Bone 143 (2021) 115652

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

Assessing causal relationship from gut microbiota to heel bone mineral density

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ARTICLE INFO

Keywords:
Mendelian randomization
Gut microbiota
Bone mineral density
Osteoporosis
Causal relationship

ABSTRACT

Recent studies have demonstrated the important role played by gut microbiota in regulating bone development, but the evidence of such causal relationship is still sparse in human population. The aim of this study is to assess the causal relationship from gut microbiota to bone development and to identify specific causal bacteria taxa via a Mendelian randomization (MR) approach. A genome-wide association study (GWAS) summary statistic based two-sample MR analysis was performed. Summary statistics of microbiome GWAS (MGWAS) in 1126 twin pairs of the TwinsUK study was used as discovery sample, and the MGWAS in 984 Dutch participants from the LifeLines-DEEP cohort was used as replication sample. Estimated heel bone mineral density (eBMD) GWAS in 426,824 participants from the UK biobank (UKB) cohort was used as outcome. Bacteria were grouped into taxa features at both order and family levels. In the discovery sample, a total of 25 bacteria features including 9 orders and 16 families were analyzed. Fourteen features (5 orders + 9 families) were nominally significant, including 5 orders (*Bacteroidales*, *Clostridiales*, *Lactobacillales*, *Pasteurellales* and *Verrucomicrobiales*) and 9 families (*Bacteroidaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Mogibacteriaceae*, *Pasteurellaceae*, *Porphyromonadaceae*, *Streptococcaceae*, *Verrucomicrobiaceae* and *Veillonellaceae*). One order *Clostridiales* and its child taxon, family *Lachnospiraceae*, were successfully replicated in the replication sample (*Clostridiales* $P_{discovery} = 3.32 \times 10^{-3}$ $P_{replication} = 7.29 \times 10^{-3}$; *Lachnospiraceae* $P_{discovery} = 0.03$ $P_{replication} = 7.29 \times 10^{-3}$). Our findings provided evidence of causal relationship from microbiota to bone development, as well as identified specific bacteria taxa that regulated bone mass variation, thus providing new insights into the microbiota mediated bone development mechanism.

1. Introduction

Osteoporosis is a systematic skeletal metabolic disease among the elderly, triggering in enhanced bone fragility and osteoporotic fracture. It is characterized by reduced bone mineral density (BMD) and degenerated microstructure of bone tissue [1]. In 2010, over 10 million (overall prevalence of 10.3%) older adults had osteoporosis in the United States. Based on the overall 43.9% low bone mass prevalence, 43.4 million older adults had low bone mass [2]. Besides, fractures and osteoporosis are projected to incur over $25 billion in costs by 2025 [3].

BMD is determined by genetics, environment and their interplay [4–6]. In the recent years, it has been increasingly recognized that gut microbiota play an important role in shaping complex traits and diseases including BMD [7–9]. In a recent mice model study, mice raised in a sterile environment exhibit reduced number of osteoclasts and

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https://doi.org/10.1016/j.bone.2020.115652
Received 27 May 2020; Received in revised form 4 September 2020; Accepted 16 September 2020
Available online 21 September 2020

increased bone content compared with conventionally raised mice, whereas the bone mass returned to normal after colonization with normal gut microbiota [10]. In a population study, the diversity of the gut microbiota distribution negatively associates with risk of fracture and frailty at various age groups [11]. Numerous pathogenic factors leading to osteoporosis and fracture, such as deficiency of various vitamins and micronutrients and sex steroid metabolism, are related to the disturbance of gut microbiota distribution [12,13]. Nonetheless, the evidence towards the causal relationship from microbiota to bone metabolism is still sparse, especially in human populations.

Mendelian randomization (MR), in contrast to traditional randomized controlled trial (RCT), is an efficient approach to investigate causal relationship from an exposure to an outcome in a cross-sectional study [14,15]. MR analysis relies on three basic assumptions: i) instrumental variable (IV) is strongly associated with exposure; ii) IV is independent of any observed and un-observed confounders of exposure-outcome associaton; and iii) IV is associated with outcome only through exposure. The reason that genetic variants are selected as IV is that they are allocated at random at birth according to the Mendel's second law and are fixed throughout the life, therefore are independent of confounders (e.g. socioeconomic factors) by conception.

In a recent study, Sanna et al. performed a MR study in 952 normoglycemic individuals for whom genome-wide genotyping, gut metagenomic sequence and fecal short-chain fatty acid (SCFA) levels were available, and identified one type of SCFA propionate that was causally associated with an increased risk of type 2 diabetes, demonstrating the efficacy of microbiota oriented causal inference by MR analysis [16].

Conventional MR analysis relies on individual-level information at both exposure and outcome sides. Restricted by limited experiment expense, individual-level data are usually small in sample size, resulting in limited statistical power. As an alternative, two-sample MR based on summary statistics has advantages over traditional MR [17,18]. It is equally effective to individual-level MR analysis without the additional recruitment of samples and experimental designs [19,20]. Moreover, thanks to the increasingly expanded number of genome-wide association studies (GWAS) for both microbiota and complex traits, large-scale GWAS summary statistics are becoming readily available [21–26], making it possible to implement summary statistics based MR analysis with largely enhanced statistical power over conventional individual-level based MR analysis.

In the present study, aiming to investigate the causal relationship from microbiota to estimated heel BMD (eBMD) and to identify specific causal bacteria taxa, we conduct a GWAS summary statistic based two-sample MR study. Specifically, summary statistics from 2 microbiota GWAS serve as exposure (discovery + replication) while summary statistics from the UK Biobank (UKB) eBMD GWAS serve as outcome.

2. Materials and methods

2.1. Data sources

We conducted a GWAS summary statistics based MR study. The data were composed of microbiome and eBMD GWAS summary statistics that were released to the public by previous studies that were approved by respective institutional review boards (IRBs). No new IRB approval was required. A flowchart briefly describes the whole procedure in Fig. 1a.

The discovery gut microbiota study was the TwinsUK study [23], a cohort of adult volunteer twins from the TwinsUK Registry in British. The data used in this study came from 1126 twin pairs, as described elsewhere [23]. In brief, 3261 fecal samples were collected from all participants. Microbiome 16S rRNA was sequenced by the Illumina Miseq 2 × 250 bp platform, followed by operational taxonomic units (OTUs) picking. Host genome was genotyped by Illumina HumanHap610 Quad Chip and was imputed into the 1000 Genomes project (phase 3) reference panel. Genetic associations were examined between 945 bacteria taxa and 1.3 million imputed host SNPs. A total of 307 host SNPs were associated with 61 bacteria taxa (1 kingdom + 6 phyla + 9 classes + 9 orders + 16 families + 16 genera + 4 species) at a FDR < 0.2. The P values at these SNPs ranged from 4.94×10^{-9} to 7.33×10^{-5}. The summary statistics of these significant SNPs were assessed through the supplemental table of the study publication [23] (Supplementary Table 1).

The replication gut microbiota study was the LifeLines-DEEP study [24], a general prospective population cohort in the northern Netherlands. In brief, fecal samples from 984 participants aged 18 to 86 years were collected. Host genome was genotyped by the HumanCytoSNP-12 BeadChip and the Immunochip, was imputed into the Genome of the Netherlands (GoNL) reference panel. Metagenomic sequencing was performed by the Illumina HiSeq sequencing platform. A total of 83 SNPs from 9 host genomic loci were associated with 29 microbial taxonomies (2 kingdom + 4 phyla + 5 classes + 6 orders + 7 families + 5 genera + 4 species) at the significance level $\alpha = 5 \times 10^{-5}$. The summary statistics of these significant SNPs were assessed through the supplemental table of the study publication [24] (Supplementary Table 2).

As outcome, the GWAS summary statistics of eBMD in 426,824 UKB cohort participants were used, which is the largest GWAS study of BMD to date [5]. In brief, the UKB cohort is a prospective and population-based cohort of ~500,000 participants from across the United Kingdom, aged between 40 and 69 at recruitment. Heel BMD was estimated based on quantitative ultrasound speed of sound (SOS) and broadband ultrasound attenuation (BUA). Genome-wide genotypes were available for all participants at 784,256 genotyped autosome markers, and were imputed into UK10K haplotype, 1000 Genomes project phase 3 and Haplotype Reference Consortium (HRC) reference panels. GWAS was performed in 426,824 qualified participants. The GWAS summary statistics were publicly available at the GEFOS website (http://www.gefos.org).

2.2. Instrumental variable selection

Both discovery and replication samples adopted same criteria for IV selection. Specifically, bacteria taxa were analyzed at both order and family levels. A feature was defined as a distinct order or family. All SNPs associated with each feature were selected as IVs for that feature. For SNP with multiple signals within one feature, the strongest signal was selected for that feature. In accordance with Sanna et al. [16], SNP association threshold was set to be 1.0×10^{-5}. To account for the linkage disequilibrium (LD) pattern, SNPs within each feature were clumped with PLINK (v1.9) to retain independent SNPs only. The LD threshold was set to be $r^2 < 0.1$ and the clumping window was set to be 500 kb. LD was estimated based on the 1000 genomes project sequencing data (phase 3).

To examine horizontal pleiotropy effects, the MR-PRESSO Global test and Outlier test [27] were applied. The MR-PRESSO Outlier test calculates for each SNP a P-value for its pleiotropy significance, and the MR-PRESSO Global test calculates a P-value for overall horizontal pleiotropy. SNPs were sorted in an ascending order in terms of their MR-PRESSO Outlier test P-values and were then removed one-by-one. Each time when a SNP was removed from the list, the MR-PRESSO Global test was performed on the remaining SNPs. The recursion repeated until the Global test P-value was not significant ($P > 0.05$). The list of remaining SNPs after removal of pleiotropic ones was used for subsequent MR analysis.

2.3. Effect size estimate

The eBMD GWAS summary statistics were derived from a standardized phenotype (i.e., mean 0 and variance 1), therefore we could estimate the proportion of phenotypic variance explained by SNP from summary statistics with the formula $2f(1 - f)\beta^2$, where f is effect allele

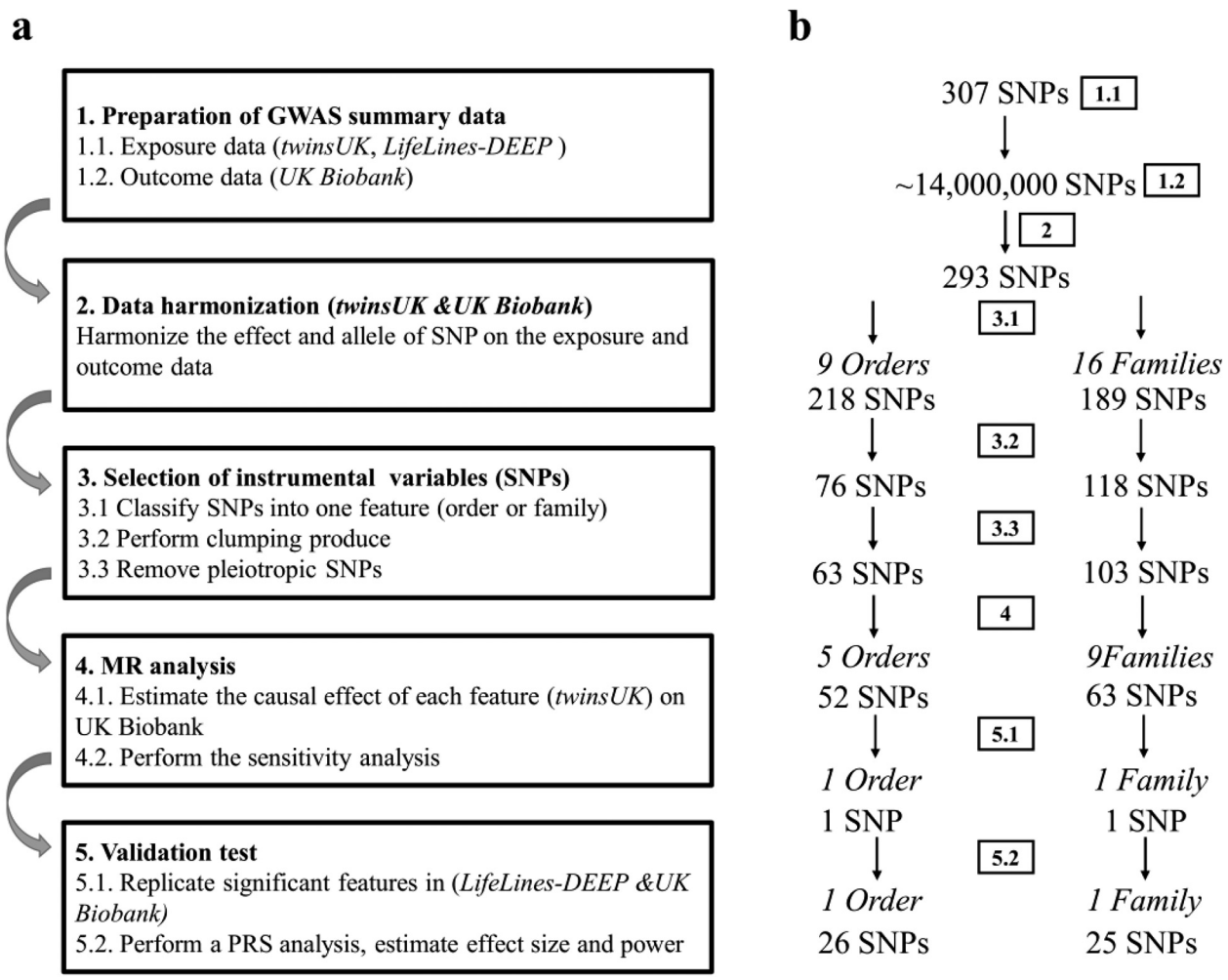

Fig. 1. Diagrammatic description of MR analysis in the discovery and replication.
(a) The whole workflow of MR analysis. (b) The main results and the change in the number of SNPs were displayed.

frequency and β is regression coefficient for eBMD. For microbiome data, we were not able to derive the proportion of phenotypic variance explained by SNP because the phenotype being analyzed may not be standardized.

2.4. Power assessment

The statistical power to assess causal effect was calculated by an online tool mRnd (http://cnsgenomics.com/shiny/mRnd) [28]. The power was derived using the non-centrality parameter of a chi-squared distribution to calculate statistical power of a continuous outcome in two-sample MR approach. Several major parameters determining power calculation include sample size (N = 2252 in the present study), the strength of association of instrumental variable with exposure (R^2) and the strength of association of exposure with outcome (β_2). We evaluated the effect of R^2 and β_2 on power with the following two settings: i) sample size = 2252, β_2 = 0.2, R^2 varied; and ii) sample size = 2252, R^2 = 0.01, β_2 varied.

2.5. MR analysis

We performed MR analysis to examine the causal effect from microbiome feature to the BMD outcome. Specifically, we tested the association of the identified IVs within each microbiome feature with BMD. Four popular MR methods, including the inverse-variance weighted (IVW) test [29], the MR-Egger regression [30], the weighted median estimator [31] and the MR-PRESSO [27], were used for the MR analysis. Each statistical method has its own model assumption, and any violation of the assumption may make the method inferior or even totally invalid. Specific to the 4 studied methods: 1) the IVW method assumes no horizontal pleiotropy [29]; 2) the MR-Egger assumes the presence of pleiotropy in > 50% SNPs [30]; 3) the weighted median method assumes the presence of pleiotropy in < 50% SNPs [31]; and 4) the MR-PRESSO assumes the presence of pleiotropy but will remove pleiotropic SNPs intrinsically [27]. The IVW method is reported to be slightly more powerful than the others under certain conditions [31]. Therefore, the results were mainly based on the IVW method while the other 3 methods served as its complement.

For features containing only one IV for which the multiple-SNP tests were not applicable, the Wald ratio test was used [32]. The potential heterogeneity was examined by the IVW test and the MR-Egger regression. Significant features (including order or family) identified in the discovery TwinsUK study were subjected to be replicated in the replication LifeLines-DEEP study. MR analysis procedure was same as that in the discovery study.

All the above analyses including sensitivity analysis and MR analyses were performed with the R packages *TwoSampleMR* (https://github.com/MRCIEU/TwoSampleMR) [33] and *MRPRESSO* (https://github.com/rondolab/MR-PRESSO) [27].

2.6. Validation in the UKB individual-level data

We validated the identified associations in the UKB individual-level data. Specifically, we constructed a weighted genetic risk score (GRS) for the identified bacterial taxa and then examined the association of GRS with eBMD in the UKB individual-level data. UKB participants who were genetically determined as "Caucasian" and who had both genotype data and eBMD value were included. Participants who had a self-reported gender inconsistent with the genetic gender, whose sex chromosome was aneuploid or who withdraw their consents were excluded. An unrelated sub-sample was then inferred with the software KING [34]. The final sample used for the GRS analysis was the unrelated homogeneous "Caucasian" sub-sample (N = 263,342).

The GRS was constructed by the sum of trait-raising alleles weighted by their regression coefficients, with the following equation

$$GRS = \sum_{i=1}^{l} \beta_i g_i,$$

where β_i is the regression coefficient of SNP-bacteria association and g_i is allele dosage of the ith SNP. l denotes the total number of IVs (26 or 25). The association of the computed GRS with eBMD was examined in a linear regression model in software R (v3.6.1). The covariates included age, sex and the top 10 principal components (PCs).

3. Results

In the discovery TwinsUK sample, there are a total of 260 SNPs associated with gut microbiome at the significance level $P < 1.0 \times 10^{-5}$. After clumping, there are 76 and 118 SNPs left for orders and families, categorized into 9 bacteria orders and 16 families, respectively (Supplemental Table 3). Fig. 1b displays the change in the number of SNPs throughout the whole procedure. The order with the largest number of SNPs is *Clostridiales* (30 SNPs), followed by *Bacteroidales* (30 SNPs). There are 3 orders, *Bifidobacteriales*, *Enterobacteriales* and *Lactobacillales*, each containing only one SNP. At the family level, the family with the largest number of SNPs is *Ruminococcaceae* (32 SNPs), followed by *Bacteroidaceae* (30 SNPs) and *Lachnospiraceae* (28 SNPs). There are 4 families each containing only one SNP, *Bifidobacteriaceae*, *Enterobacteriaceae*, *Streptococcaceae* and *Veillonellaceae*. Of note, family is a sub-category of order, therefore the sets of SNPs contained in family and its relevant order may heavily overlap. For example, the family *Ruminococcaceae* belongs to the order *Clostridiales* and the SNPs contained in them are identical.

Sensitivity was evaluated at all the included orders and families containing multiple IVs in the discovery TwinsUK sample. For the orders *Clostridiales* and *Bacteroidales*, a total of 4 out of 30 IVs and 9 out of 30 IVs were detected as outliers using the MR-PRESSO outlier test. Likewise, at the family level, 3 out of 28 IVs in family *Lachnospiraceae*, 9 out of 30 IVs in family *Bacteroidacea*e and 3 out of 32 IVs in family *Ruminococcaceae* were detected as outliers. After removing these pleiotropic SNPs, no evidence of horizontal pleiotropy was observed (both MR-PRESSO Global test $P > 0.05$ and MR-Egger regression $P > 0.05$).

The statistical power is evaluated in Supplemental Fig. 1. Obviously, the power increases with increased R^2 or β_2. When R^2 or β_2 is fixed at 0.01 or 0.2, a considerable power rate 80% is achieved when $\beta_2 > 2.03$ or$R^2 > 0.083$. In reality, neither R^2 nor β_2 could achieve as high as the desired level, resulting in a much inferior power rate. Even with limited statistical power, microbiome MR analysis still has the capacity of establishing causal relationship [16].

3.1. MR analysis

In the discovery sample, after removing SNPs of potentially horizontal pleiotropy effects, the IVW MR analysis identified 4 orders causally associated with eBMD at the nominal significance level ($P < 0.05$): *Bacteroidales* ($P = 0.02$), *Clostridiales* ($P = 3.32 \times 10^{-3}$), *Pasteurellales* ($P = 7.95 \times 10^{-3}$) and *Verrucomicrobiales* ($P = 0.04$). Most of these significant results are validated by the other 3 alternative MR tests (Supplemental Table 4). One additional order *Lactobacillales* containing only one IV was significant by the Wald ratio test ($P = 0.02$).

At the family level, 6 bacteria taxa are causally associated with eBMD at the nominal level using the IVW test, including *Bacteroidaceae* ($P = 0.02$), *Clostridiaceae* ($P = 3.18 \times 10^{-3}$), *Lachnospiraceae* ($P = 0.03$), *Mogibacteriaceae* ($P = 6.94 \times 10^{-4}$), *Pasteurellaceae* ($P = 7.95 \times 10^{-3}$) and *Verrucomicrobiaceae* ($P = 0.04$). Again, most of them are validated by the the 3 alternative MR tests, demonstrating the robustness across tests (Supplemental Table 4). Another 3 families each containing one IV are significant using the Wald ratio test: *Porphyromonadaceae* ($P = 8.04 \times 10^{-3}$), *Streptococcaceae* ($P = 0.02$) and *Veillonellaceae* ($P = 1.28 \times 10^{-4}$).

In total, a total of 14 features (5 orders + 9 families) are causally associated with eBMD in the discovery sample. Because family is a sub-category within order, certain families and orders are heavily redundant. For example, the family *Bacteroidaceae* is within the order *Bacteroidales*. Because no other family within this order is included, both features contain exactly the same set of IVs and consequently result in the exact same P values.

These 14 features are subjected to be replicated in the LifeLines-DEEP replication sample. A total of 57 SNPs are significant at the same 1.0×10^{-5} level in the replication sample, 30 of which map to 2 of the above 14 features, while no SNP maps to the remaining 12 features. These 2 features include order *Clostridiales* and family *Lachnospiraceae* (Table 1). Family *Lachnospiraceae* belongs to order *Clostridiales*, and they map to the same 4 SNPs which are in the high LD with each other. Only one single SNP rs10743315 is left after clumping by PLINK (v1.9), whose P-value is 6.39×10^{-6} and which is located in the locus 12p12.3. Using the Wald ratio test, the MR P-value is 7.29×10^{-3} for both features. The consistent negative effect direction between both discovery and replication samples strengthens the confidence of their

Table 1
Causal estimations of gut microbiome on eBMD in the discovery and replication cohorts.

Stage	MR test	Order			Family		
		Clostridiales			*Lachnospiraceae*		
		No. SNP	b_{xy}	P-value	No. SNP	b_{xy}	P-value
Discovery	IVW	26	−0.0004	**3.32×10^{-3}**	25	−0.0009	**0.03**
	MR-Egger		−0.0004	**0.02**		−0.001	0.09
	Weighted median		−0.0004	**0.01**		−0.001	0.06
	MR-PRESSO		−0.0004	**7.04×10^{-3}**		−0.0009	**0.03**
Replication	Wald ratio test	1	−0.002	**7.29×10^{-3}**	1	−0.002	**7.29×10^{-3}**

Notes: No. SNP is the number of SNPs being used as IVs. bxy is the estimated effect coefficient. Significant P-values were marked in bold. IVW: inverse-variance weigh.

true association. The difference in effect estimate between the discovery and replication samples could be due to different numbers of SNPs or different exposure sample sizes.

In the discovery sample, the 26 and 25 SNPs of the 2 identified features collectively explained 0.007% and 0.005% variance in eBMD, respectively (Supplemental Table 5). All multiple-SNP based MR methods gave consistent effect estimates (*Clostridiales*, all beta = −0.0004; *Lachnospiraceae*, beta = −0.0009 or −0.001) and slightly different *P*-values. While this difference was not unexpected, we noticed that the IVW method gave smallest *P*-values in both cases. In our analysis, we considered horizontal pleiotropy as a severe confounding source and had explicitly excluded pleiotropic SNPs before the MR analysis. Our data fitted the IVW assumption best, which may partially explain why the IVW method gave the smallest *P*-value. Additionally, though the 2 identified features were not significant in the discovery stage after the Bonferroni correction, they were successfully replicated in the replication stage even after conservative multiple testing correction.

Both the IVW test and the MR-Egger regression show no evidence of heterogeneity at the 2 identified features (*Clostridiales* P_{IVW} = 0.14; $P_{MR\text{-}Egger}$ = 0.12; *Lachnospiraceae* P_{IVW} = 0.52; $P_{MR\text{-}Egger}$ = 0.47). Forest plots across various tests are displayed in Fig. 2, and the scatter plots are displayed in Fig. 3.

To check the influence of potential confounding effect, we listed several potential confounders, including diet (coffee intake, processed meat intake, tea intake and variation in diet), behavior (alcohol drinker status, smoking and sleep disorders) and exercise activity (moderate physical activity, vigorous physical activity and walk) categories. We then retrieved the associations of the identified IVs with these confounders in the UKB cohort sample through the GeneATLAS website (http://geneatlas.roslin.ed.ac.uk/phewas). The results, as listed in the Supplementary Table 6, show that none of the associations is significant after correcting for multiple testing, indicating limited influence of confounder.

3.2. Validation in UKB individual-level data

The 2 identified features were validated by the GRS analysis in the UKB individual level data. Both features are significantly associated with eBMD (*Clostridiales* beta = −0.0002, *P* = 0.002; *Lachnospiraceae* beta = −0.0003, *P* = 0.04) (Table 2). Moreover, the effect direction is consistent with that estimated based on summary statistics.

4. Discussion

In this study, we performed a MR analysis to evaluate causal relationship from gut microbiome to bone density. Using summary statistics from 2 microbiome GWAS (MGWAS) and one BMD GWAS, we discovered and replicated one bacteria order *Clostridiales* and one family *Lachnospiraceae* within this order that were causally associated with heel BMD. The negative effect direction implied a reverse regulation pattern.

The gut microbiota is a complex and dynamic group of ecological microbial communities that are colonized in the human gut, even called a "forgotten organ" [35]. Infants have distinctive and unstable microbiota, whose dominant anaerobes involve *Bifidobacterium*, *Bacteroides*, *Clostridia* and *Parabacteroides* [36,37]. The diversity of intestinal bacteria increases with age, while the phylogenetic composition and function tend to be stable during growth. In adults, a healthy and balanced bacterial composition of gut epithelial barrier is mainly maintained by four phyla of *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria* [38]. The bacteria taxa *Clostridiales* and *Lachnospiraceae* identified in this study are members of the *Firmicutes* phylum, which are present mainly in the mammalian and human gut microbiota. All members of *Lachnospiraceae* are strictly anaerobic [39]. In the vitro and animal experiments, *Lachnospiraceae* inhibits bone regeneration and remodeling by reducing intestinal IL-6 levels using the membrane-anchored IL-6 receptor [40,41]. A microbiome analysis indicated that *Firmicutes/Bacteriodetes* ratio negatively correlated with bone mass through exercise altered high fat diet, as did class *Clostridia* and family *Lachnospiraceae* in mice experiments [42]. Though it remains to be explained for the mechanism underlying the regulator path from *Lachnospiraceae* to bone development, previous study showed that small intestine microbiota overgrowth may be an important cofactor [43]. Recently, the gut-bone signaling axis is receiving increasing attention including the regulation of prebiotics, probiotics and production of metabolites [44–46].

Evidence from population research remained inconsistent for the relationship between *Lachnospiraceae* and bone development. Wang et al. found that two genera *Blautia* and *Ruminococcaceae* from family *Lachnospiraceae* had significant decreased abundance in osteoporosis patients than in normal controls [47]. Whereas, Li et al. reported that the abundance of *Lachnospiraceae* was found to be positively correlated with BMD and T-score [48]. Our results revealed a negative causal relationship. There are possible reasons for the contradicted results.

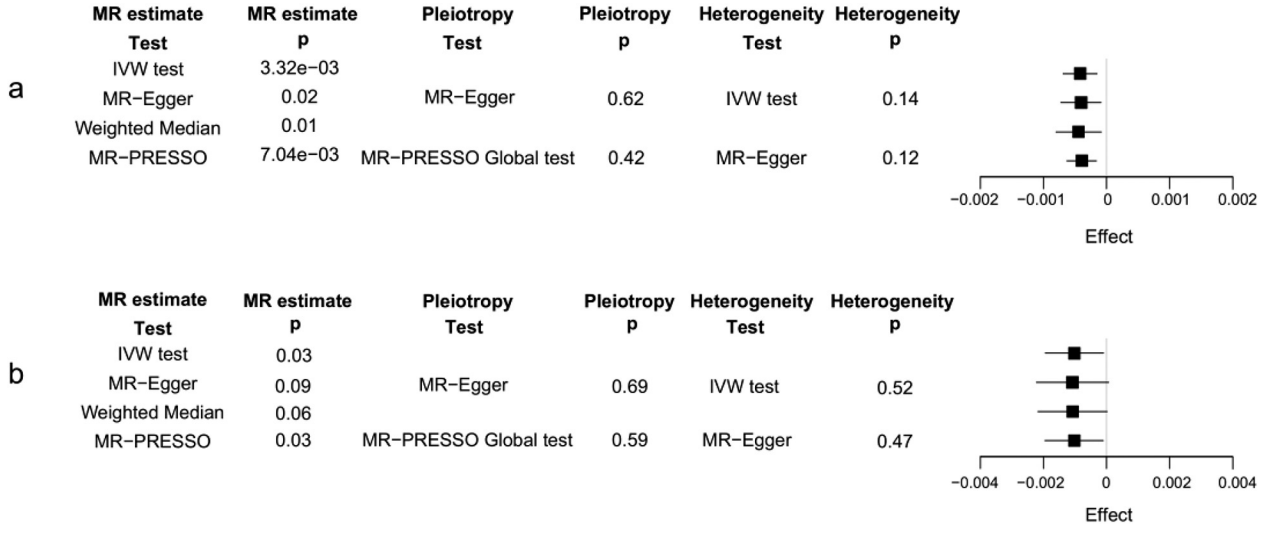

Fig. 2. Forest plots of the two identified features.
Forest plots of the 4 MR tests at the order *Clostridiales* (a) and the family *Lachnospiraceae* (b). Causal effect size, pleiotropy and heterogeneity significance were displayed for each test, if applicable.

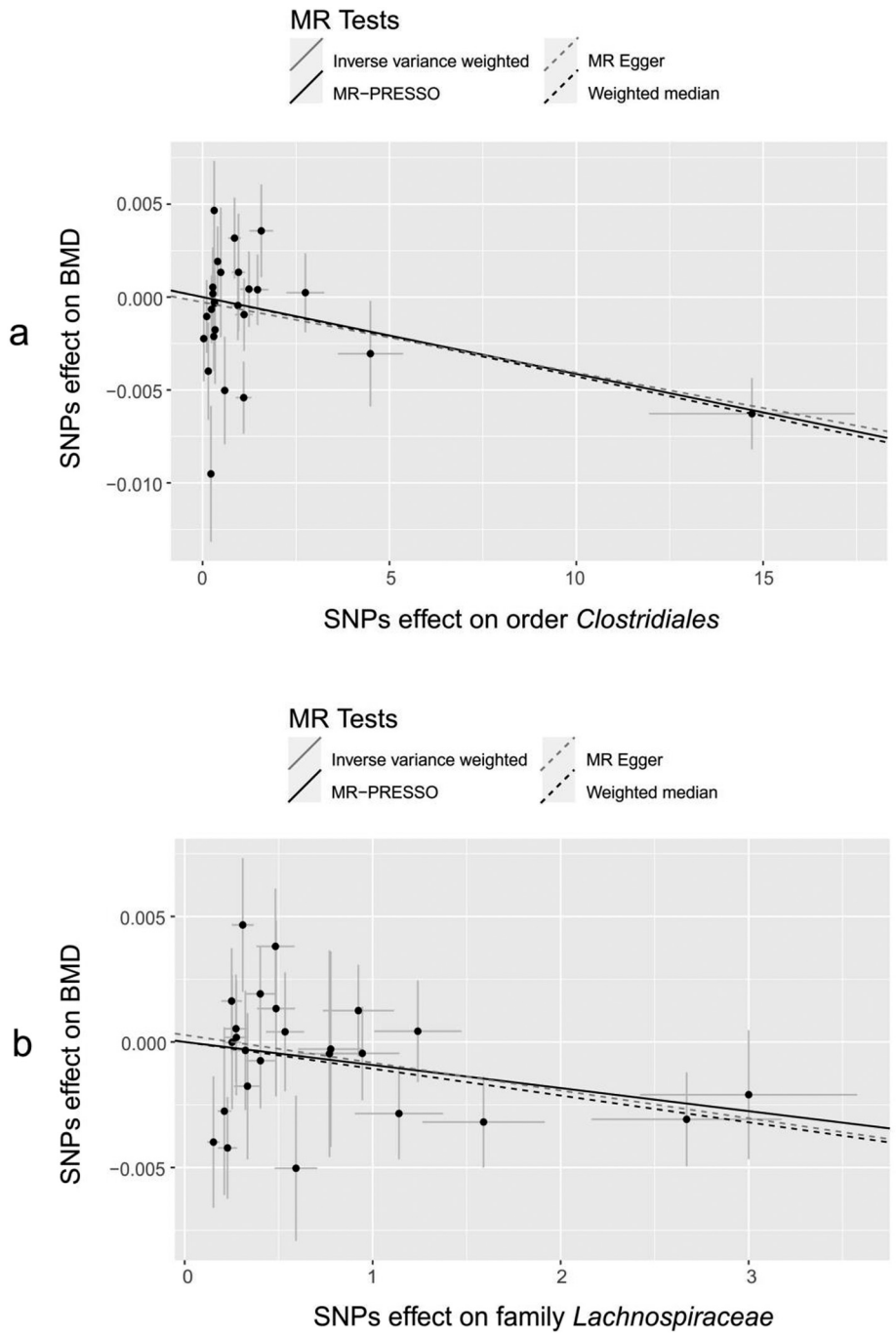

Fig. 3. Scatter plot of the 4 MR tests at the order *Clostridiales* (a) and the family *Lachnospiraceae* (b).
SNP effects were plotted into lines for the IVW test (*grep solid line*), MR-Egger regression (*black solid line*), weighted median estimator (*grep dotted line*), and MR-PRESSO (*black dotted line*). The slope of the line corresponded to the causal estimation of that test.

Besides the difference of population and experimental factors, *Lachnospiraceae* contains many other child-taxa [49], whose relationship with BMD is unclear. Moreover, BMD variation is influenced by many factors and which factor plays a dominant role remains still unknown. However, further functional investigation is warranted to validate this hypothesis, which is out of the scope of this study.

In a previous similar study, Cheng et al. performed a polygenetic risk score (PRS) based MR analysis in the UKB and reported weak relevance of gut microbiota on the development of BMD [50]. The present study differs from that study in the following three aspects: i) taxa features: Cheng et al. analyzed the gut microbiota as a whole, while the present study divided into multiple features that may be more homogeneous; ii) sample size: Cheng et al. used individual-level dual energy X-ray absorptiometry (DXA) BMD in a relatively small sample (N = 5065), while the present study used summary statistics derived from a much larger sample (N = 426,824); iii) evidence of association: Chen et al. evaluated 31 traits in one single discovery stage, and the smallest P-value was 0.0437 for pelvis BMD, which was indeed not significant at all when taking multiple testing of 31 traits into account. In contrast, the present study was composed of both discovery and replication stages.

In the replication stage in which only 1 SNP was identified for each feature, the Wald ratio test was the only method that was applicable. Though the 1-IV test seems to be less robust than multiple-IVs tests, the outcome could still be valuable and reliable. For instance, Iraia et al.

Table 2
The GRS analysis of the two identified features with eBMD in the UKB sample.

Feature	No. SNP	Beta	SE	*P*-value	N
Order *Clostridiales*	26	−0.0002	0.00005	**0.002**	263,342
Family *Lachnospiraceae*	25	−0.0003	0.0002	**0.04**	263,342

Notes: No. SNP is the number of SNPs being used as IVs. Beta is the estimated effect coefficient of GRS analysis. SE is the standard error of estimate coefficient. Significant *P*-values were marked in bold. N is the sample size.

[51] recently identified 8 microbiota features causally associated with Celiac disease. All the significant signals were derived from the Wald ratio test of 1-IV.

Our study was advantageous in several aspects. First, we offered a novel attempt to infer the causal relationship from gut microbiome to BMD, thus providing a new approach to screen candidate gut microbiota for subsequent functional studies. Second, the identified associations were successfully replicated in an independent sample, strengthening the confidence towards true causal relationship. At last, the approach was based on large-scale GWAS summary statistics that are publicly available, thus offers an efficient option to mine reliable genetic information without additional experiment costs.

Obviously, there are also certain limitations in our study. First, gut microbiota GWAS is still in its infancy, therefore the choice of genetic instrumental variables was very limited. Second, bacteria taxa were only studied at order or family level, but not at more specialized level such as genus or species. Third, the genome-wide summary statistics of MGWAS were not publicly available for a reverse MR analysis.

In conclusion, by conducting a two-sample MR analysis using publicly available GWAS summary data, we evaluated the causal link from gut microbiome to BMD as well as identified potentially causal bacteria taxa for bone development and regulated bone mass variation. Our results may be helpful in providing new insights into the microbiota mediated bone development mechanism.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2020.115652.

Abbreviations

BMD	bone mineral density
MR	mendelian randomization
RCT	randomized controlled trial
IV	instrumental variable
SCFA	fecal short-chain fatty acid
GWAS	genome-wide association study
eBMD	estimated heel BMD
UKB	UK Biobank
IRB	institutional review board
SOS	speed of sound
BUA	broadband ultrasound attenuation
HRC	Haplotype Reference Consortium
LD	linkage disequilibrium
IVW	inverse-variance weighted
GRS	genetic risk score
PC	principal component
MGWAS	microbiome GWAS
PRS	polygenetic risk score
DXA	dual energy X-ray absorptiometry
OTU	operational taxonomic unit
GoNL	Genome of the Netherlands

Funding

YFP and LZ were partially supported by the funding from National Natural Science Foundation of China (31771417 and 31571291) and a project funded by the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions. The numerical calculations in this paper have been done on the supercomputing system of the National Supercomputing Center in Changsha.

CRediT authorship contribution statement

Jing-Jing Ni: Conceptualization, Formal analysis, Data curation, Writing - original draft. **Xiao-Lin Yang:** Software, Formal analysis, Data curation. **Hong Zhang:** Validation, Writing - original draft. **Qian Xu:** Formal analysis, Writing - original draft. **Xin-Tong Wei:** Resources, Writing - original draft. **Gui-Juan Feng:** Data curation, Writing - original draft. **Min Zhao:** Investigation, Writing - original draft. **Yu-Fang Pei:** Conceptualization, Writing - review & editing, Supervision. **Lei Zhang:** Conceptualization, Methodology, Software, Data curation, Writing - review & editing, Supervision.

Declaration of competing interest

The authors state that there is no conflict of interest.

Acknowledgments

We appreciate all the volunteers who participated in this study. We are grateful to the TwinsUK study and the LifeLines-DEEP study for releasing the gut microbiota GWAS summary statistics, and the UKB eBMD study for releasing the BMD GWAS summary statistics. The weighted genetic risk score analysis was conducted using the UK Biobank resource under application number 41542.

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