no significant differences between the mean rank of the RANKL, and sclerostin levels based on blood groups as demonstrated in Figures 2A and B.

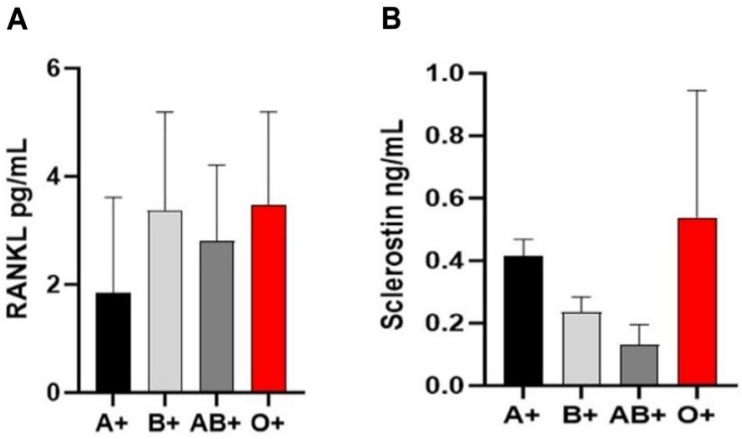

Figure 2. A) Serum levels of RANKL based on blood groups. B) Serum levels of sclerostin based on blood groups. Data are presented as Mean ± standard error (SE). No significant difference between the mean rank of the RANKL based on blood groups. No significant difference between the mean rank of sclerostin, based on blood groups as demonstrated.

Comparison of serum levels of RANKL and sclerostin based on gender, cigarette smoking, age, and BMI

Also, our results found no significant difference between studied parameters when distributed according to gender, cigarette smoking, age, and BMI as illustrated in Tables 7 and 8.

Table 7. RANKL levels according to sociodemographic data in CD patients.

Features		RANKL (pg\mL) (Mean±SE)	P value
Age	40≤	3.905 ±1.048	0.311*
	40>	2.199 ±1.070	
Gender	Male	3.574±1.048	0.718*
	Female	2.984±1.237	
Cigarette smoking	Smoking	3.791±1.208	0.405*
	Non-smoking	2.914±1.061	
BMI	Under weight	3.694±3.624	0.964*
	Normal	2.992±1.259	
	Overweight	2.667±1.379	
	Obesity	4.132±2.080	

* Non-significant differences

Table 8. Sclerostin levels according to sociodemographic data in CD patients.

Features		Sclerostin (ng/mL) (Mean±SE)	P value
Age	40≤	0.561±0.281	0.353*
	40>	0.150±0.068	
Gender	Male	0.556±0.694	0.373*
	Female	0.161±0.073	
Cigarette smoking	Smoking	0.206±0.000	0.169*
	Non-smoking	0.327±0.191	
BMI	Under weight	0.110±0.000	0.595*
	Normal	0.690±0.309	
	Overweight	0.310±0.210	
	Obesity	0.132±0.123	

* Non-significant differences

Correlation of RANKL and sclerostin levels with Vit B12, Zinc, and Vit D3 in CD patients

A Pearson correlation coefficient was also calculated to analyze the linear correlation between RANKL, and sclerostin levels in CD patients and Vit B12, Zinc, and Vit D3 levels. Table 9 shows a negative connection between RANKL levels and zinc levels. Sclerostin was found to be negatively associated with Vit D3 in this research as shown in Table 9.

Table 9. Correlation of RANKL and sclerostin levels with Vit B12, Zinc, and Vit D3 in CD patients.

	B12 ng/ml	Zinc mg/dl	D3 ng\mL
RANKL (Pearson correlation)	-0.169	-0.744*	-0.187
Sig. (2-tailed)	0.476	0.022	0.431
Sclerostin (Pearson correlation)	-0.446	-0.556	-.0678*
Sig. (2-tailed)	0.229	0.120	0.045

*Significant at probability level p≤0.05

DISCUSSION

The results showed that fungus infections, particularly those caused by *Candida* spp., which are the most prevalent pathogens in CD patients, significantly affect the levels of RANKL and sclerostin in CD patients. These results may be attributed to *Candida*'s inflammatory properties. The pro-inflammatory effects of *Candida albicans* in mouse colitis models are evidence linking between fungal microbiome and CD [24].

Candida is assumed to be able to infiltrate the gut's compromised epithelial barrier and induce invasive illness, which makes sense in the situation of IBD, especially with

concurrent immunosuppression [25]. Other studies have shown that alteration in the gut microbiota has a significant effect on bone loss [26]. Since intestinal microbes indirectly stimulate or repress osteoblasts and osteoclasts, they might alter the balance between bone formation and resorption. Furthermore, gut microbes affect bone metabolism by altering the immunological condition of bones or controlling signaling proteins that promote tissue healing, inflammation, cell division, and survival (growth factors)., thereby influencing bone mass [27]. Dysbiotic gut microbial flora identified in IBD may have an indirect effect on bone by several possible mechanisms. T cells activated by the gut microbiota may serve as "inflammatory shuttles" between the intestine and bone. Secondly, microbe-associated molecular patterns released into the circulation in IBD may activate immunological responses in the bone marrow by immune cells as well as osteocytes, osteoblasts, and osteoclasts, resulting in decreased bone production and increased resorption [28]. Bone homeostasis may be impacted by gut microbiota, which is recognized to be crucial in controlling the host's health and physiology. By boosting the synthesis of circulating cytokines including interleukin (IL)-17, TNF, and receptor activator of nuclear factor (NF)-kB ligand (RANKL), it may contribute to the pathophysiology of osteoporosis [29]. The results of this study indicated that there was a highly significant rise in $P<0.001$ in the level of RANKL in CD patients compared to the control. This elevation in RANKL levels may be attributed to increased RANKL expression in response to proinflammatory cytokines, specifically TNF and interleukins 1 and 17, emphasizing the importance of inflammation in RANKL-mediated effects on bone [30]. Thus, proinflammatory cytokines could impact bone metabolism by increasing RANKL expression. Osteoclasts express RANKL, which binds to an osteoclast precursor that expresses the RANKL receptor, RANK (receptor activator of NF-B) receptors, and the osteoprotegerin (OPG) receptor. Osteoclasts grow and differentiate when RANKL binds to RANK receptors, which increases bone loss [31]. It is well documented that RANKL is secreted by various immune system cell types, including T and B cells, dendritic cells, and macrophages. Notably, RANKL production is influenced by a variety of variables, including proinflammatory cytokines [32].

On the other hand, our results show a slight increase in $P<0.05$ in sclerostin levels in CD patients compared to control. Numerous cell types contain the Wnt signaling pathway, a signaling system that controls a range of biological processes (including bone remodeling, cell differentiation, and tissue regeneration). The Wnt/-catenin pathway has been shown to have anti-inflammatory properties in IBD, and its role is presently being investigated [33]. Bone homeostasis is mediated by Wnt signaling. Sclerostin (SOST) is the endogenous suppressor of the Wnt pathway. SOST, a monomeric glycoprotein with a cysteine-knot pattern is produced by osteoblasts. By binding to low-density lipoprotein-related proteins 5 and 6 (LRP5 and LRP6), Seclerostin improves its suppressive effects on the Wnt pathway and blocks the canonical Wnt signaling [34]. Through this mechanism, SOST dose-dependently decreases osteoprotegerin (OPG), increasing the receptor activator of the nuclear factor-kB ligand/OPG mRNA ratio. This catabolic action is achieved by encouraging the osteocyte to produce and activate osteoclasts [35].

In inflammatory conditions like IBD, active T lymphocytes produce TNF-α, which also stimulates the formation of sclerostin [36]. In addition, decreased SOST levels are linked to increased osteoblast activity through stimulation of the Wnt/β-catenin signaling pathway [37]. Furthermore, sclerostin may increase the production of RANK-L, and the binding of RANKL to its receptor RANK is a critical step in the formation of osteoclasts from hematopoietic progenitor cells, as well as the activation of mature osteoclasts [38]. Shifts in the diversity of fungal microbiota in CD patients are linked to inflammation of

the mucosa [39]. Inflammatory conditions can affect both bone production and resorption, although they most frequently affect both [40]. A slightly significant difference $p \leq 0.05$ in RANKL levels between patients and the control group with *Candida* spp. This difference may be attributed to the small number of patient cases with *Candida* spp. only compared to control or due to environmental factors like lifestyle and diet which contribute to osteoporosis and bone loss [41]. The present study shows that sclerostin negatively correlated with Vit D3 ($r = -0.678$, $p = 0.05$). Vitamin D has systemic effects; it regulates innate and adaptive immunological responses and affects calcium homeostasis, which is implicated in bone metabolism. Vitamin D deficiency has negative effects on the immune system in IBD patients, causing dysregulation and inflammation-related loss of bone mineral density BMD [42]. Serum Vitamin D has been linked to alterations in the gut microbiome associated with inflammatory immune responses [43]. It has been reported that TNF-α acts as an activator for sclerostin expression [44]. Vitamin D therapy has been shown to inhibit the TNF pathway in IBD patients [45], which may have an impact on sclerostin levels.

A Pearson correlation coefficient was computed to assess the linear relationship between RANKL and Zinc. There was a strong negative correlation between the two assessed variables ($r = -.744$, $p = 0.022$). Further, the trace element zinc (Zn) is absorbed in the small intestine and serves as a cofactor for a number of growth-related enzymes, immunological function, and tissue repair. Zn functions as an antioxidant and regulates the stability of biological membranes [46]. Patients with IBD frequently suffer from zinc deficiency with prevalence rates ranging from 15% to 40%. In IBD patients, zinc insufficiency could be contributing to mucosal inflammation, which is a hallmark of CD disease [47]. Zn also stimulates osteoblastic cells and inhibits osteoclast activity in bone tissue via the zinc-regulated RANKL/RANK/OPG pathway [48]. Thus, alterations in zinc levels may have an effect on RANKL serum levels.

CONCLUSIONS

CD disease is associated with bone metabolism alterations. However, the signals that influence bone metabolism are not fully understood. The current results suggest that alterations in gut microbiota could affect the systemic immune response and provide signals that impact bone metabolism via RANKL and sclerostin levels. One significant limitation of the study was the CD sample size. Also, the causes of CD remain unknown despite its long history.

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AUTHOR CONTRIBUTIONS

HMA designed outlines and drafted the manuscript. RHKA and ASH performed the experiments and analyzed the data. RHKA and AKI wrote the initial draft of the manuscript. HMA and RHKAR reviewed the scientific contents described in the manuscript. All authors read and approved the final submitted version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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