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ORIGINAL RESEARCH

The Gut Microbiota and the Nerve-Bone Axis: Insights from a Mendelian Randomization and Mediation Analysis

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Background: This study aims to explore the interconnections among gut microbiota, autonomic nervous system (ANS), and bone through the proposed gut-nerve-bone axis using Mendelian randomization (MR) mediation analysis.

Methods: Genetic variants associated with gut microbiota were extracted from the MiBioGen consortium. Summary statistics for bone mineral density (BMD) were derived from a UK Biobank genome-wide association study dataset. We used heart rate variability (HRV) to represent the activity of ANS in the MR analysis. Initially, we employed a two-sample MR approach to evaluate the causal impact of gut microbiota on BMD. Subsequently, we used an additional mediation analysis to assess the effect of HRV on these associations, and sensitivity analysis was used to ensure the reliability of our results.

Results: *Coprococcus* 2 (β=0.03, 95% confidence interval, CI: 0.00 to 0.05, *P*=0.02), *Lachnospiraceae* NC2004 (β=0.01, 95% CI: 0.00 to 0.03, *P*<0.05), and another 11 genetically predicted taxa exhibited correlations with BMD. Among three types of HRV, pvRSA/HF and RMSSD were both associated with gut microbiota and BMD. The effect of *Lachnospiraceae* NC2004 on BMD occurs through RMSSD with a mediated proportion of 40.0% (95% CI: 21.1 to 58.9%, *P*=0.03) of the total effect. Sensitivity analyses reinforced the validity of our interpretations.

Conclusion: In summary, the present research supports a genetic correlation among gut microbiota, ANS, and BMD, which reinforces the concept of a gut-nerve-bone axis. Notably, the interplay between gut microbiota and BMD may be mediated by ANS. Additional mechanistic and clinical investigations are required to corroborate our findings.

Plain Language Summary: Autonomic nervous system (ANS) disorders are closely related to osteoporosis. But there is no evidence to approve its association. This manuscript is the first to assess the association between ANS and bone mineral density (BMD). Furthermore, it may be the first to explore causal links among gut-nerve-bone axis.

Keywords: gut microbiota, autonomic nervous system, bone mineral density, heart rate variability, Mendelian randomization

Introduction

The autonomic nervous system (ANS) exhibits widespread distribution throughout the human body and controls the functions of nearly all organ systems.[1] It is essential in governing a multitude of physiological functions, encompassing the regulation of heart rate, blood pressure, and the rate of respiration.[1] An increasing body of evidence suggests an association between the ANS and osteoporosis, particularly in the context of bone remodeling processes.[2,3] Recently, considerable research interest has been generated by the concept of the nerve-bone axis. Mounting evidence suggests that sympathetic signals contribute to the disruption of bone homeostasis primarily by influencing mesenchymal stem cells

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(MSCs) and their progeny, while also impacting hematopoietic stem cell-derived osteoclasts.[4] A recent review has encapsulated the growing body of evidence indicating the existence of the axis, which is implicated in a range of pertinent pathophysiological conditions, including osteoarthritis, osteoporosis, and bone-related tumors.[5]

The gut microbiota is a complicated and vast microbial community existing in the gastrointestinal tract of humans. Researches have demonstrated that the gut microbiota affects bone metabolism and regulates bone density.[6] The gut microbiota is able to influence bone metabolism by regulating metabolic products such as short-chain fatty acids, 5-HT, and bile acids.[7] It can also affect material absorption, and consequently bone formation, by lowering the pH value and enhancing mineral solubility.[8] Additionally, it can contribute to the adjustment of bone metabolic processes and overall health by regulating the endocrine system.[7] Increasing evidence suggests the existence of a gut-bone axis.[6,9]

The intricate bidirectional relationship network known as the gut-brain axis weaves together neural, hormonal, and immune signaling pathways, bridging the digestive tract with nerve. Meanwhile, the gut microbiota possesses the ability to interact with the immune cells that coat the intestinal barrier, provoking relative responses.[7] These interactions give rise to various metabolites that circulate in the bloodstream, capable of penetrating the blood-brain barrier. Once in the nervous system, these metabolites can directly affect neural processes. As a result, this interplay indirectly influences bone metabolism through a complex pathway known as the brain-bone axis. Given this, we speculate that the nerve-bone axis and the gut-bone axis may be interconnected by the gut-nerve-bone axis. Understanding the cause-and-effect relationships within the gut-nerve-bone axis requires a detailed investigation into ANS to ascertain the correlation of the gut microbiota with bone health.

Traditional observational research into risk factors for bone mineral density (BMD) is prone to confounding variables and reverse causation.[10] However, Mendelian randomization (MR) employs genetic variants as instrumental variables (IVs) in its analytical approach, provides a robust method for establishing causal relationships.[11] This investigation uses a two-sample MR approach to clarify the causal connections between gut microbiota, ANS, and BMD. Heart rate variability (HRV) denotes the fluctuation in the duration between consecutive heartbeats, serving widely as a delicate, non-intrusive indicator of ANS function across the sympathetic (SNS) and parasympathetic (PNS) divisions.[12] Unlike direct nerve recordings or catecholamine assays (invasive/labile), HRV allows scalable measurement in large cohorts—essential for genetic instrumental variable analyses. Subsequently, we used HRV to represent ANS activity in the MR analysis, an approach that has been adopted by several studies.[12–14] Our study offers a comprehensive view of the interactions between gut microbiota, ANS, and BMD. The findings of this research not only contribute to our understanding of these complex relationships but also have significant implications for clinical interventions and public health approaches aimed at alleviating the impact of osteoporosis.

Methods

Study Design

Figure 1 depicts the research design. The bidirectional association of gut microbiota with BMD was evaluated by a two-sample MR method. Additionally, we performed a mediation analysis utilizing a two-step MR framework to investigate whether ANS might act as a mediator in the pathway from gut microbiota to BMD. This study adheres to the Strengthening the Reporting of Observational Studies in Epidemiology Using Mendelian Randomization (STROBE-MR) guidelines.[15]

Data Source

Genetic data associated with HRV were gathered through a two-phase meta-analysis of genome-wide association study (GWAS), encompassing a total of 53,174 individuals of European descent.[16] HRV parameters were assessed during resting conditions through electrocardiograms that spanned a range of time intervals, from several seconds up to 90 minutes, or extending between 2 and 12 hours over the course of a day.[16] We selected the standard deviation of the normal-to-normal inter-beat intervals (SDNN), the root mean square of the successive differences of inter-beat intervals (RMSSD), and peak-valley respiratory sinus arrhythmia or high-frequency power (pvRSA/HF) as IVs for HRV.[17] SDNN represents global ANS influence (combined sympathetic-parasympathetic activity).[18] RMSSD captures

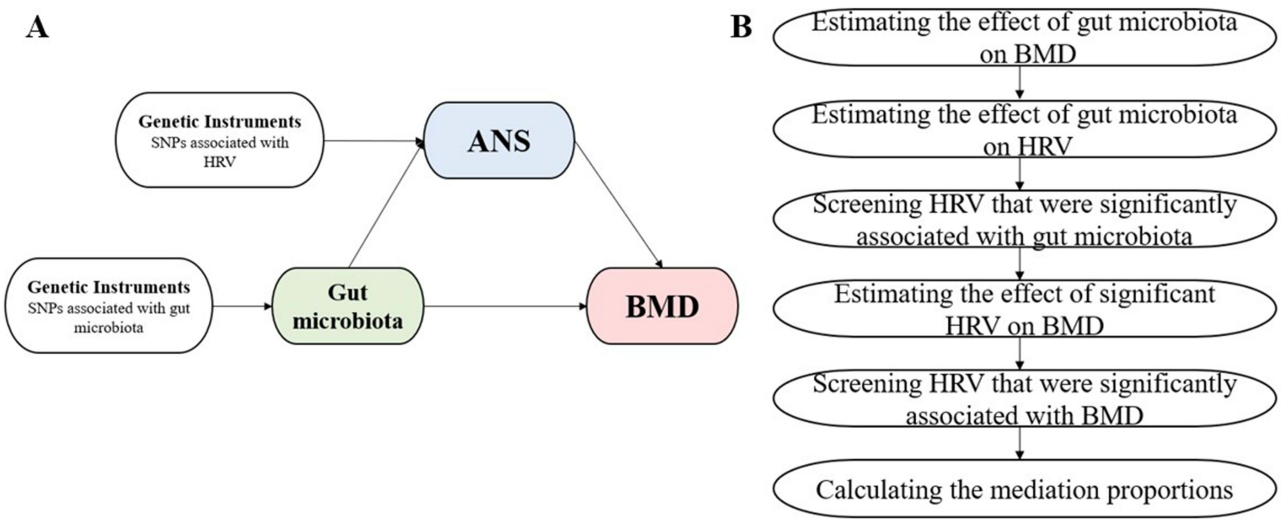

Figure 1 Overview of the study design. (**A**) The two-step MR framework. (**B**) The flowchart for implementing the two-step MR approach.
Abbreviations: BMD, bone mineral density; HRV, heart rate variability.

high-frequency parasympathetic modulation and strongly correlates with HF power ($r > 0.90$).[19] pvRSA/HF (0.15–0.40 Hz) was prioritized as a direct measure of parasympathetic (vagal) activity validated through pharmacological blockade studies.[18,20] These parameters exhibit robust genome-wide associations (>100 significant loci) in large-scale GWAS,[21,22] unlike controversial metrics (eg, LF/HF ratio) with disputed physiological interpretations.[23] All three are endorsed as primary HRV metrics by international guidelines with established measurement protocols across cohorts.[18] Finally, we confined our analyses to genetic variants associated with each biomarker that achieved genome-wide significance ($P<5\times10^{-8}$) and were mutually independent (linkage disequilibrium [LD] $r^2<0.001$ within 10,000 kb).

Using data from the UK Biobank Resource, investigators conducted an analysis of heel BMD data from a comprehensive cohort of 583,314 European individuals, examining approximately 12 million single nucleotide polymorphisms (SNPs) in the process.[24] The information was sourced from IEU OpenGWAS repository (accessible at https://gwas.mrcieu.ac.uk), with the GWAS identifier for "BMD" being "ebi-a-GCST90029004".

Summary data regarding gut microbiota were gathered through the genome-wide association meta-analysis carried out by the MiBioGen consortium (https://mibiogen.gcc.rug.nl/), marking it as one of the most comprehensive investigations to date into the genetic variability of the human gut microbiome.[25] This exhaustive research included sequencing information from 16S ribosomal RNA genes, encompassing a total of 211 distinct categories of gut bacteria.

Statistical Analysis

MR Analysis to Estimate the Effects of Gut Microbiota on BMD

We utilized two-sample MR analyses to probe the associations between gut microbiota and BMD. The primary method chosen for causal inference was inverse-variance weighted (IVW) regression.[26] To complement and enhance the reliability of the results, additional methods such as MR-Egger, weighted median, weighted mode, and simple mode were also used. When only one genetic instrument was available, the Wald ratio method was employed for MR analysis.

Mediation MR Analysis Linking Gut Microbiota with BMD via HRV

We conducted a mediation analysis with a two-step MR approach to investigate whether HRV might play a mediating role in the pathway between specific gut microbiota and BMD. Initially, we used a two-sample MR approach to quantify the impact of gut microbiota on HRV. Next, we applied the two-sample MR method to assess the influence of HRV that demonstrated statistically significant correlations with specific gut microbiota on BMD. The overall effect identified in the MR analysis can be broken down into two distinct components: an indirect effect mediated through the identified

HRV and a direct effect that operates independently of HRV (Figure 1A).[27] By dividing the indirect effect by the total effect, we calculated the proportion of the total effect that is attributable to the mediating variables.

Sensitivity Analysis

To assess heterogeneity among IVs, we conducted Cochran's Q test, which offered insights into the potential variation in causal effects across different SNPs. We also performed the MR-Egger intercept test to examine horizontal pleiotropy, thereby enhancing the robustness of our causal effect estimates. MR-PRESSO adjusted for horizontal pleiotropy through the elimination of outliers. Furthermore, we employed a leave-one-out approach to examine the influence of specific SNPs on the comprehensive inferences, pinpointing variants that might exert a disproportionate impact on the observed associations. These sensitivity analyses were crucial in refining our understanding of the connections among gut microbiota, HRV, and BMD, ensuring a more dependable conclusion. All analyses were performed using the R (version 4.4.2) with the packages "TwoSampleMR", "MendelianRandomization", and "MRPRESSO".

Results

Effect of Gut Microbiota on BMD

The IVW method findings indicate that several genus level groups, including *Coprococcus* 2 (β=0.03, 95% confidence interval, CI: 0.00 to 0.05, *P*=0.02), *Oscillibacter* (β=0.02, 95% CI: 0.00 to 0.03, *P*=0.01), *Lachnospiraceae* NC2004 (β=0.01, 95% CI: 0.00 to 0.03, *P*<0.05), and two unnamed genera (IDs 1000006162 and 2755) (β=0.02, 95% CI: 0.01 to 0.03, *P*<0.01; β=0.01, 95% CI: 0.00 to 0.03, *P*<0.05, respectively), as well as an unnamed family (ID 1000006161) (β=0.02, 95% CI: 0.01 to 0.03, *P*<0.01), and the order level groups *NB1n* (β=0.02, 95% CI: 0.01 to 0.03, *P*<0.01) and *Rhodospirillales* (β=0.02, 95% CI: 0.00 to 0.04, *P*=0.02), all show positive correlations with BMD (Figure 2).

The genera *Ruminococcaceae* NK4A214 (β=−0.03, 95% CI: −0.05 to −0.02, *P*<0.01), *Romboutsia* (β=−0.02, 95% CI: −0.04 to −0.01, *P*=0.02), and *Catenibacterium* (β=−0.02, 95% CI: −0.04 to −0.01, *P*=0.03), along with the families XI (β=−0.01, 95% CI: −0.02 to 0.00, *P*=0.02) and *Acidaminococcaceae* (β=−0.03, 95% CI: −0.05 to −0.01, *P*=0.01) exhibited negative correlations with BMD (Figure 2). The findings were corroborated through a sensitivity analysis conducted with the MR-PRESSO method. No horizontal pleiotropy was identified across instruments in terms of the gut microbiota's impact on BMD (*P*>0.05). Utilizing the MR-PRESSO global test and the MR-Egger as references, we ruled

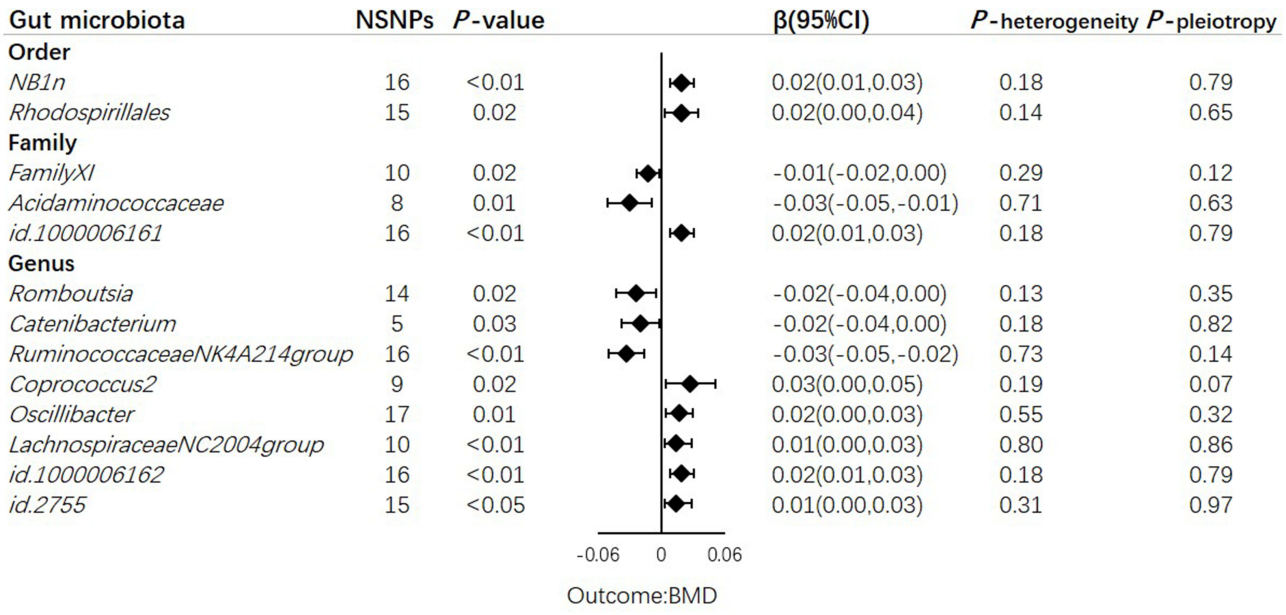

Figure 2 The effects of gut microbiota on BMD by two-sample Mendelian randomization.
Abbreviations: NSNP, Number of SNP; CI, confidence interval; BMD, bone mineral density.

out the possibility of significant heterogeneity in the relationships. Moreover, no SNPs were found to significantly affect the estimates in the leave-one-out analysis (Supplementary Figure 1).

Mediation Effects of HRV on the Gut-Bone Axis

We estimated the effect of gut microbiota on HRV and the effect of HRV on BMD. Only the genera *Coprococcus* 2 and *Lachnospiraceae* NC2004 were significantly associated with both HRV and BMD. We noticed an increase in pvRSA/HF due to *Coprococcus* 2 (β=0.15, 95% CI: 0.01 to 0.29, *P*=0.03) and an increase in RMSSD due to *Lachnospiraceae* NC2004 (β=0.05, 95% CI: 0.00 to 0.09, *P*<0.05) in the IVW model (Table 1). The findings received validation through the application of the MR-PRESSO.

We further estimated whether HRV was significantly associated with the effect of gut microbiota on BMD. In the IVW model, we observed that pvRSA/HF had a positive association with BMD (β=0.06, 95% CI: 0.03 to 0.08, *P*<0.01). RMSSD had a positive association with BMD (β=0.08, 95% CI: 0.04 to 0.12, *P*<0.01) (Table 1). The genetic variants for pvRSA/HF and RMSSD were all strong (F statistics >10) (Supplementary Tables 1 and 2). In sensitivity analysis, although the heterogeneity test yields a statistically significant result for RMSSD (Q=30.71, *P*=0.01), the use of random effects models in MR analyses can mitigate the heterogeneity. Furthermore, no evidence of horizontal pleiotropy was identified (*P*>0.05).

We performed mediation analyses to identify whether the impact of gut microbiota on BMD was channeled through HRV. Analyses of *Coprococcus* 2 with BMD showed no significant mediation effects acting through pvRSA/HF (*P*=0.39). An examination of *Lachnospiraceae* NC2004 relative to BMD revealed a substantial mediating impact via RMSSD with a mediated proportion of 40.0% (95% CI, 21.1−58.9%, *P*=0.03), indicating a dependent association between gut microbiota and BMD (Figure 3).

Table 1 The Effect of Gut Microbiota on HRV and the Effect of HRV on BMD by Two-Sample Mendelian Randomization

Exposure	Outcome	NSNPs	Method	β(95% CI)	*P*-value
Coprococcus2	pvRSA/HF	6	MR Egger	0.81(−0.58,2.20)	0.32
		6	Weighted median	0.20(0.03,0.38)	0.02
		6	Inverse variance weighted	0.15(0.01,0.29)	0.03
		6	Simple mode	0.21(−0.04,0.46)	0.17
		6	Weighted mode	0.21(−0.04,0.46)	0.17
LachnospiraceaeNC2004group	RMSSD	8	MR Egger	0.03(−0.23,0.29)	0.83
		8	Weighted median	0.05(−0.01,0.11)	0.10
		8	Inverse variance weighted	0.05(0.00,0.09)	<0.05
		8	Simple mode	0.08(−0.03,0.18)	0.18
		8	Weighted mode	0.07(−0.02, 0.17)	0.18
pvRSA/HF	BMD	7	MR Egger	0.07(−0.02,0.16)	0.18
		7	Weighted median	0.05(0.01,0.08)	0.01
		7	Inverse variance weighted	0.06(0.03,0.08)	<0.01
		7	Simple mode	0.01(−0.05,0.08)	0.69
		7	Weighted mode	0.06(0.02,0.10)	0.03
RMSSD	BMD	15	MR Egger	0.12(0.03,0.22)	0.02
		15	Weighted median	0.12(0.08,0.16)	<0.01
		15	Inverse variance weighted	0.08(0.04,0.12)	<0.01
		15	Simple mode	0.12(0.05,0.18)	<0.01
		15	Weighted mode	0.12(0.08,0.16)	<0.01

Abbreviations: NSNP, Number of SNP; CI, confidence interval; pvRSA/H, peak-valley respiratory sinus arrhythmia or high-frequency power; RMSSD, the root mean square of the successive differences of inter-beat intervals; BMD, bone mineral density; MR, Mendelian randomization; HRV, heart rate variability.

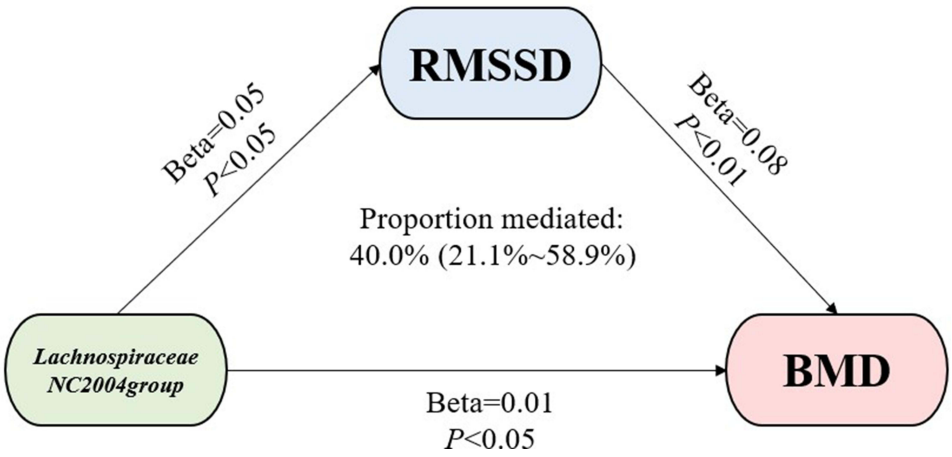

Figure 3 Mediation effect of gut microbiota on BMD via HRV.
Abbreviations: RMSSD, the root mean square of the successive differences of inter-beat intervals; BMD, bone mineral density; HRV, heart rate variability.

Discussion

As far as we are aware, this research constitutes one of the pioneering efforts to methodically assess the associations between the ANS and BMD from a genetic standpoint. Furthermore, it may be the first study to explore the links among gut microbiota, ANS, and BMD. Genetically predicted pvRSA/HF and RMSSD were both associated with gut microbiota and BMD. The effect of *Lachnospiraceae* NC2004 on BMD occurs through RMSSD with a mediated proportion of 40.0% (95% CI: 21.1 to 58.9%, *P*=0.03). In this work, we introduce and provide evidence for the existence of the gut-nerve-bone axis. The notion of the gut-nerve-bone axis posits that the gut microbiota impacts bone metabolism by adjusting ANS. In this comprehensive MR study, involving 583,314 individuals with detailed BMD data, we observed a significant positive association between genetic determinants of gut microbiota and BMD levels. The robustness of our findings is further supported by consistent results across multiple sensitivity analyses. Moreover, our mediation analysis suggested that the relationship between *Lachnospiraceae* NC2004 and BMD was mediated by ANS. This innovative mediational MR study not only provides strong evidence for the existence of the nerve-bone and gut-nerve-bone axes but also sheds light on the connections among gut microbiota, ANS, and bone.

Multiple studies have demonstrated that gut microbiota could regulate BMD, thereby affecting osteoporosis. Similarly to our results, Yuan et al[28] found that the *Acidaminococcaceae* and XI families were negatively correlated with BMD. Zeng et al[29] discovered that unknown family ID.1000006161, unknown genus ID.1000006162, and Order *NB1n* were linked to a lower risk of osteoporosis. In an analysis by Hefang et al[6] the genus *Coprococcus* 2 indicated a negative correlation (odds ratio, OR =0.50, 95% CI: 0.27 to 0.92, *P*=0.03), highlighting the intricate association between the diversity of gut microbiota and the likelihood of developing postmenopausal osteoporosis. Several possible mechanisms have been proposed for the gut microbiota's impact on BMD. The gut microbiota enhances inorganic salt solubility for improved absorption, crucial for bone mineralization.[2] It bolsters the mineral absorption of the gut surface by promoting enterocyte and colonocyte proliferation, supporting gut microbiota homeostasis, and increasing levels of short-chain fatty acids (SCFAs) for bone homeostasis.[30] A healthy gut epithelium barrier prevents inflammation and bone degradation.[31] SCFAs suppress osteoclastogenesis and the resorption of bone, thereby enhancing bone density. Probiotics and SCFAs reduce oxidative stress, preventing excessive bone remodeling.[30] The gut microbiota, acts as an endocrine organ, modulating growth factors and hormones, including IGF-1 and cortisol, and influencing bone cell differentiation and gut serotonin production, which may regulate bone mass.[32]

In our study, gut microbiota was associated with the activity of ANS. For instance, the metabolic product of *Lachnospiraceae* butyrate could activate intestinal vagal nerves directly and independently of cholecystokinin.[33,34] Metabolic products produced by gut microbiota, such as SCFAs, could affect the activity of ANS. These metabolic products may act on the nervous system through the bloodstream, thereby influencing neural signals that regulate bone metabolism. The lipid content within the intestines activates vagal afferent pathways, potentially resulting in

alterations to the expression of certain brain regions and an upsurge in parasympathetic activity emanating from diverse areas of the brain, thereby altering the autonomic influence on the body.[35] Alterations in gut permeability can lead to increased concentrations of SCFAs in the bloodstream, resulting in enhanced activation of the sympathetic nervous system by SCFAs.[36] Gut microbiota could also affect the secretion of intestinal hormones, which, like ghrelin, may influence ANS. In a human study, ghrelin inhibits SNS activity with a moderate effect on PNS activity.[37]

A significant observation was that genetically predicted gut microbiota could enhance BMD by influencing the activity of ANS. This study could be the first to assess the associations between ANS and BMD using MR analysis. Another study analyzed ANS activity through HRV and found a significant relationship between BMD and ANS activity.[2] The results indicated that the BMD of the group with higher overall ANS activity was significantly higher than that of the group with lower ANS activity.[2] ANS exerts its influence on bone metabolism through various pathways, thereby regulating bone density. Specific neurotransmitters, such as neuropeptide Y, are regulated by ANS. NPY secretion in osteocytes promotes fat production and inhibits bone formation, thereby affecting bone density.[38] Additionally, ANS can influence bone density by modulating the differentiation and function of bone marrow MSCs through nerve innervation and neurotransmitter release.[39]

The use of probiotics in the modulation of osteoporosis has gained significant traction in clinical settings. Studies on clinical trials have indicated that probiotic supplementation bolsters bone metabolism and augments the presence of bacterial species that produce SCFAs, thereby mitigating bone loss. For example, supplementation with probiotics has shown reduced bone loss in postmenopausal women.[40] Targeting gut microbiota presents a promising alternative approach for the treatment of patients with osteoporosis.

This study has some limitations. The contextual specificity of our findings suggests the need for validation across diverse real-world settings. Although we have strived to pinpoint potential gut microbiota, our focus was confined to particular gut microbiota. It is plausible that other significant factors and pathways affecting the nerve-bone relationship have not been explored. The omission of certain mediators may result in an incomplete elucidation of the mechanisms underlying the association. Furthermore, this study did not account for epigenetic factors, which might modulate the interactions and represent important avenues for future research directions. Since all datasets were derived from European populations, the generalizability of the findings to other ancestries may be limited. Although the mediation analysis involving *Coprococcus* 2 did not achieve statistical significance, future research could delve into potential explanations, such as insufficient statistical power to detect a smaller effect size, or the possibility of alternative biological pathways being involved.

Conclusion

In summary, the present research supports a genetic correlation among gut microbiota, ANS, and BMD, which reinforces the concept of a gut-nerve-bone axis. Notably, the interplay of gut microbiota with BMD is potentially mediated by ANS. Targeting gut microbiota such as *Lachnospiraceae* NC2004 presents a promising alternative approach for the treatment of osteoporosis. Future studies could explore the specific molecular pathways and signaling mechanisms underlying the gut-nerve-bone axis, conduct longitudinal animal studies to observe the long-term effects of gut microbiota interventions on bone health, and perform clinical trials to evaluate the efficacy and safety of modulating gut microbiota in osteoporosis treatment.

Ethics Approval and Consent to Participate

This study utilizes aggregated data rather than individual-level data. The data involved all originate from publicly published GWAS summary databases, which complies with the conditions for exemption from review as stated in the "Ethical Review Measures for Life Sciences and Medical Research Involving Humans".

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Disclosure

The authors declare that they have no conflicts of interest for this work.

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