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Assessment of osteoporotic fractures and risk prediction

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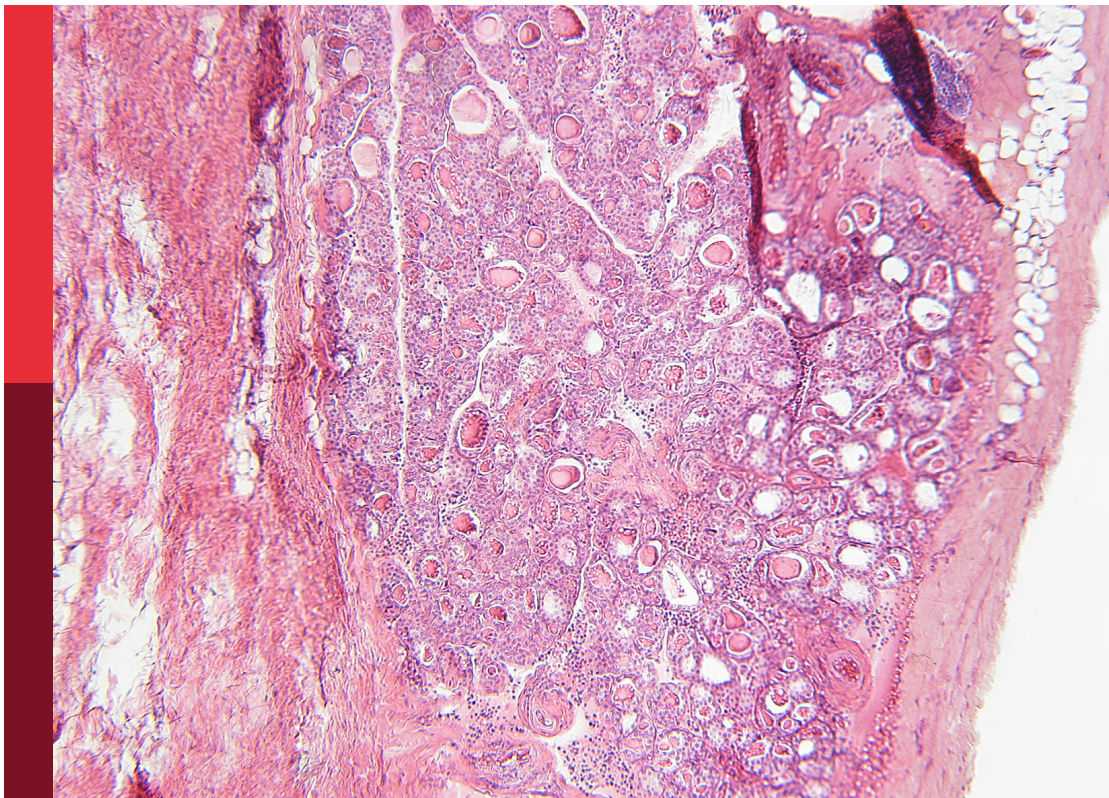

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Assessment of osteoporotic fractures and risk prediction

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Editorial: Assessment of osteoporotic fractures and risk prediction

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Editorial on the Research Topic
Assessment of osteoporotic fractures and risk prediction

Osteoporosis is a metabolic skeletal disorder that is characterized by low bone mineral density (BMD), a deterioration of the microstructure of bone tissue, and a decrease in bone strength, leading to an increase in bone fragility and the risk of fractures (1). Symptomatic vertebral and hip fragility fractures are severe osteoporotic fractures that limit the quality of life and increase morbidity and mortality [(2), Shen et al, (3)]. Currently, a total of 10.9 million men and 49.3 million women in China are estimated to have osteoporosis (4). Meanwhile, it has been estimated that world–wide, there were 158 million individuals aged 50 years or older at high fracture risk in 2010, and that number is expected to double by 2040, predominantly in Asia (5). Therefore, early screening for osteoporosis has a significant role in controlling the disease and lowering the prevalence of osteoporotic fractures.

Although great advances have been achieved in surgical strategies for the treatment of osteoporotic fractures, information on the early assessment of osteoporotic fractures remains limited. Therefore, we organized this special issue that aims to provide insight into the etiology and pathogenesis of osteoporotic fractures, such as the connections between bone mineral density, bone mineral content, and muscle, focusing on clinical research related to the diagnosis, prevention, treatment, and monitoring of osteoporotic fracture. We received more contributions on this topic than originally anticipated, so we have expanded the special issue into a two–volume collection.

Among the contributions in this collection, a retrospective study by Li and colleagues provides clear evidence that modifiable body composition indicators such as body mass index (BMI), body fat percentage (BFP), and skeletal muscle index (SMI) are significantly associated with osteoporosis (6). In a study of the relationships between anthropometric variables and osteoporotic fracture risk, Wu et al. report that body surface area (BSA) may be a potential new risk factor for osteoporotic fractures (7). Moreover, based on their BSA stratification, the authors conclude that BSA may be a risk factor for clinically severe osteoporotic fractures in men with the risk significantly increased by 41–55% when BSA ≤ 1.6895 m^2. Regarding vertebral fractures, Liu et al. have investigated the prevalence of vertebral fractures in middle–aged and elderly Chinese individuals (8). Based on the China Action on Spine and Hip Status

(CASH) study, the authors concluded that the prevalence of vertebral fractures increased rapidly in women after age 50, but comparatively slowly in men. In addition, participants under the age of 50 with a grade 1 vertebral fracture had normal bone mass compared with non–fractured participants (6). The authors' conclusions are consistent with another recently published report (9). In a study of hip fractures, Wang et al. found substantial differences in total and cortical volume as well as cortical thickness between fractured and non–fractured women across the proximal femur. The study of three–dimensional bone geometry and soft tissue is of particular interest in hip fracture research (10–12). Mao et al. have constructed a convolutional neural network model for screening primary osteopenia and osteoporosis based on lumbar radiographs, which may help improve the low rate of diagnosis of osteoporosis (13). Kou et al. have investigated possible diagnostic markers for the early diagnosis of osteoporosis on untargeted gas chromatography (GC)/liquid chromatography (LC)–mass spectrometry (MS) and identified 18 differential metabolites that are potential biomarkers of osteoporosis in postmenopausal women.

Other studies in this special issue investigated risk factors affecting bone mineral density, such as hyperglycemia [Wang et al.], serum amino acid levels [Cui et al.], non–alcoholic fatty liver disease and the degree of hepatic steatosis [Xie and Liu], MicroRNAs in Serum Exosomes [Shi et al.], milk intake [Chen et al.], Neuropeptide Y [Chen and Zhang], nitrates [Liu et al.], menopause–related cortical bone loss (14).

In conclusion, the articles included in this two–volume collection offer fresh perspectives into the etiology and pathogenesis of osteoporotic fractures. With more research in this critical area, we anticipate that many of these discoveries will find their way into clinical practice.

Author contributions

XC, Z–FS, and XW contributed to conception of the study. XC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Relationship Between Serum Amino Acid Levels and Bone Mineral Density: A Mendelian Randomization Study

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Background: This study aimed to explore the association between serum amino acids (AAs) levels and bone mineral density (BMD).

Methods: We performed a two-sample Mendelian randomization (MR) analysis to analyze the associations between the levels of eight AAs and BMD values by using summary-level genome-wide association study (GWAS) data. We applied the MR Steiger filtering method and MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO) global test to check for and remove single nucleotide polymorphisms (SNPs) that were horizontally pleiotropic. The associations were estimated with the inverse variance weighted (IVW), MR-Egger, weighted median and MR Robust Adjusted Profile Score (MR.RAPS) methods.

Results: Our study found that genetically increased isoleucine (Ile) [IVW: effect = 0.1601, 95% confidence interval (CI) = 0.0604 ~ 0.2597, p = 0.0016] and valine (Val) levels (IVW: effect = 0.0953, 95% CI = 0.0251 ~ 0.1655, p = 0.0078) were positively associated with total body BMD (TB-BMD). The results also revealed that genetically increased tyrosine (Tyr) levels were negatively associated with TB-BMD (IVW: effect = -0.1091, 95% CI = -0.1863 ~ -0.0320, p = 0.0055).

Conclusions: In this study, associations between serum AA levels and BMD were established. These findings underscore the important role that serum AAs play in the development of osteoporosis and provide evidence that osteoporosis can be prevented and treated by the intake of certain AAs.

Keywords: amino acid, bone mineral density – BMD, Mendelian randomization, valine, tyrosine, isoleucine

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INTRODUCTION

Osteoporosis is the most common bone disease, and it is characterized by low bone mass, bone tissue deterioration and bone structure disruption (1). Osteoporosis is also the reason for fragility fractures, and the most common fracture sites are the spine, hip and distal forearm. The one-year estimated mortality of hip fractures in mainland China is 13.96% (2). Therefore, osteoporosis is a

major threat to an enormous number of people and exacts a terrible toll on elderly adults, who constitute a rapidly growing population in the world. The measurement of bone mineral density (BMD) has been proven to be an effective method for diagnosing osteoporosis and assessing the risk of fragility fracture (3). Although osteoporosis is an important and common public health problem, the mechanisms and risk factors underlying osteoporosis and BMD are still poorly understood.

Optimal intake of certain nutrients, such as calcium and selenium, has a substantial impact on BMD and is positively correlated with BMD (4). Dietary proteins are important nutrients for maintaining musculoskeletal health. Both bone and muscle are lost with age, with up to 1% lost per year after age 50, and increased dietary protein intake with age is recommended to ameliorate this loss (5). A systematic review and meta-analysis performed in 2009 of published papers from January 1966 to July 2008 showed that protein supplementation had a significant positive influence on lumbar spine BMD in human adults; moreover, nearly all published cross-sectional studies demonstrated a positive association between dietary protein intake and bone health (6). As the main components of proteins, amino acids (AAs) also play an important role in regulating bone metabolism. However, a clear consensus has not been reached on the role of AAs in bone health because AAs may have competing effects on bone. A recent study in monozygotic twins demonstrated the genetically independent benefit of several specific AAs for bone health (7). One large-scale cohort study by Su et al. (8) suggested that a specific AA profile is correlated with greater BMD and lower subsequent fracture risk, independent of diet and lifestyle factors. Male patients with idiopathic osteoporosis also presented changes in free AA profiles, which indicated the role of AA utilization in osteoporosis (9). However, observational studies that estimated causal inference have numerous inherent limitations, such as reverse causality and confounding effects, thus making the interpretation of these associations difficult and their meaning uncertain (10).

Mendelian randomization (MR) analyses can overcome the limitations of conventional studies by using single nucleotide polymorphisms (SNPs) as instrumental variables (IVs) for assessing the causal effect of a risk factor (exposure) on an outcome (11). MR relies on three assumptions: (a) the genetic variant is associated with the exposure; (b) the genetic variant is not associated with confounders; and (c) the genetic variant influences the outcome only through the exposure. A two-sample MR obtains IV-exposure and IV-outcome associations from two different sets of participants. The IVs used in MR are derived from genome-wide association studies (GWAS) and available due to the development of high-throughput genomic technologies. Therefore, in this study, we used the MR approach to explore the causal effect of circulating AA levels on total body and site-specific BMD. This approach can provide estimates of the effects of traits while reducing bias due to confounding and reverse causation. The design strategy for the two-sample MR in our study is shown in **Figure S1** in the Supplementary Material.

METHODS

We performed a two-sample MR analyses to study the effect of AA levels on BMD values. Our approach relied upon summary-level GWAS data to obtain MR estimates (12, 13). We selected SNPs with a genome-wide association ($p<5E-08$), with independent inheritance ($R^2<0.001$), and without linkage disequilibrium (LD) with each AA as IVs. Proxy SNPs ($R^2>0.9$) from LDlink (https://ldlink.nci.nih.gov/) were used when the SNPs were not available for the outcome (14). To estimate the LD level, we selected the reference sample formed by European ancestral individuals from the 1000 Genomes Project (15). Palindromic SNPs with intermediate allele frequencies (palindromic SNPs refer to SNPs with the A/T or G/C alleles and "intermediate allele frequencies" refer to 0.01<allele frequency<0.30) were excluded from the selected instrument SNPs. We also calculated the F statistics for the SNPs to measure the strength of the instruments. IVs with an F statistic less than 10 were excluded and frequently labeled "weak instruments" (16). SNPs with a minor allele frequency (MAF) of < 0.01 were also excluded to avoid potential the statistical bias from the original GWAS since they usually carry with low confidence. Moreover, we used the PhenoScanner tool (17, 18) to check whether any of the selected SNPs were associated with potential confounders at risk of affecting BMD. We set the threshold at genome-wide significance ($p<5E-08$) when using the PhenoScanner tool.

The summary data for the associations between SNPs and AAs were retrieved from the Nightingale Health UK Biobank Initiative. The Finnish innovator of an internationally recognized blood biomarker technology for studying chronic diseases will analyze the biomarker profiles of 500,000 blood samples from the UK Biobank. Nightingale's biomarker profiling technology will be used to analyze the UK Biobank blood samples by measuring metabolic biomarkers found by recent studies. The UK Biobank recruited 502,639 European participants aged 37~70 years in 22 assessment centers across the UK. All study participants reached the assessment centers by their own means, and enrollment was not performed at nursing homes. All participants provided written informed consent, and ethical approval was obtained from the North West Multicenter Research Ethics Committee. Blood samples were drawn at baseline between 2007 and 2010 (19). A random subset of nonfasting baseline plasma samples from 118,466 individuals and 1,298 replication samples were measured using high-throughput nuclear magnetic resonance spectroscopy (Nightingale Health Plc; biomarker quantification version 2020), which provided simultaneous quantification of 249 metabolic biomarkers, including AAs, routine lipids, lipoprotein subclass, fatty acid composition, and other low-molecular weight metabolites, such as ketone bodies and glycolysis metabolites quantified in molar concentration units, in a single assay (20). The metabolic biomarker dataset from the Nightingale Health UK Biobank Initiative was made available for the research community through the IEU GWAS database, which is a database of genetic associations in the GWAS summary datasets (https://gwas.mrcieu.ac.uk/) (13). We only focused on the particular set of AAs and extracted summary statistics about eight single AAs, namely, alanine (Ala), glutamine (Gln), histidine (His), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), tyrosine (Tyr) and valine (Val),

because we wanted to investigate whether AA metabolism could be associated with BMD values.

We extracted summary statistics on femoral neck (FN), lumbar spine (LS) and forearm (FA) BMD (g/cm^2) from the GEnetic Factors for OSteoporosis Consortium website (21). Genetic variants with large effects on BMD were identified in 53,236 individuals of European ancestry. Genetic variants with a minor allele frequency (MAF)>0.5% were tested for their effects on femoral neck, lumbar spine (L1-4), and forearm BMD, and the values were adjusted for sex, age, age^2, weight and standardized to have a mean of zero and a standard deviation of one because different dual-energy X-ray absorptiometry (DXA) machines have known systematic differences in BMD measurements. The summary-level data for total body BMD (TB-BMD) (g/cm^2) were extracted from one GWAS meta-analyses including 30 epidemiological studies comprising individuals from populations across America, Europe, and Australia, with a variety of designs and participant characteristics (22). Most participants in the study were from population-based cohorts of European ancestry, two cohorts comprised African American individuals, and four cohorts included individuals with a mixed background. TB-BMD was also measured by DXA following standard manufacturer protocols. TB-BMD values were corrected for age, weight, height, and genomic principal components. The detailed characteristics of the GWAS associated with exposures and outcomes are shown in **Table S1** in the Supplementary Material.

We applied the MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO) global test (23) to remove SNPs that were horizontal pleiotropic outliers to reduce heterogeneity in the estimate of the causal effect. We conducted this analysis by using the MR-PRESSO R package (https://github.com/rondolab/MR-PRESSO). The number of distributions was set to 1,000 and the threshold was set to 0.05. Moreover, we applied MR Steiger filtering (24) as implemented in the TwoSampleMR R package to test the causal direction of each of the extracted SNPs on the exposures and outcomes. This approach calculated the variance explained in the exposure and the outcome by the instrument SNPs and tests whether the variance in the outcome is less than the exposure. A "TRUE" MR Steiger result suggested causality in the expected direction, while a "FALSE" result suggested causality in the reverse direction. We excluded SNPs with "FALSE" results, which meant that it showed evidence of primarily affecting outcomes rather than exposures.

We conducted the MR with inverse variance weighted (IVW) (13, 25), MR-Egger (26, 27) and Weighted median estimate methods (28). The IVW method uses a meta-analysis approach to combine the Wald ratios of the causal effects of each SNP and can provide the most precise estimates (13, 25). The Weighted median estimate provides a reliable effect estimate of the causal effect when at least 50% of the weight in the analysis comes from effective IVs (28). MR-Egger regression is used to create a weighted linear regression of the outcome coefficients with the exposure coefficients. The slope of the weighted regression line provides an asymptotically unbiased causal estimate of the exposure on the outcome if the INSIDE (instrument strength is independent of direct effect) assumption is met. In addition, the intercept of the MR-Egger regression line was used to quantify the amount of horizontal pleiotropy present in the data averaged across the genetic instruments (26, 27). Under the INSIDE assumption, the MR-Egger intercept test identifies horizontal pleiotropy if the intercept from the MR-Egger analysis is not equal to zero (27). We also calculated the MR Robust Adjusted Profile Score (MR.RAPS) to estimate the causal effects because it can lead to considerably higher statistical power than conventional MR analysis (29). MR.RAPS considers the measurement error in SNP-exposure effects, and it is unbiased when weak instruments are used and robust to systematic and idiosyncratic pleiotropy (29). The MR.RAPS method can also alleviate but cannot solve the problem of horizontal pleiotropy (29). We used the IVW (30) method to detect heterogeneity, which was quantified by the Cochran Q statistic. Moreover, we also performed multivariable MR (MVMR) analysis to control for genetic associations of AAs with some BMD potential risk factors, such as alcohol consumption, BMI and education attainment to adjust for the effect of confounders. The summary-level data of alcohol consumption were extracted from the GWAS study in the UK Biobank (UKB) sample of white British individuals (31), BMI were extracted from the meta-analysis of GWAS in European adults (32) and education attainment were extracted from the GWAS conducted in a discovery sample of 101,069 individuals and a replication sample of 25,490 (33). We estimated the power of our study according to a method suggested by Brion et al. (34). This method uses a noncentrality parameter to calculate the statistical power of the continuous outcome and an approximate linear model on the observed binary scale adapted for binary outcome. The method required several parameters to estimate the power. For the continuous outcomes, the first parameter was the proportion of phenotypic variation (r2) explained by IV SNPs, which was estimated on the original GWAS. The second was the effect size of the exposure to the outcome at the epidemiological level, which was estimated from another independent observational cohort (8). Addition parameters included the sample size and standard deviation (SD) of exposure and outcome. The summary-level MR analysis was performed by the TwoSampleMR package (version 0.5.0) in R (version 3.6.1, the R foundation). The statistical tests of the MR analysis were two-sided, and the results of the MR analyses regarding the causal effects of AAs on BMD were considered statistically significant at a Bonferroni-corrected p<0.0125 (e.g., 0.050/4 outcomes). Relationships for which the p value was below 0.05 but above 0.0125 were considered nominally significant.

RESULTS

According to the SNP selection criteria, we first extracted 36, 46, 17, 10, 17, 9, 38 and 22 significant genome-wide and independently inherited SNPs associated with eight AAs. When extracting the corresponding SNPs for outcomes, we

had to exclude some SNPs that were absent and no proxy SNPs in high LD (R^2>0.9) found from LDlink in the summary statistics of outcomes. We also removed the palindromic SNPs when harmonizing the effect of IVs and excluded the SNPs with false causal direction identified by the MR Steiger filtering. Moreover, when using the PhenoScanner tool, we excluded some SNPs that were associated with confounders, which were proved to be causally associated with BMD such as body mass index (BMI), weight, calcium and low density lipoprotein (LDL) (4, 35), which might violate the second assumption of MR. We also excluded the horizontal pleiotropic outliers through the MR-PRESSO global test. The selection process and the reasons for selecting the SNPs are described in detail in **Figures S2–9**. The final numbers of SNPs included in the MR are presented in **Tables 1–4**. For all the included IVs, the F statistics were above 10 (e.g., Ala: ranging from 30.0542 to 249.5155 for FN-BMD; from 30.0542 to 249.5155 for LS-BMD; from 30.8591 to 249.5155 for FA-BMD and from 30.0542 to 249.5155 for TB-BMD), which indicated that they satisfy the strong relevance assumption of MR and that weak instrument bias would not substantially influence the estimations of causal effects. We also confirmed the true causal direction for the included SNPs with the MR Steiger method. The proportion of phenotypic variation explained by each genetic variant was also calculated. The detailed characteristics of the included SNPs are shown in **Tables S2–9**.

Figure 1 and **Tables 1–4** display the causal effects of AAs on BMD based on IVW, MR-Egger, Weighted median, MR.RAPS and MR-PRESSO methods. At the Bonferroni-corrected p threshold of 0.0125, the results provided evidence that genetically increased Ile (e.g., IVW: effect = 0.1601, 95% confidence interval [CI] = 0.0604 ~ 0.2597, p = 0.0016) and Val levels (e.g., IVW: effect = 0.0953, 95% CI = 0.0251 ~ 0.1655, p = 0.0078) were positively associated with TB-BMD (**Table 4**). The results revealed that genetically increased Tyr levels were negatively associated with TB-BMD (e.g., IVW: effect = -0.1091, 95% CI = -0.1863 ~ -0.0320, p = 0.0055) (**Table 4**). We did not observe the statistically significant associations between AAs and site-specific BMD (FN, LS and FA-BMD) (**Tables 1–3**), although Phe levels were negatively associated with FA-BMD at a nominal threshold (p<0.050) (e.g., IVW: effect = -0.2717, 95% CI=-0.4816 ~ -0.0619, p = 0.0111) (**Table 3**). For the other AAs, such as Ala and His, significant causal effects were not observed on FN, LS, FA and TB-BMD based on IVW, MR-Egger, Weighted median, MR.RAPS and MR-PRESSO methods (**Tables 1–4**).

We conducted heterogeneity analyses using the IVW method and conducted the pleiotropy analyses using the MR-Egger intercept test (**Tables 1–4**). Heterogeneity was not observed in the MR analyses for the causal associations of AAs on BMD changes (e.g, for the causal association between Ile and the TB-BMD, Q = 3.6813, p = 0.8157) (**Table 4**). Based on the MR-Egger intercept test, we did not find evidence of directional pleiotropy between the AA levels and BMD (e.g., Ile: intercept = -0.0041, p = 0.7601 for FN-BMD; intercept = -0.0095, p = 0.4520 for LS-BMD; intercept = 0.0023, p = 0.9089 for FA-BMD; and intercept = -0.0041, p = 0.5808 for TB-BMD) (**Tables 1–4**). The MR-PRESSO global test also revealed that no horizontal pleiotropic outliers were identified in the MR analyses (e.g., Ile: p = 0.1655 for FN-BMD; p = 0.3640 for LS-BMD; p = 0.7540 for FA-BMD; and p = 0.8277 for TB-BMD) (**Tables 1–4**). The results of MVMR adjusted for alcohol consumption, BMI and educational attainment were similar to the univariable MR results, with significant direct associations identified between Ile, Tyr, Val and TB-BMD (e.g., Ile: p = 0.0001 adjusted for alcohol consumption; p = 0.0097 adjusted for BMI; and p = 0.0058 adjusted for educational attainment) (**Tables S10–12**). The sample sizes of BMD traits in the current analysis are presented in **Table S13**. We calculated the proportions of AA variation explained by IVs ranging from 0.0056 to 0.0394. Under the current sample size and exposure variations, we provided the minimum and maximum detectable causal effects required to achieve 80% statistical power for the MR analysis, and they were located in the CI of our results. Therefore, our study had 80% power to detect a causal effect of 0.1535 g/cm^2 increase in TB-BMD per 1-SD increase of Ile levels, 0.0967 g/cm^2 increase in TB-BMD per 1-SD increase of Val levels and 0.1003 g/cm^2 decrease in TB-BMD per 1-SD increase of Tyr levels.

DISCUSSION

Molecular mechanism analyses have suggested that a number of AAs may be associated with BMD. Bone marrow stromal cells were demonstrated to express both intracellular and extracellular nutrient-sensing pathways for AAs, and certain AAs were described as potent stimulators of an increase in intracellular calcium, suggesting that AAs were important signaling molecules for normal cell function (36). Osteoblasts can express specialized AA receptors and transporters that enable the adjustment of cellular bioenergetics according to fluctuations in AA availability (37). Some AA was a potent stimulus of growth hormone secretion, which in turn results in an increase in circulating levels of insulin-like growth factor-1 (IGF-1), a known anabolic stimulus for osteoblasts. Bone mass can be elevated by AA-induced increases in calcium absorption efficiency, osteoblast proliferation and bone mineralization, synthesis of type I collagen, circulating levels of IGF-1, reduced bone resorption, osteoclast attachment and suppressed osteoclast differentiation (38). AAs can also enhance intestinal calcium absorption *in vivo*, increase the secretion of Alk Phos and decrease the production of interleukin-6 from osteoblasts *in vitro*. Increases in Alk Phos and decreases in interleukin-6 levels may result in increases in bone collagen synthesis and bone formation and reduced bone resorption (38).

In the present study, we reported for the first time the causal associations between AAs and BMD through a MR analysis. We provided evidence to support the causal effects of Ile,Val and Tyr on TB-BMD. In the MR study, we used strong IVs from the summary statistics of the largest GWAS conducted for AAs and BMD. We employed a range of methods known to control for pleiotropy and checked the heterogeneity, and we obtained highly consistent results. Pleiotropic effects were detected by using the MR-Egger intercept and MR-PRESSO method. Using

TABLE 1 | MR estimates of the causal effects of AAs on FN-BMD using various analysis methods.

Exposures	Methods	Number of SNPs	Effect	95% CI	MR p-value	Cochran Q statistic	Heterogeneity p-value	MR-Egger		MR-PRESSO
								Intercept	Intercept p-value	Global test p-value
Ala	IVW	25	0.0056	-0.0938~0.1050	0.9126	32.4024	0.1173			
	MR-Egger	25	0.1210	-0.1252~0.3672	0.3454			-0.0053	0.3257	
	Weighted median	25	0.0860	-0.0402~0.2121	0.1817					
	MR.RAPS	25	0.0124	-0.0855~0.1102	0.8045					
	MR-PRESSO	25	0.0056	-0.0938~0.1050	0.9135					0.1015
Gln	IVW	27	-0.0457	-0.1068~0.0153	0.1417	29.7737	0.2771			
	MR-Egger	27	-0.0434	-0.1290~0.0422	0.3293			-0.0002	0.9395	
	Weighted median	27	-0.0507	-0.1172~0.0158	0.1350					
	MR.RAPS	27	-0.0431	-0.1081~0.0218	0.1931					
	MR-PRESSO	27	-0.0457	-0.1068~0.0153	0.1537					0.3661
His	IVW	8	0.1150	-0.0247~0.2547	0.1067	4.7754	0.6874			
	MR-Egger	8	0.2536	-0.4301~0.9372	0.4946			-0.0094	0.6990	
	Weighted median	8	0.0494	-0.1309~0.2298	0.5910					
	MR.RAPS	8	0.1159	-0.0292~0.2609	0.1174					
	MR-PRESSO	8	0.1150	-0.0004~0.2304	0.0918					0.6517
Ile	IVW	7	0.1409	-0.0285~0.3103	0.1030	9.0653	0.1699			
	MR-Egger	7	0.2172	-0.2811~0.7154	0.4320			-0.0041	0.7601	
	Weighted median	7	0.1532	-0.0397~0.3461	0.1195					
	MR.RAPS	7	0.1734	-0.0037~0.3506	0.0550					
	MR-PRESSO	7	0.1409	-0.0285~0.3103	0.1541					0.1655
Leu	IVW	10	0.0411	-0.0900~0.1722	0.5391	10.5394	0.3086			
	MR-Egger	10	0.0454	-0.2660~0.3569	0.7821			-0.0003	0.9762	
	Weighted median	10	0.0406	-0.1056~0.1869	0.5862					
	MR.RAPS	10	0.0528	-0.0726~0.1783	0.4092					
	MR-PRESSO	10	0.0411	-0.0900~0.1722	0.5543					0.4418
Phe	IVW	7	-0.0081	-0.1285~0.1122	0.8948	8.5048	0.2034			
	MR-Egger	7	-0.0904	-0.3683~0.1875	0.5518			0.0060	0.5432	
	Weighted median	7	-0.0380	-0.1586~0.0826	0.5368					
	MR.RAPS	7	-0.0107	-0.1306~0.1093	0.8615					
	MR-PRESSO	7	-0.0081	-0.1285~0.1122	0.8991					0.3286
Tyr	IVW	25	-0.0111	-0.0867~0.0644	0.7724	26.5534	0.3257			
	MR-Egger	25	-0.0026	-0.1189~0.1138	0.9657			-0.0007	0.8490	
	Weighted median	25	-0.0119	-0.1116~0.0877	0.8143					
	MR.RAPS	25	-0.0111	-0.0871~0.0649	0.7739					
	MR-PRESSO	25	-0.0111	-0.0867~0.0644	0.7749					0.3703
Val	IVW	12	0.0719	-0.0294~0.1732	0.1640	8.2676	0.6892			
	MR-Egger	12	0.1122	-0.0689~0.2934	0.2525			-0.0028	0.6103	
	Weighted median	12	0.0393	-0.0887~0.1673	0.5469					
	MR.RAPS	12	0.0738	-0.0310~0.1785	0.1675					
	MR-PRESSO	12	0.0719	-0.0159~0.1597	0.1367					0.6874

AAs, amino acids; Ala, alanine; Gln, glutamine; His, histidine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Tyr, tyrosine; Val, valine; BMD, bone mineral density; FN-BMD, femoral neck BMD; SNPs, single nucleotide polymorphisms; MR, Mendelian randomization; IVW, inverse variance weighted; MR.RAPS, MR Robust Adjusted Profile Score; MR-PRESSO, MR Pleiotropy RESidual Sum and Outlier; Effect, the causal effects of 1-SD increase of AAs on BMD; SD, standard deviation; CI, confidence interval.

TABLE 2 | MR estimates of the causal effects of AAs on LS-BMD using various analysis methods.

Exposures	Methods	Number of SNPs	Effect	95% CI	MR p-value	Cochran Q statistic	Heterogeneity p-value	MR-Egger		MR-PRESSO
								Intercept	Intercept p-value	Global test p-value
Ala	IVW	20	-0.0517	-0.1676~0.0642	0.3822	22.0655	0.2810			
	MR-Egger	20	0.0576	-0.2223~0.3375	0.6914			-0.0052	0.4107	
	Weighted median	20	-0.0465	-0.2118~0.1189	0.5818					
	MR.RAPS	20	-0.0585	-0.1856~0.0687	0.3674					
	MR-PRESSO	20	-0.0517	-0.1676~0.0642	0.3931					0.3001
Gln	IVW	25	0.0134	-0.0543~0.0810	0.6988	23.0452	0.5171			
	MR-Egger	25	-0.0147	-0.1069~0.0775	0.7574			0.0027	0.3890	
	Weighted median	25	0.0084	-0.0749~0.0917	0.8434					
	MR.RAPS	25	0.0107	-0.0592~0.0806	0.7641					
	MR-PRESSO	25	0.0134	-0.0530~0.0797	0.6964					0.5956
His	IVW	8	-0.0944	-0.2569~0.0681	0.2548	3.7915	0.8035			
	MR-Egger	8	0.1204	-0.6743~0.9151	0.7765			-0.0145	0.6078	
	Weighted median	8	-0.0442	-0.2510~0.1627	0.6757					
	MR.RAPS	8	-0.0950	-0.2630~0.0731	0.2680					
	MR-PRESSO	8	-0.0944	-0.2140~0.0252	0.1657					0.8308
Ile	IVW	7	0.1744	0.0082~0.3405	*0.0397*†	6.4597	0.3737			
	MR-Egger	7	0.3534	-0.1097~0.8165	0.1950			-0.0095	0.4520	
	Weighted median	7	0.1818	-0.0308~0.3943	0.0937					
	MR.RAPS	7	0.1795	0.0086~0.3505	*0.0396*					
	MR-PRESSO	7	0.1744	0.0082~0.3405	0.0854					0.3640
Leu	IVW	9	0.1255	-0.0320~0.2831	0.1183	9.7951	0.2797			
	MR-Egger	9	-0.0909	-0.4174~0.2357	0.6025			0.0136	0.1887	
	Weighted median	9	0.0762	-0.0876~0.2400	0.3618					
	MR.RAPS	9	0.1206	-0.0559~0.2972	0.1804					
	MR-PRESSO	9	0.1255	-0.0320~0.2831	0.1569					0.3687
Phe	IVW	7	-0.1068	-0.2243~0.0108	0.0750	2.5878	0.8585			
	MR-Egger	7	-0.1174	-0.3757~0.1409	0.4139			0.0008	0.9315	
	Weighted median	7	-0.1132	-0.2502~0.0238	0.1053					
	MR.RAPS	7	-0.1070	-0.2281~0.0141	0.0832					
	MR-PRESSO	7	-0.1068	-0.184~-0.0296	*0.0351*					0.9067
Tyr	IVW	24	-0.0063	-0.0914~0.0787	0.8837	24.2904	0.3879			
	MR-Egger	24	-0.0047	-0.1315~0.1220	0.9422			-0.0001	0.9731	
	Weighted median	24	0.0414	-0.0715~0.1543	0.4721					
	MR.RAPS	24	-0.0006	-0.0903~0.0891	0.9898					
	MR-PRESSO	24	-0.0063	-0.0914~0.0787	0.8850					0.3875
Val	IVW	10	0.1042	-0.0167~0.2250	0.0911	4.6955	0.8600			
	MR-Egger	10	0.1185	-0.0996~0.3367	0.3180			-0.0011	0.8807	
	Weighted median	10	0.0920	-0.0496~0.2335	0.2027					
	MR.RAPS	10	0.1045	-0.0201~0.2292	0.1002					
	MR-PRESSO	10	0.1042	0.0169~0.1914	*0.0441*					0.8787

AAs, amino acids; Ala, alanine; Gln, glutamine; His, histidine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Tyr, tyrosine; Val, valine; BMD, bone mineral density; LS-BMD, lumbar spine BMD; SNPs, single nucleotide polymorphisms; MR, Mendelian randomization; IVW, inverse variance weighted; MR.RAPS, MR Robust Adjusted Profile Score; MR-PRESSO, MR Pleiotropy RESidual Sum and Outlier; Effect, the causal effects of 1-SD increase of AAs on BMD; SD, standard deviation; CI, confidence interval.

†*the italic MR p-value was considered nominally significant at $p<0.05$.*

TABLE 3 | MR estimates of the causal effects of AAs on FA-BMD using various analysis methods.

Exposures	Methods	Number of SNPs	Effect	95% CI	MR p-value	Cochran Q statistic	Heterogeneity p-value	MR-Egger		MR-PRESSO
								Intercept	Intercept p-value	Global test p-value
Ala	IVW	24	0.0389	-0.1381~0.2159	0.6668	19.2259	0.6880			
	MR-Egger	24	0.0722	-0.3677~0.5122	0.7506			-0.0015	0.8726	
	Weighted median	24	0.0222	-0.2239~0.2683	0.8599					
	MR.RAPS	24	0.0466	-0.1374~0.2305	0.6198					
	MR-PRESSO	24	0.0389	-0.1230~0.2007	0.6421					0.7131
Gln	IVW	31	-0.0081	-0.1267~0.1106	0.8941	35.6282	0.2205			
	MR-Egger	31	-0.0385	-0.2100~0.1330	0.6632			0.0027	0.6294	
	Weighted median	31	-0.0068	-0.1519~0.1383	0.9266					
	MR.RAPS	31	-0.0129	-0.1401~0.1143	0.8425					
	MR-PRESSO	31	-0.0081	-0.1267~0.1106	0.8950					0.2835
His	IVW	9	0.1224	-0.1562~0.4011	0.3890	6.5817	0.5824			
	MR-Egger	9	0.0813	-0.7800~0.9425	0.8585			0.0026	0.9239	
	Weighted median	9	0.0325	-0.3272~0.3923	0.8594					
	MR.RAPS	9	0.1123	-0.1770~0.4016	0.4467					
	MR-PRESSO	9	0.1224	-0.1303~0.3752	0.3701					0.5349
Ile	IVW	7	0.2292	-0.0542~0.5126	0.1129	3.4614	0.7491			
	MR-Egger	7	0.1861	-0.5722~0.9443	0.6508			0.0023	0.9089	
	Weighted median	7	0.1504	-0.1960~0.4968	0.3949					
	MR.RAPS	7	0.2304	-0.0628~0.5235	0.1235					
	MR-PRESSO	7	0.2292	0.0140~0.4445	0.0819					0.7540
Leu	IVW	9	0.1661	-0.0865~0.4187	0.1976	5.9488	0.6530			
	MR-Egger	9	0.0499	-0.5036~0.6033	0.8648			0.0073	0.6577	
	Weighted median	9	0.0755	-0.2373~0.3884	0.6361					
	MR.RAPS	9	0.1608	-0.1005~0.4222	0.2277					
	MR-PRESSO	9	0.1661	-0.0518~0.3839	0.1735					0.6342
Phe	IVW	7	-0.2717	-0.4816~-0.0619	***0.0111****	4.1909	0.6509			
	MR-Egger	7	-0.1617	-0.6234~0.3000	0.5230			-0.0080	0.6224	
	Weighted median	7	-0.2880	-0.5314~-0.0446	*0.0204*†					
	MR.RAPS	7	-0.2726	-0.4893~-0.0560	*0.0137*					
	MR-PRESSO	7	-0.2717	-0.4471~-0.0964	*0.0229*					0.7533
Tyr	IVW	26	-0.0384	-0.1897~0.1130	0.6192	27.9032	0.3123			
	MR-Egger	26	0.0401	-0.1850~0.2652	0.7303			-0.0063	0.3643	
	Weighted median	26	-0.0729	-0.2789~0.1330	0.4876					
	MR.RAPS	26	-0.0626	-0.2117~0.0865	0.4107					
	MR-PRESSO	26	-0.0384	-0.1897~0.1130	0.6236					0.2910
Val	IVW	13	0.0429	-0.1769~0.2627	0.7018	15.4376	0.2184			
	MR-Egger	13	0.2316	-0.1739~0.637	0.2868			-0.0124	0.3024	
	Weighted median	13	0.0880	-0.1717~0.3477	0.5065					
	MR.RAPS	13	0.0991	-0.1241~0.3224	0.3840					
	MR-PRESSO	13	0.0429	-0.1769~0.2627	0.7085					0.2297

AAs, amino acids; Ala, alanine; Gln, glutamine; His, histidine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Tyr, tyrosine; Val, valine; BMD, bone mineral density; FA-BMD, forearm BMD; SNPs, single nucleotide polymorphisms; MR, Mendelian randomization; IVW, inverse variance weighted; MR.RAPS, MR Robust Adjusted Profile Score; MR-PRESSO, MR Pleiotropy RESidual Sum and Outlier; Effect, the causal effects of 1-SD increase of AAs on BMD; SD, standard deviation; CI, confidence interval.

**the bold and italic MR p-value was considered statistically significant at a Bonferroni-corrected $p < 0.0125$.*

†the italic MR p-value was considered nominally significant at $p < 0.05$.

TABLE 4 | MR estimates of the causal effects of AAs on TB-BMD using various analysis methods.

Exposures	Methods	Number of SNPs	Effect	95% CI	MR p-value	Cochran Q statistic	Heterogeneity p-value	MR-Egger		MR-PRESSO
								Intercept	Intercept p-value	Global test p-value
Ala	IVW	27	-0.0227	-0.0961~0.0507	0.5441	31.4677	0.2113			
	MR-Egger	27	-0.1454	-0.3275~0.0366	0.1300			0.0055	0.1628	
	Weighted median	27	0.0254	-0.0741~0.1249	0.6173					
	MR.RAPS	27	-0.0251	-0.1033~0.0531	0.5292					
	MR-PRESSO	27	-0.0227	-0.0961~0.0507	0.5494					0.2137
Gln	IVW	29	-0.0168	-0.0622~0.0285	0.4669	30.0697	0.3599			
	MR-Egger	29	-0.0469	-0.1095~0.0157	0.1536			0.0026	0.1898	
	Weighted median	29	-0.0230	-0.0774~0.0313	0.4062					
	MR.RAPS	29	-0.0165	-0.0641~0.0311	0.4960					
	MR-PRESSO	29	-0.0168	-0.0622~0.0285	0.4729					0.3548
His	IVW	10	0.0659	-0.0724~0.2043	0.3501	16.4419	0.0582			
	MR-Egger	10	0.3130	-0.1051~0.7311	0.1804			-0.0151	0.2560	
	Weighted median	10	0.1106	-0.0209~0.2422	0.0992					
	MR.RAPS	10	0.0973	-0.0140~0.2085	0.0866					
	MR-PRESSO	10	0.0659	-0.0724~0.2043	0.3745					0.0786
Ile	IVW	8	0.1601	0.0604~0.2597	***0.0016****	3.6813	0.8157			
	MR-Egger	8	0.2386	-0.0433~0.5204	0.1482			-0.0041	0.5808	
	Weighted median	8	0.1442	0.0207~0.2677	*0.0221*†					
	MR.RAPS	8	0.1608	0.0573~0.2643	***0.0023***					
	MR-PRESSO	8	0.1601	0.0878~0.2323	***0.0034***					0.8277
Leu	IVW	12	0.0759	-0.0104~0.1622	0.0847	11.2878	0.4195			
	MR-Egger	12	0.0693	-0.1446~0.2832	0.5398			0.0004	0.9482	
	Weighted median	12	0.1117	-0.0002~0.2237	0.0505					
	MR.RAPS	12	0.0925	0.0041~0.1808	*0.0402*					
	MR-PRESSO	12	0.0759	-0.0104~0.1622	0.1126					0.4493
Phe	IVW	8	-0.0406	-0.1168~0.0355	0.2956	1.9472	0.9627			
	MR-Egger	8	-0.0667	-0.2198~0.0864	0.4259			0.0018	0.7139	
	Weighted median	8	-0.0629	-0.1571~0.0313	0.1906					
	MR.RAPS	8	-0.0407	-0.1190~0.0376	0.3084					
	MR-PRESSO	8	-0.0406	-0.0808~-0.0005	0.0878					0.9229
Tyr	IVW	20	-0.1091	-0.1863~-0.0320	***0.0055***	20.5048	0.3648			
	MR-Egger	20	-0.0037	-0.1268~0.1194	0.9536			-0.0066	0.0596	
	Weighted median	20	-0.0477	-0.1623~0.0669	0.4147					
	MR.RAPS	20	-0.1142	-0.1932~-0.0351	***0.0046***					
	MR-PRESSO	20	-0.1091	-0.1863~-0.0320	*0.0121*					0.3302
Val	IVW	15	0.0953	0.0251~0.1655	***0.0078***	10.0451	0.7589			
	MR-Egger	15	0.0820	-0.0534~0.2174	0.2563			0.0010	0.8260	
	Weighted median	15	0.0918	0.0007~0.1829	*0.0484*					
	MR.RAPS	15	0.0979	0.0254~0.1705	***0.0082***					
	MR-PRESSO	15	0.0953	0.0358~0.1548	***0.0072***					0.8128

AAs, amino acids; Ala, alanine; Gln, glutamine; His, histidine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Tyr, tyrosine; Val, valine; BMD, bone mineral density; TB-BMD, total body BMD; SNPs, single nucleotide polymorphisms; MR, Mendelian randomization; IVW, inverse variance weighted; MR.RAPS, MR Robust Adjusted Profile Score; MR-PRESSO, MR Pleiotropy RESidual Sum and Outlier; Effect, the causal effects of 1-SD increase of AAs on BMD; SD, standard deviation; CI, confidence interval.

**the bold and italic MR p-value was considered statistically significant at a Bonferroni-corrected $p<0.0125$.*

†the italic MR p-value was considered nominally significant at $p<0.05$.

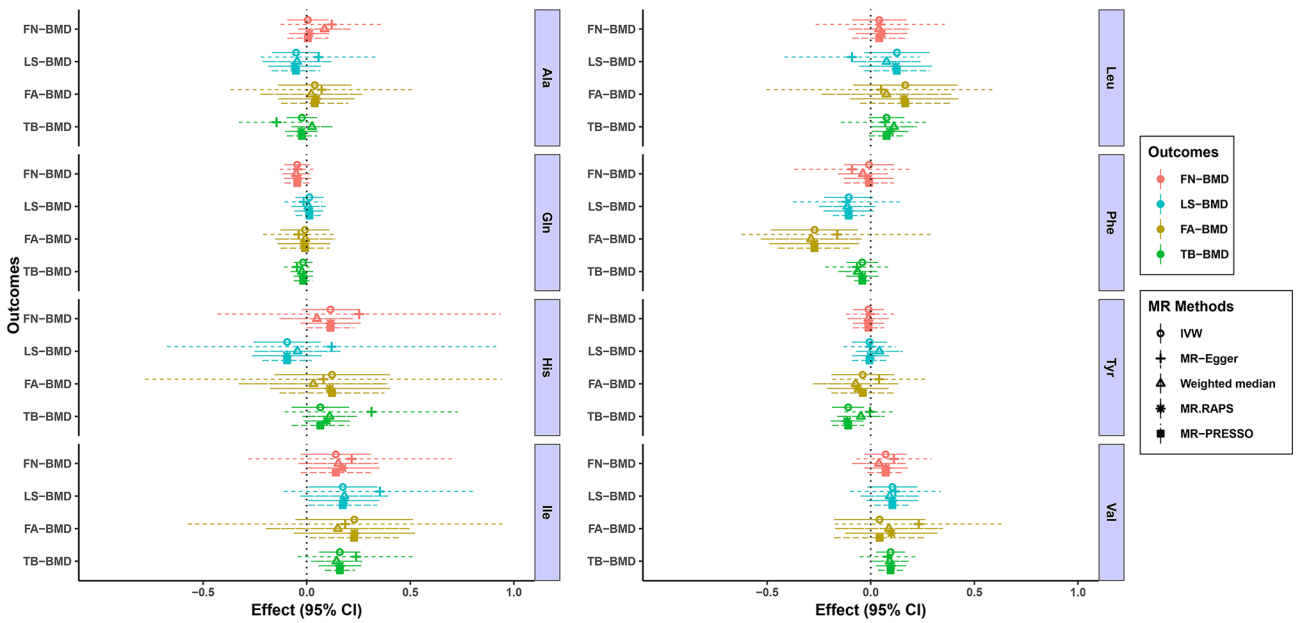

FIGURE 1 | MR estimates of the associations between eight AA levels and BMD. The x-axis is the effects of AAs on BMD values. The vertical dashed line is the reference at effect = 0. The y-axis presents different BMD types, which are highlighted in different colors. Different MR methods are displayed with different line types. AAs, amino acids; BMD, bone mineral density; CI, confidence interval; IVW, inverse variance weighted; MR.RAPS, MR Robust Adjusted Profile Score; MR-PRESSO, MR Pleiotropy RESidual Sum and Outlier; Ala, alanine; Gln, glutamine; His, histidine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Tyr, tyrosine; Val, valine; FN-BMD, femoral neck BMD; LS-BMD, lumbar spine BMD; FA-BMD, forearm BMD; TB-BMD, total body BMD.

the MR design, we could mitigate the confounding factors due to the application of Mendel's second law of the random assortment of alleles. Reverse causality was also prevented because genetic variants were fixed at conception and could not be affected by disease processes. The results above showed that the presence of pleiotropic SNPs was minimal. Besides the univariable MR analysis, we also conducted the MVMR analysis taking into account the effect of some potential risk factors of BMD. We found the results were stable after adjusting for these risk factors. In addition, we also calculated the power of MR. Taken together, our MR results have high precision and stability to support the evidence.

Val, Leu, and Ile are branched-chain AAs (BCAAs) that are critical for the maintenance of bone strength and density and associated with greater muscle and fat mass (39). BCAAs have a direct effect on the initiation of mRNA translation and are the most potent stimulator of muscle protein synthesis, which is critical for the maintenance of adequate bone strength and density (40). In our study, we found that Ile and Val were positively associated with TB-BMD. Tyr, Phe and His are aromatic amino acids (AAAs) involved in protein synthesis. AAAs and their metabolites are involved in the synthesis of various secondary metabolites, including pigment compounds, plant hormones and biological polymers (41). The molecular mechanisms underlying the associations between AAAs and bone metabolism have been partially revealed. AAAs reduced the expression of the calcitonin receptor, carbonic anhydrase II and cathepsin K in osteoclasts *in vitro*, which may suppress osteoclast differentiation (42). Increasing the intake of AAAs might stimulate an increase in the circulating levels of IGF-1 and influence calcium homeostasis, which is involved in the stimulation of mature osteoblasts and regulates skeletal growth (43, 44). However, Le et al. (45) suggested that dietary AAA intake was not significantly associated with hip fractures, hip BMD, or any measurements of body composition. Our study support the negative causal effects of Tyr and Phe on BMD, although the findings indicated that Phe was negatively associated with FA-BMD at a nominal threshold. The negative associations between AAs and BMD were surprising, although some AAs were reported to cause bone loss and increase the risk of fracture. Higher homocysteine (Hcy) was associated with significant BMD decline and independently associated with a higher risk of fracture (8). The MR results from Wang et al. (46) also revealed a negative association between Hcy and BMD. However, they also indicated that decreased plasma Hcy was not associated with FN-BMD, LS-BMD and the risk for bone fracture. *In vitro* studies have revealed that Hcy might also promote collagen accumulation in bone, contribute to decreased bone strength and reduce bone blood flow (47), thus suggesting a pathogenic role of Hcy in bone health. A cross-sectional study involving a total of 773 Taiwanese women revealed that elevated Gln was significantly associated with low BMD (48). Gln might convert to glutamate, which would lead to bone resorption through the expression of glutamate receptors on bone cells, especially osteoclasts. This finding explained the association between elevated Gln and low BMD (48). However,

we did not identify causal associations between Gln and BMD. Currently, only one clinical study has been published about the association between Ala and BMD. A cross-sectional study (49) of 103 patients with spinal cord injury found that higher alanine levels were not related to BMD after controlling for confounders, including demographic and injury-related characteristics and calcium intake. Our results did not support the causal associations between Ala and BMD. Although the causal associations were found between AAs and TB-BMD, we still did not support the causal associations between AAs and site-specific BMD (FN, LS and FA-BMD). This result also suggested that the effects of circulating AAs on bone metabolism might be systemic rather than local. The molecular mechanism of function of AAs on the bone metabolism also supported the hypothesis (36–38).

Although the design of MR analyses makes this method less susceptible to confounders than other observational studies, limitations still exist. First, we only evaluated the association between a single AA and BMD and did not consider the interactions between the AAs and the interactions with other nutritional factors, such as calcium, which might lead to potential pleiotropy. This limitation might cause the inconclusive causal associations between serum AA levels and BMD. However, we assessed potential pleiotropy using the MR-Egger method and MR-PRESSO method. We also used the PhenoScanner tool to exclude the SNPs associated with confounders. Hence, although the risk of a residual horizontal pleiotropic effect cannot be ruled out, it likely did not change the conclusions of this study in a clinically meaningful way. Second, we did not perform age and gender stratification for the population, which are two essential factors that can affect BMD (1). However, excluding these processes likely did not have a large effect on our analyses because of the large sample size included for AAs and BMD, which might have reduced the bias. Second, most of the population in the original GWAS were from European ancestry, but the participants in the TB-BMD GWAS were of mixed ancestry. The population stratification may not have been completely ruled out and may have influenced the causal estimates, although most participants were from population-based cohorts of European ancestry in the TB-BMD GWAS (22). Last, we did not thoroughly explore the mechanism underlying the causality between AAs and BMD. Therefore, mechanistic research should focus on specific AAs at cellular and individual levels in the future.

CONCLUSION

In summary, we provided precise evidence that the levels of certain AAs in the serum, namely, Ile and Val, were positively associated with TB-BMD; and Tyr was negatively associated with TB-BMD. We did not observe the statistically significant associations between AAs and site-specific BMD (FN, LS and FA-BMD). These findings underscore the important role that serum AAs play in the development of osteoporosis and provide evidence that osteoporosis can be treated and prevented by supplementing certain AAs. Future studies are needed to investigate the potential mechanisms by which AAs influence bone metabolism and to examine the potential role of these mechanisms in the treatment of osteoporosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YT, JH, and ZC conceptualized and designed the study. ZC and BH provided the package codes in R language and analyzed the data in the study. ZC drafted the manuscript. HF gave constructive suggestions when writing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.763538/full#supplementary-material

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Hyperglycemia Is Not Associated With Higher Volumetric BMD in a Chinese Health Check-up Cohort

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Background and Purpose: Type 2 diabetes mellitus patients have an increased fracture risk despite having higher areal bone mineral density (aBMD) measured by DXA. This apparent paradox might be explained by the overestimation of BMD by DXA due to the higher fat mass in type 2 diabetes mellitus patients. Volumetric BMD (vBMD) as assessed by quantitative CT (QCT) is not influenced by fat mass. We assessed the association of vBMD and fasting plasma glucose in a large cohort of Chinese subjects and compared the vBMD in healthy and diabetic subjects. In addition, we compared the relation between aBMD, vBMD, glucose and fat mass in a subset of this cohort.

Materials and Methods: 10309 participants from the China Biobank project underwent QCT based on chest low dose CT to compute vBMD of L1 and L2 vertebrae and FPG measurements between 2018 and 2019. Among them, 1037 subjects also had spine DXA scans. Data was analyzed using linear regression models.

Results: In the total cohort (5889 men and 4420 women, mean age 53 years, range 30-96), there was no significant association between vBMD and FPG after adjustment for age (women: p=0.774; men: p=0.149). 291 women and 606 men fitted the diagnostic criteria of diabetes. Both women and men with diabetes had lower vBMD compared to non-diabetic subjects, but this became non-significant after adjusting for age in the total cohort (women: p=0.817; men: p=0.288) and after propensity score matching based on age (women: p=0.678; men: p=0.135). In the DXA subcohort, aBMD was significantly higher in men with diabetes after adjusting for age and this difference disappeared after further adjusting for total fat area (p=0.064).

Conclusion: We did not find any effect of fasting plasma glucose or diabetes on the volumetric BMD measured with QCT after adjustment for age. Therefore, vBMD

measured with QCT might be a more reliable measurement to diagnose osteoporosis and assess fracture risk than aBMD measured with DXA in diabetic patients.

Keywords: fasting plasma glucose (FPG), type 2 diabetes, osteoporosis, areal bone mineral density (aBMD), volumetric bone mineral density (vBMD)

1 INTRODUCTION

Although type 2 diabetes mellitus and osteoporosis are common diseases in the ageing society, the relationship between these is less clear (1). Accumulating data has shown that the risk of osteoporotic fractures is increased in DM patients (2–5); a recent meta-analysis showed an increase in the risk of hip fracture in diabetes (type 1: relative risk (RR) 4.93, CI 3.06-7.95 and type 2: RR 1.33, CI 1.19-1.49) and for non-vertebral fractures (type 1: RR 1.92, CI 0.92-3.99 and type 2: RR 1.19, CI 1.11-1.28) (2). Contrary to the association between low bone mineral density (BMD) and diabetes consistently observed in type 1 DM patients, there is increasing evidence from recent studies indicating that type 2 diabetes mellitus patients have higher BMD compared to healthy subjects (1, 5, 6). Since higher BMD is associated with lower fracture risk in the general population, this apparent paradox might be explained by the overestimation of areal BMD (aBMD) by dual energy X-ray absorptiometry (DXA), the standard measurement method of BMD in clinical practice due to the higher fat mass in type 2 diabetes mellitus patients. Fracture risk prediction in type 2 diabetes mellitus becomes more challenging, since most fracture risk calculators, such as the FRAX tool, therefore underestimate fracture risk for individuals with diabetes due to this higher BMD (6). Furthermore, the associated under-treatment of bone fragility in type 2 diabetes mellitus patients could lead to inadequate fracture prevention (7).

DXA is a projectional method thus aBMD measurements are subject to variations in soft tissue thickness and composition. Algorithms used in commercial DXA scanners are based on assumptions about the homogenous disposition of fat in the body that are not generically valid (8). For example, obesity increases the likelihood of vertebral fracture but aBMD is known to increase with body weight in subjects with higher BMI. Volumetric BMD (vBMD in units of mg/cm^3) measured by quantitative computed tomography (QCT) is a three-dimensional measure that is much less affected by body size and soft tissue composition. However, vBMD has been sparsely applied in the investigation of the relationship between BMD and type 2 diabetes mellitus because it is less frequently performed in the clinical investigation of osteoporosis. It is still unknown whether vBMD measured with QCT is a better indicator of true skeletal status than aBMD in patients with diabetes.

Therefore, in the present study we investigate the relation between vBMD measured with QCT and fasting plasma glucose in a large cohort of Chinese subjects and compare the vBMD between subjects with and without diabetes. In addition, we aim to directly compare the association of vBMD and aBMD in subjects with and without diabetes and we hypothesize that body fat influences the association of aBMD and diabetes more than vBMD.

2 MATERIALS AND METHODS

2.1 Participants

Participants included in this study were a subset of the China Biobank project, a prospective nationwide multi-center cohort study studying osteoporosis, obesity, and fatty liver (6). This cohort has been registered with the US clinical trials database (clinicaltrials.gov; trial identifier: NCT03699228). Subjects in the present study were originally referred to the health management centers of the affiliated Yijishan Hospital of Wannan Medical University (4142 women, 5501men), and the affiliated hospital of Guiyang Medical University (278 women, 388 men), as part of their employers' health check-up programs, and received a low dose chest CT (LDCT) scan for lung cancer screening. A total of 5889 men and 4420 women were included in the study, which involved the post-scan processing of CT (QCT full cohort). No additional radiation was involved. Among the study participants, 444 women and 593 men had DXA scans of the lumbar spine (DXA subcohort). The study was approved by the ethics committee of Beijing Jishuitan hospital and each participant gave written informed consent for their data to be used.

2.2 Blood Sampling and Laboratory Analysis

The blood sampling and laboratory analysis are part of the health checkup procedure and were described in detail previously (9). After an overnight fast, blood samples were drawn and fasting plasma glucose (FPG) concentration was measured using the hexokinase method. All tests and analyses were conducted in a certified clinical examination center at each of the collaborating medical centers. Diabetes was defined as FPG ≥ 7.0 mmol/L according to the diagnostic criteria of the American Diabetes Association (10) and/or use of antihyperglycemic medication and/or self-reported diagnosis of diabetes.

2.3 Anthropometry and Other Covariates

Weight (kg) and height (m) were measured using calibrated digital scales and stadiometers and body mass index was calculated [BMI = weight (kg)/height (m)^2]. Information on antidiabetic medication was restricted to insulin and/or oral antidiabetic medications or no medication use. Total abdominal fat area (TFA) was determined at the level of the 2nd lumbar vertebra (L2) by CT.

2.4 QCT and DXA Scans

The details of the China Biobank study protocol have been published elsewhere (9). LDCT scans were conducted on an Optima CT540 CT scanner (GE Healthcare, WI, USA) at the Wannan center and a Supria CT scanner (Hitachi, Tokyo, Japan) at the Guiyang center. The LDCT was performed according to the same protocol at both centers. Mindways QCT Pro (Mindways Software, Inc., Austin, TX, USA) was used for all QCT vBMD measurements and all CT scans were

acquired at 120 kVp. LDCT is now the standard for lung cancer screening and the subsequent analysis of these CT scans enabled evaluation of vBMD at L1 and L2 using the Mindways QCT Pro software calibrated with a QCT asynchronous phantom (Mindways, Austin, TX, USA). Osteoporosis was defined by an average vBMD at L1 and L2 < 80 mg/cm^3. The European spine phantom (ESP 145) was scanned 10 times on each QCT system for quality control. The quality assurance (QA) results showed the ESP vBMD measured at each center differed by less than 5 mg/cm^3 on average. Therefore, the original vBMD was used for further analysis. Based on 10 repeated scans of the ESP at each participating center the median coefficient of variation (%CV) for the L1–L3 ESP vBMD was 0.48% (range, 0.31% to 1.20%) (11). All data were transferred to the Data Management Center (Beijing Jishuitan hospital) for data cleaning and analysis.

DXA measurements of aBMD and lumbar spine projected area were conducted using GE Lunar DXA (GE Lunar Prodigy and DPX Bravo DXA scanners, GE Healthcare, WI, USA) systems, GE Lunar Encore software and GE Lunar positioning devices to enable consistency and accuracy of patient positioning. The lumbar spine (L1–L4) scan was performed at the Wannan Centre and Guiyang Centre. DXA and LDCT were performed on the same day. Osteoporosis was defined as a T-score < -2.5. All data were transferred to the Data Management Centre (Beijing Jishuitan Hospital) for data cleaning and analysis.

2.5 Statistical Analysis

Continuous data were described by the mean and standard deviation (SD), and percentages were calculated for categorical variables. Differences between DM and controls groups were analyzed using student-t tests or Mann-Whitney U tests for continuous variables, and the Chi-square test for categorical variables. General linear models were fitted using the method of least squares to evaluate associations of glucose and vBMD. Both sex-specific continuous variables of glucose and vBMD were evaluated in unadjusted and adjusted general linear models, adjusted by age. To control for the potentially confounding factor of age, propensity score matching (PSM) was applied to match subjects for diabetic patients. The propensity score was calculated with logistic regression and matched using the method of nearest neighbor matching with a caliper of 0.1. The balance test of propensity score matching was carried out by using standard difference. Wilcoxon matched pairs signed rank test was used for the comparison after PSM. Because the age distribution of the study population differed from that of the Chinese population as a whole, the sex-specific prevalence of osteoporosis was standardized using the China Biobank study prevalence for each 2-year age group and the most recent Chinese population data (2010 China Census Data) (11). All statistical analyses were performed using the IBM SPSS Statistic 24 and R 3.64 software. A p-value < 0.05 was taken to be statistically significant.

3 RESULTS

3.1 Baseline Parameters

3.1.1 QCT Full Cohort

Baseline characteristics of the subjects are presented in **Table 1**. Of the 4420 women, 291 fitted the diagnostic criteria of diabetes (49 by FPG >7.0 mmol/L and 242 by health check records). Women with diabetes were significantly older (63 versus 51 years) and had a higher BMI (24.6 vs 23.1) than the non-diabetes women. Of the 5889 men, 606 fitted the diagnostic criteria of diabetes (163 by FPG >7.0 mmol/L and 443 by health check records). The men with diabetes were significantly older (59 versus 52 years) and had a slightly but significantly higher BMI (25.0 vs 24.5) than the non-diabetes men. Women had a mean vBMD of 135.8 mg/cm^3 and 12.1% of the women met the definition of osteoporosis (OP), men had a mean vBMD of 130.7 mg/cm^3 and 6.5% met the definition of osteoporosis. The prevalence of OP was significantly higher in women with diabetes (37.5% vs 10.4%), but following age-standardization using the 2010 China Census Data (11), the estimated prevalence of osteoporosis was similar, the adjusted OP rates for DM women being 12.8% and non-DM women 12.1%.

TABLE 1 | Characteristics of participants with and without diabetes mellitus (DM) in the QCT full cohort.

	Women				Men			
	Total	**Non-DM**	**DM**	**P**	**Total**	**Non-DM**	**DM**	**P**
N	4420	4129	291		5889	5283	606	
Age	51.5 ± 11.2	50.6 ± 10.6	63.8 ± 11.2	<0.001	52.9 ± 11.8	52.1 ± 11.6	59.4 ± 11.7	<0.001
Height	157.6 ± 5.5	157.7 ± 5.5	155.8 ± 5.6	<0.001	168.3 ± 5.7	168.4 ± 5.6	167.1 ± 5.8	<0.001
Weight	57.6 ± 7.9	57.4 ± 7.8	59.8 ± 9.2	<0.001	69.7 ± 9.4	69.6 ± 9.4	69.8 ± 9.4	0.731
BMI	23.2 ± 3.0	23.1 ± 2.9	24.6 ± 3.2	<0.001	24.6 ± 2.9	24.5 ± 2.9	25.0 ± 3.0	<0.001
FPG	5.1 ± 1.0	4.9 ± 0.5	7.6 ± 2.5	<0.001	5.3 ± 1.3	5.0 ± 0.6	7.9 ± 2.4	<0.001
vBMD	135.8 ± 44.2	138.4 ± 43.4	99.1 ± 38.4	<0.001[a]; 0.817[b]	130.7 ± 34.0	131.7 ± 35.2	121.7 ± 30.8	<0.001[a]; 0.288[b]
N of Osteopenia(%)	1059(24.0)	959(23.2)	100(34.4)	<0.001[a] 0.484[c]	1850(31.4)	1593(30.2)	257(42.4)	<0.001[a] 0.105[c]
N of OP(%)	537(12.1)	428(10.4)	109(37.5)	<0.001[a]; 0.287[d]	383(6.5)	334(6.3)	49(8.1)	0.095[a]; <0.001[d]

[a]unadjusted; [b]adjusted for age; [c]adjusted for age using the QCT population age' (adjusted Osteopenia rates for Non-DM women 24.0%, DM women 23.4%, Non-DM men 31.1% and DM men 32.5%, respectively); [d]adjusted for age using the QCT population age, (adjusted OP rates for Non-DM women 12.1%, DM women 12.8%, Non-DM men 6.9% and DM men 4.5% respectively).

For the total population, among the men 1.5% were underweight (BMI<18.5), 54.6% were normal (BMI 18.5-24.9), 40.3% were overweight (BMI 25-29.9) and 3.6% were obese (BMI 30-39). Among the women, the percentages were 2.9%, 72.4%, 22.3% and 2.4%, respectively.

N, number; BMI, body mass index; FPG, fasting plasma glucose; vBMD, volumetric bone mineral density; OP, osteoporosis.

3.1.2 DXA Subcohort

Baseline characteristics of the subjects are presented in **Table 2**. Of the 444 women, 32 fitted the diagnostic criteria of diabetes. Women with diabetes were significantly older (61 versus 52 years) and had a higher BMI (25.6 vs 23.4) and abdominal total fat area (304 versus 242 cm^2) than the non-diabetes women. Of the 593 men, 80 fitted the diagnostic criteria of diabetes. The men with diabetes were significantly older (55 versus 50 years) and, although the BMI was similar, the total fat area of the abdomen was significantly higher (295 vs 264 cm^2) than in the non-diabetes men. Women had a mean aBMD of 1.00 g/cm^2 and 14.2% of the women met the definition of osteoporosis (OP), men had a mean aBMD of 1.06 g/cm^2 and 4.9% met the definition of osteoporosis. The prevalence of OP was significantly higher in women with diabetes (40.6%), but following age-standardization using the 2010 China Census Data (11), the estimated prevalence of osteoporosis was similar, the adjusted OP rates for DM women being 10.6% and non-DM women 11.1%.

3.2 Association of BMD With FPG

3.2.1 QCT Full Cohort

There was no significant association between vBMD and FPG after adjustment for age (men: p=0.149; women: p=0.774) (**Figure 1** and **Figures S1**, **S2**).

3.2.2 DXA Subcohort

After adjustment for age, a significant association with FPG was observed for aBMD in men (p=0.011) but not in women (p=0.203) or for vBMD (men: p=0.775; women: p=0.403) (**Figure 2**).

3.3 Comparison of BMD Between Diabetes Patients and Healthy Subjects

3.3.1 QCT Full Cohort

Table 1 shows that subjects with diabetes have lower vBMD compared to healthy subjects in both men and women. However, after adjusting for age, no significant difference was observed. To investigate this further, we used propensity score matching. After PSM using age, 277 women with type 2 diabetes mellitus were matched with 277 healthy controls and 592 type 2 diabetes mellitus men with 592 healthy controls (**Table 3**). **Table 3** also shows that, as expected, type 2 diabetes mellitus patients have higher BMI and FPG although the differences are small. In the 277 women with FPG concentrations in the diabetic range (7.6 ± 2.5 mmol/L) compared to women with FPG concentrations in the normal range (5.0 ± 0.5 mmol/L) (**Table 3**), vBMD was not significantly different (99.8 vs 99.6 mg/cm^3, p=0.678). In 592 men matched for age with fasting plasma glucose levels in the normal (5.1 ± 0.6 mmol/L) versus in the diabetic (7.9 ± 2.3 mmol/L) range, vBMD was also not significantly different (119.4 vs 121.5 mg/cm^3, p=0.135).

3.3.2 DXA Subcohort

Table 2 demonstrates comparisons of participants with DXA-derived aBMD and QCT-derived vBMD between DM and non-DM. Before adjusting for age, interestingly both vBMD and aBMD were significantly lower in women with diabetes compared to non-diabetic women whereas, as expected according to our hypothesis, vBMD was lower but aBMD was higher in men with diabetes compared to men without diabetes. After adjusting for age, only the aBMD remained significantly higher in men with diabetes. But finally, after adjusting for total fat area, also this difference disappeared.

After PSM using age, 29 women with type 2 diabetes mellitus were matched with 29 healthy controls and 79 type 2 diabetes mellitus men with 79 healthy controls (**Table 4**). **Table 4** also shows that, as expected, type 2 diabetes mellitus patients have higher BMI and FPG although the differences are small. In the 277 women with FPG concentrations in the diabetic range (7.6 ± 2.5 mmol/L) compared to women with FPG concentrations in the normal range (5.0 ± 0.5 mmol/L) (**Table 3**), vBMD as well as aBMD were not significantly different (vBMD 100.5 vs 98.4 mg/cm^3, p=0.738; aBMD 0.91 vs. 0.91,

TABLE 2 | Comparisons of participants with DXA-derived aBMD and QCT-derived vBMD between DM and Non-DM in DXA subcohort.

	Women						Men					
	Total	**Non-DM**	**DM**	**P**	**p1**	**p2**	**Total**	**Non-DM**	**DM**	**P**	**p1**	**p2**
N	444	412	32				593	513	80			
Age(years)	52.7 ± 10.1	52.0 ± 9.9	61.2 ± 9.7	<0.001			50.5 ± 9.9	49.9 ± 9.6	54.7 ± 10.3	<0.001		
Height	156.0 ± 5.6	156.3 ± 5.5	152.6 ± 5.6	<0.001			168.5 ± 5.7	168.7 ± 5.8	167.3 ± 6.2	0.043		
Weight	57.3 ± 8.2	57.1 ± 8.2	59.6 ± 8.6	0.100			70.6 ± 9.5	70.5 ± 9.4	71.1 ± 10.2	0.616		
BMI	23.6 ± 3.2	23.4 ± 3.2	25.6 ± 2.9	<0.001			24.8 ± 3.0	24.8 ± 3.0	25.4 ± 3.1	0.094		
FPG	5.05 ± 0.84	4.90 ± 0.51	7.04 ± 1.51	<0.001			5.42 ± 1.67	4.97 ± 0.58	8.30 ± 2.94	<0.001		
TFA(cm^2)	246 ± 96	242 ± 95	304 ± 93	<0.001			268 ± 100	264 ± 99	295 ± 104	0.01		
vBMD(L1-2)	128 ± 43	131 ± 42	94 ± 35	<0.001	0.139	0.254	130 ± 31	131 ± 31	125 ± 27	0.124	0.471	0.367
aBMD(L1-2)	1.00 ± 0.17	1.01 ± 0.16	0.89 ± 0.16	<0.001	0.292	0.195	1.06 ± 0.14	1.06 ± 0.13	1.09 ± 0.15	0.034	0.022	0.064
N of OP(%)	63(14.2)	50(12.1)	13(40.6)	<0.001[a]; 0.459 [b]			29(4.9)	25(4.9)	4(5.0)	0.961[a]; <0.001[b]		

[a]*unadjusted;* [b]*adjusted for age using the QCT population age, (adjusted OP rates for Non-DM women 11.1%, DM women 10.6%, Non-DM men 5.9%, DM men 2.2%, respectively).*
p1: adjusted for age.
p2: adjusted for age and TFA.
TFA, total fat area of the abdomen at L2 level; aBMD, areal bone mineral density.

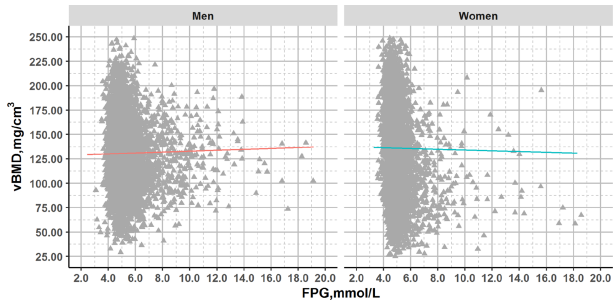

FIGURE 1 | Plots of FPG and vBMD in QCT full cohort with glucose concentrations across the range from normal to diabetes. Association lines (adjusted for age): Men: y=0.431x+128.501, R^2 = 0.000, p>0.05; Women: y=-0.125x+136.574, R^2 = 0.000, p>0.05.

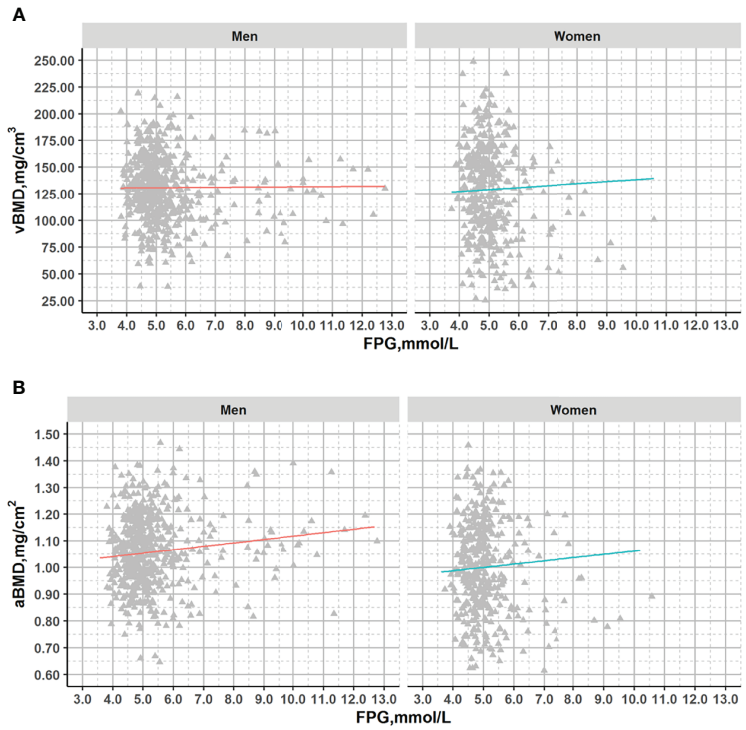

FIGURE 2 | Plots of vBMD and aBMD with fasting glucose across the range from normal to diabetes in DXA subcohort. Association lines (adjusted for age): **(A)**. Men: y=-0.190x+131.714, R^2 = 1.49*10^{-4}, p=0.775; Women: y=1.964x+119.133,R^2 = 0.003,p=0.304; **(B)**. Men: y=0.009x+1.013, R^2 = 0.011,p=0.016; Women: y=0.012x+0.944, R^2 = 0.004,p=0.203;.

TABLE 3 | Characteristics of matched participants with and without DM using propensity score in the QCT full cohort.

	Women			Men		
	Non-DM	**DM**	**P**	**Non-DM**	**DM**	**P**
N	277	277		592	592	
Age	63.6 ± 10.9	63.6 ± 10.9	1.000	59.3 ± 11.6	59.3 ± 11.6	1.000
BMI	23.7 ± 3.0	24.6 ± 3.2	0.001	24.1 ± 2.9	25.0 ± 3.0	<0.001
FPG	5.0 ± 0.5	7.6 ± 2.5	<0.001	5.1 ± 0.6	7.9 ± 2.3	<0.001
vBMD	99.8 ± 42.2	99.6 ± 38.7	0.678	119.4 ± 33.7	121.5 ± 30.8	0.135
N of OP (%)	96(34.7)	104(37.5)	0.479	62(10.5)	48(8.1)	0.161

Propensity score matching by age.

TABLE 4 | Characteristics of matched participants with and without DM using propensity score in the DXA subcohort.

	Matched population[1]						Matched population[2]					
	Women			Men			Women			Men		
	Non-DM	DM	P	Non-DM	DM	P	Non-DM	DM	P	Non-DM	DM	P
N	29	29		79	79		30	30		79	79	
age	59.8 ± 8.7	59.8 ± 8.7	1.000	54.3 ± 9.7	54.3 ± 9.7	0.705	61.0 ± 8.6	60.8 ± 9.7	0.665	55.3 ± 10.6	54.5 ± 10.2	0.359
BMI	23.26 ± 2.61	25.73 ± 2.85	0.003	24.42 ± 2.98	25.40 ± 3.09	0.048	24.29 ± 2.83	25.35 ± 2.87	0.075	24.76 ± 2.62	25.33 ± 3.12	0.173
FPG	5.10 ± 0.68	6.85 ± 1.60	<0.001	4.97 ± 0.63	8.19 ± 2.94	<0.001	5.08 ± 0.56	6.66 ± 1.52	<0.001	5.08 ± 0.64	8.15 ± 2.95	<0.001
TFA	260.67 ± 82.51	314.76 ± 84.49	0.048	243.59 ± 98.27	296.13 ± 104.63	0.005	292.22 ± 83.79	293.75 ± 86.00	0.781	280.01 ± 97.88	293.03 ± 103.14	0.395
vBMD	100.53 ± 43.38	98.39 ± 34.13	0.738	127.35 ± 29.75	125.52 ± 28.78	0.642	104.88 ± 34.31	94.09 ± 36.37	0.141	127.46 ± 34.31	125.71 ± 28.69	0.528
aBMD	0.91 ± 0.20	0.91 ± 0.16	0.430	1.05 ± 0.14	1.09 ± 0.16	0.074	0.92 ± 0.15	0.89 ± 0.17	0.658	1.08 ± 0.15	1.09 ± 0.16	0.903

[1]*PSM by age;* [2]*PSM by age and TFA.*

p=0.430). In 79 men matched for age with fasting plasma glucose levels in the normal (5.0 ± 0.6 mmol/L) versus in the diabetic (8.2 ± 2.9 mmol/L) range, vBMD was not significantly different (vBMD: 119.4 vs 121.5 mg/cm$^{3)}$, however the aBMD showed a trend towards higher aBMD in in men (aBMD 1.05 vs. 1.09, p=0.074). In addition, we used PSM with age and BMI to clarify the role of fat tissue and in men, the difference indeed disappeared (aBMD 1.08 vs. 1.09, p=0.903).

4 DISCUSSION

As a main result we did not find any association of vBMD with fasting plasma glucose across the healthy to diabetic range in this large cohort of >10.000 subjects. Also, when comparing subjects with and without diabetes, vBMD was similar after adjustment for age in men and women. Considering that diabetes nowadays is a prevalent condition worldwide with ever increasing numbers, it is important to be able to adequately predict fracture risk, initiate treatment and prevent fractures in these patients (12). This study confirms that vBMD measured with QCT is not affected by diabetes or fasting plasma glucose concentration i.e. does not overestimate BMD and therefore could be used as a reliable estimate of BMD to assess fracture risk in diabetes.

Although the diagnosis of diabetes is defined by a fasting plasma glucose above a threshold of 7.0 mmol/L, there is a continuum of fasting plasma glucose concentrations from normal to impaired fasting glucose (IFG) to diabetic where the risk of diabetes complications is progressively increasing with increasing fasting plasma glucose concentrations (13, 14). In addition, many patients are unaware of their diabetes for years and are often diagnosed by screening or based on the manifestation of complications. Indeed, a recent study showed that in 170.000 Chinese subjects with a mean age of 44 years, the rate of diabetes based on HbA1c measurements was 10.9% of which only 4% was previously diagnosed and 38% fitted the diagnosis of prediabetes (15).

Most studies assessing BMD and diabetes/glucose measured areal BMD with DXA instead of QCT and reported a higher BMD in diabetes subjects (1, 3, 5, 6, 16–18). In our QCT full cohort, a subgroup of subjects also underwent DXA scanning in addition to QCT. QCT results in this DXA subcohort were comparable to those in the full cohort. Interestingly, in these subjects, we found a higher aBMD only in men but the number of women with diabetes was small (N=29) precluding any conclusions from these data. In men (N=79), we confirmed the higher aBMD, also after adjustment for age. Since QCT scans were available of these patients, we could measure total fat area of the abdomen (TFA) with this state-of-the-art technique (19) and we showed that after adjustment for TFA the higher aBMD in men was indeed no longer significantly different between diabetic and non-diabetic subjects. This result, although obtained in a small group of subjects, indeed supports the common notion of the overestimation of aBMD due to overlying soft tissue. A very recent study showed that diabetes increased aBMD by increasing obesity-related indexes (20). Several other studies also indicated

that aBMD was associated with BMI and that differences in aBMD between diabetic and nondiabetic subjects disappeared after adjustment for BMI (21–23).

In the large Diabetes Heart Study BMD was measured by DXA and QCT (22). There was a very weak correlation of vBMD measured at the lumbar spine with BMI but in the total cohort there was no difference in age adjusted vBMD of the lumbar spine between diabetic (T2DM, n=808) and non diabetic (n=106) subjects. In contrast the age adjusted aBMD difference of the lumbar spine and total hip was significant and in agreement with our results and the studies cited above disappeared after adjustment for BMI.

Our results are similar to another recent QCT-based study in a Chinese population of 4000 subjects of which 600 had diabetes, showing that, without adjustment for age, vBMD was lower in impaired fasting glucose and diabetic patients. Unfortunately, age adjusted data were not presented in that study although there was a significant age difference between the groups (normal 47 years, IFG 53, diabetes 55 years) (24) and many studies including the current one have shown significant vBMD decreases with age (**Figure S1**).

In contrast to the existing literature and to our results in men, we did not find a positive association between areal BMD and glucose in women or a higher areal BMD in women with diabetes, although the number of women in the latter analysis was small (N=29) and needs to be interpreted with caution. This is a limitation of our study. Several factors could explain this sex difference and/or incongruity within the literature; I] sex and menopausal status; BMD accrual, peak bone mass and bone loss are different between men and women and menopause has a profound effect on bone remodeling, therefore analyses should be stratified for age and menopausal status. II] ethnicity and BMI; although the prevalence of diabetes is comparable in Western and Chinese societies, the BMI at which patients develop diabetes is very different and perhaps more importantly BMI does not capture differences in body composition (15, 25). Only adjustment for TFA but not for BMI eliminated aBMD differences between men with and without diabetes. It is important to note that characteristics of the DM population in our study were similar to DM patients across China, which were characterized in a recent study (15). III] age is an important determinant of BMD and diabetes becomes more likely with aging. Therefore adjusting analyses for age is of paramount importance IV] diabetes duration, treatment and glycemic control; many complications of diabetes tend to become more frequent with longer duration of diabetes and poorer glycemic control (7, 13, 26). In addition, diabetes treatment such as insulin or thiazolidinediones can also impact on bone mass. It is another limitation of our study that data on the duration or diabetic treatment were missing in our cohort, but we do conclude from our data that most patients were well controlled considering their mean fasting plasma glucose of 7.6 mmol/L. V] location of BMD measurement; BMD is commonly measured at the spine or hip, however these sites are not interchangeable and can be differentially affected in certain disease states depending on the effect on trabecular (spine) or cortical (hip) bone of the underlying disease (4, 27). As another limitation, in this study, we had only spine BMD data available. Given the fact that vertebral fracture risk in type 2 diabetes mellitus diabetes is not or only marginally increased, the hip may be the preferred anatomical site to assess. VI] type of diabetes; type 1 diabetes patients have a lower areal and volumetric BMD and a much higher fracture risk than type 2. It is also a limitation that we did not have data available on diabetes type of our patients, type 2 diabetes is much more common than type 2 (95% versus 5% of all diabetes patients), therefore we assumed that the vast majority of our patients would be type 2.

However, strengths of our study include the large number of subjects included in this cohort (>10.000) and the state-of-the-art measurement technique of vBMD and TFA with QCT.

In conclusion, we did not find any effect of fasting plasma glucose or diabetes on the volumetric BMD measured with QCT after adjustment for age. Therefore, without additional adjustments for body composition, vBMD measured with QCT might be a more reliable measurement to assess osteoporosis and fracture risk than aBMD measured with DXA in diabetic patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data for The China Biobank are not presently available to be shared. These data might be available at a future stage. Requests to access the datasets should be directed to xiao65@263.net.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Beijing Jishuitan Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LW, XZ, LR, AV, and XC designed the study. KZ led the analysis with input from LW, HS, XZ, LR, LX, AV, KE, and YY. YL, LX, and QS did the literature search. YY, QS, and YL collected the data. YY and QS did the measurements. All authors contributed to data interpretation. LW and KZ wrote the manuscript and all authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.794066/full#supplementary-material

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The Efficacy of Nitrates for Bone Health: A Systematic Review and Meta-Analysis of Observational and Randomized Controlled Studies

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Background: Although some studies have found that nitrates were beneficial for bone health, the findings are inconsistent. To assess the efficacy of nitrates for bone health, we conducted a meta-analysis.

Methods: PubMed, EMBASE databases, Cochrane Library for relevant articles published before December 2021 were searched. All observational and randomized controlled studies that reporting bone mineral density (BMD), fractures with nitrates use were included. A meta-analysis was performed to calculate risk ratios (RRs) for fractures, change differences for bone mineral density.

Results: Four cohort studies and two case-control studies examining the association between nitrates use and fractures were identified. The nitrates use was not associated with any fracture risk (RR = 0.97; 95% CI, 0.94–1.01; I^2 = 31.5%) and hip fracture (RR = 0.88; 95% CI, 0.76–1.02; I^2 = 74.5%). Subgroup analyses revealed no differences in fracture risk, whereas two cohort studies revealed a reduced risk of hip fracture (RR = 0.71, 95% CI, 0.58–0.86, I^2 = 0.0%). There were no statistically significant differences in BMD percent changes at lumbar spine (WMD = -0.07, 95% CI,-0.78–0.65; I^2 = 0.0%), total hip (WMD = -0.42, 95% CI,-0.88–0.04; I^2 = 0.0%), femoral neck (WMD = -0.38, 95% CI,-1.02–0.25; I^2 = 0.0%), or total body (WMD = -0.17, 95% CI,-0.51–0.17; I^2 = 0.0%) in two randomized controlled trials (RCTs) compared with a placebo. Another two RCTs compared nitrates with alendronate. Nitrates were comparable to alendronate in increasing bone mineral density at lumbar spine (WMD = 0.00, 95% CI,-0.01–0.02; I^2 = 0.0%). Besides, the most common adverse effect was headache, contributing to low adherence to therapy.

Conclusion: Our meta-analysis showed no association between nitrates use and fractures in observational studies. The results of RCTs on the usage of nitrates and their effects on BMD were inconsistent. High-quality, long-term studies are needed to clarify the efficacy of nitrates for bone health.

Keywords: nitrates, bone health, fracture, bone mineral density, meta-analysis

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INTRODUCTION

Osteoporosis, defined as a decrease in bone mineral density (BMD) and an increase in bone fragility, is a major public health issue that affects both men and women around the world (1, 2). The population aged 50 or more who are at high risk of osteoporotic fracture was predicted to be 158 million in 2010, and this number is expected to double by 2040 (3). Bone fractures are connected with significant disability and morbidity, as well as a significant financial burden on injured individuals (4).

Nitrates (isosorbide mononitrate, isosorbide dinitrate, nitroglycerin), which are a type of angina medicine (5), appear to have beneficial effects on bone. These drugs, which act as nitric oxide donors, uncouple bone resorption and formation, resulting in improved bone metabolism (6). Nitric oxide has been shown to regulate osteoclasts, which are responsible for bone resorption (7). Besides, low NO levels have been shown to improve osteogenic proliferation, differentiation, and survival (8). However, higher concentrations inhibit osteoclast differentiation and survival (9). Animal studies have suggested that nitric oxide donors may increase bone mass by regulating osteoblast and osteoclast functions in ovariectomized mice (10). According to two epidemiological studies (11, 12), people who use nitrates had higher BMD and lower rates of bone turnover. However, one cohort study found no evidence that nitrate use was related to a decreased incidence of fractures or a higher BMD (13). Furthermore, the results of two randomized controlled trials (RCTs) that examined the effects of nitroglycerin ointment on BMD were contradictory (14–16).

Recently, several clinical trials evaluating the efficacy of nitrates for bone health have been reported. To our knowledge, no comprehensive meta-analysis on this topic has been performed. To determine the effect of nitrates on bone health, a comprehensive systematic review and meta-analysis based on an extensive search of observational and randomized controlled trials is required.

METHODS

Search Strategy

The Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines were used for randomized controlled trials (RCTs) (17), and the Meta-Analyses and Systematic Reviews of Observational Studies guidelines were used for observational studies (18). Two independent reviewers (Liu and Wang) systematically searched PubMed, EMBASE database, Cochrane Library for relevant articles published before December 2021. An experienced librarian was consulted to generate a list of keywords and MeSH terms to conduct the search. The detailed search strategies are described in the **Supplementary Table 1**. Additional researches were discovered by searching the references of relevant research and review publications.

Selection Criteria

Eligible studies were included if they fulfilled the following criteria (1): cohort studies, case-control studies, or randomized controlled trials (2), reported on bone mineral density (BMD), incident fractures with nitrates use (3), the reference group were non- nitrates users (3), studies provided adequate data for the efficacy estimates. The exclusion criteria were as follows (1): duplicate articles (2), molecular biology or animal research, and (3) reviews, case reports, letters, editorials, and meta-analyses. Two investigators (Liu and Wang) independently screened the articles by title and abstract after removing duplicate articles. Then, the full texts were obtained to identify the eligible studies. Disagreements in the study selection process were fully discussed and resolved through consultation with Meng.

Data Extraction and Quality Assessment

The following information was extracted from each study: the first author's name, the year of publication, the study design, the country, the interventions and co-interventions, the sample size, age, BMD, the duration of follow-up, and reported outcomes, including effect sizes (risk ratios (RRs), odds ratios (ORs), hazard ratios (HRs), BMD percent change, or BMD change) and adverse events. We extract the reported outcomes of the final time point for RCTs. If standard deviations were not reported, we used the confidence intervals to calculate the standard deviation. We used image extraction software (Engauge Digitizer) to extract data presented only in figures without corresponding numerical data.

We evaluated the quality of included RCTs using the Cochrane Risk of Bias tool (19), the quality of included observational studies was evaluated using the Newcastle–Ottawa Scale (NOS) (20). The data extraction and quality assessments were conducted independently by two authors (Liu and Wang).

Data Analysis

The Stata 12.0 software was used to conduct the analysis. ORs were used as approximations of RRs since the incidence of fracture is so low (less than 5% per year). HRs, ORs, and RRs were extracted from the included studies. The pooled risk ratios (RRs) with 95% confidence intervals (CIs) from HRs and ORs were calculated using a random-effects model. Because most RCTs provided within-group changes in BMD outcomes, we used the reported or computed difference between the nitrates and reference groups as the effect size measure in the meta-analysis for BMD outcomes. We conducted meta-analyses when data from at least two trials were sufficiently homogenous in terms. To measure heterogeneity across trials, the I^2 and Q statistics were used. $I^2 > 50\%$ and $P < 0.05$ showed high heterogeneity across the studies examined. When significant heterogeneity was detected, subgroup analyses were performed to investigate the reasons for the heterogeneity. The Begger and Egger test was used to assess the publication bias of the studies included in the final analysis.

RESULTS

After conducting a literature search, we discovered 471 possibly eligible studies. After removing duplicates from the 471 papers

retrieved, 379 were left, with 29 of them being chosen as potentially suitable after reviewing the titles and abstracts. After examining full texts, 10 were included for data extraction in our meta-analysis (four cohort studies, two case-control studies, and four RCTs). The literature search process is illustrated in **Figure 1**.

Study Characteristics

There were 10 (11, 13–16, 21–25) studies included in our meta-analysis. Detailed characteristics of the included studies are presented in **Tables 1** and **2**. They were published between 1998 and 2020, including four cohort studies, two case-control studies, and four randomized controlled trials. Three studies were conducted in North America, four in Europe, one in Oceania, one in Asia, and one in Africa. Six studies reported BMD, and six studies reported fractures. Besides, two studies compared nitrates with a placebo, and two studies compared nitrates with alendronate. As indicated in **Table 1**, the NOS scores ranged from eight to nine points, indicating that all the observational studies chosen were of good quality. We classified RCT studies as having a low, uncertain, or high risk of bias (**Table 3**). There are two studies with a low risk of bias, one study with an uncertain risk of bias, and one study with a high risk of bias.

Main Analysis

Four cohort studies and two case-control studies examining the association between nitrates use and fractures were identified. As shown in **Figure 2**, the nitrates use was not associated with any fracture risk (RR = 0.97; 95% CI, 0.94–1.01; I^2 = 31.5%) and hip fracture (RR = 0.88; 95% CI, 0.76–1.02; I^2 = 74.5%). Two RCTs compared nitrates with a placebo. As shown in **Figure 3**, there were no statistically significant differences in BMD percent change at lumbar spine (WMD = -0.07, 95% CI,-0.78–0.65; I^2 = 0.0%), total hip (WMD=-0.42, 95% CI,-0.88–0.04; I^2 = 0.0%), femoral neck (WMD=-0.38, 95% CI,-1.02–0.25; I^2 = 0.0%), or total body (WMD = -0.17, 95% CI,-0.51–0.17; I^2 = 0.0%). Two randomized controlled trials (RCTs) compared nitrates with alendronate. As shown in **Figure 4**, nitrates were comparable to alendronate in increasing bone mineral density at lumbar spine (WMD = 0.00, 95% CI,-0.01–0.02; I^2 = 0.0%).

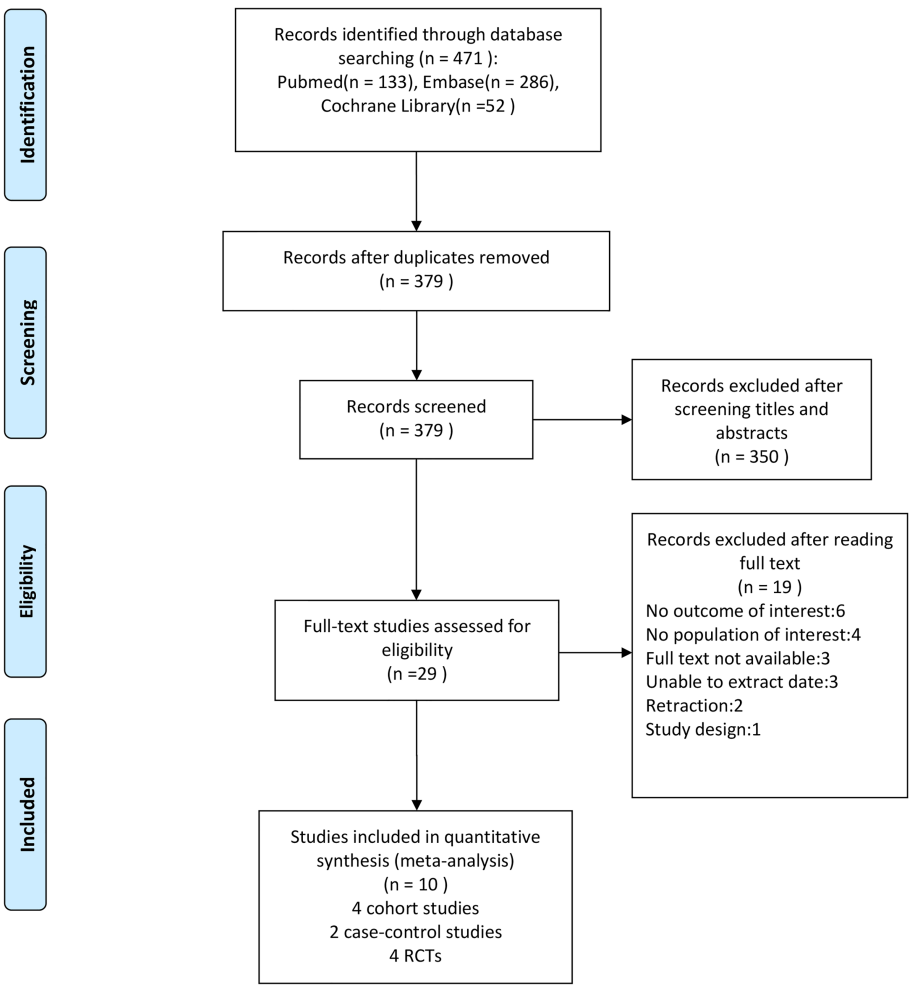

FIGURE 1 | Flow diagram of the literature search process and study inclusion.

TABLE 1 | Characteristics of the six included observational studies.

Author (year)	Study design	Country	Study population characteristics	Sample size (treatments or cases/controls)	Nitrate types	Mean age (Year) (treatments or cases/controls)		Study period	Outcomes	NOS quality score
Jamal et al. (11)	Cohort	USA	Elderly women	Daily(n=317), Intermittent(n=74)/ Nonusers(n=5827)	NG, ISDN or ISMN	79 ± 5; 77 ± 5	76 ± 5	1992-1994	BMD, Fracture risk (HR)	8
Torstensson et al. (24)	Cohort	Denmark	Aged 65 years or older	66931/725692	Nitrates	70.6 ± 8	77.3 ± 7.5	1999-2012	Fracture risk (HR)	9
Golchin et al. (13)	Cohort	USA	Postmenopausal women	137564/1647	NG, ISDN or ISMN	63.1	67.9	1993-1998	BMD, Fracture risk (HR)	8
Misra et al. (23)	Cohort	UK	60 years or older with diagnosis of ischemic heart disease	14451/14451	NG, ISDN or ISMN	72.4 ± 7.6	72.4 ± 7.6	1986-2011	Fracture risk (HR)	8
Rejnmark et al. (22)	Case-control	Denmark	Danish population	124655/373962	NG, ISDN or ISMN	42	42	1977-2000	Fracture risk (OR)	9
Pouwels et al. (21)	Case-control	Dutch	At least 18 years old	6763/26341	NG, ISDN or ISMN	>18	>18	1991-2002	Fracture risk (OR)	9

NG, nitroglycerin; ISDN, isosorbide dinitrate; ISMN, isosorbide mononitrate.

Four RCTs reported on the adverse events of nitrates use (**Table 2**). The most common adverse effect was headache (14%–31.1% incidence), contributing to low adherence to therapy. Other adverse effects included palpitations, nausea, flushing, and diaphoresis.

Subgroup Meta-Analyses

In the subgroup meta-analyses, the risk of fracture is shown in **Table 4**. When the selected studies for any fracture were grouped by study design, no significant association was seen in the three cohort studies (RR = 1.00; 95% CI, 0.97–1.03; I^2 = 0.0%). However, a negative association between the use of nitrates and any fracture risk was found only in one case-control study (RR = 0.95; 95% CI, 0.92–0.98). Two cohort and two case-control studies evaluated the association between nitrates use and hip fracture risk. The overall pooled RR for cohort studies was 0.71 (95%CI: 0.58–0.86, I^2 = 0.0%), while the pooled RR for case-control studies was 0.98 (95%CI: 0.92–1.04, I^2 = 19.3%).

Grouping of studies by NOS score revealed no significant association between the nitrates use and the any fracture risk in both the 9 point groups (RR = 0.97; 95% CI, 0.93–1.03; I^2 = 81.2%) and 8 point groups (RR =0.97; 95% CI, 0.87–1.09; I^2 = 0.0%). However, there was a significant association of nitrates with hip fracture in 8 point groups (RR, 0.71; 95% CI, 0.58–0.89; I^2 = 0.0%), but no significant association in 9 point groups (RR, 0.98; 95% CI, 0.92–1.04; I^2 = 19.3%)

When we grouped studies by region, we found no significant association between the nitrates use and the any fracture risk in North America (RR = 0.97; 95% CI, 0.87–1.09; I^2 = 0.0%) and Europe (RR = 0.97; 95% CI, 0.93–1.03; I^2 = 81.2%). The pooled RR for the hip fracture risk of North American people with nitrates was 0.81 (95%CI: 0.56–1.18), and the pooled RR for the

TABLE 2 | Characteristics of the four included randomized controlled studies.

Author (year)	Study design	Country	Intervention	Sample size (T/C)	Mean age (year) (T/C)		Mean BMD (g/cm^2) (T/C)		Duration	Reported outcomes	Risk of bias
Wimalawansa et al. (15)	RCT	USA	NG (22.5mg daily) vs placebo	93/93	56.5 ± 4.2	55.3 ± 4.2	1.1 ± 0.1	1.1 ± 0.1	36 months	BMD percent change, body bone mineral content, height, adverse event	Unclear risk
Bolland et al. (14)	RCT	New Zealand	ISMO(20mg daily), ISMN(30mg/ 60mg daily) NG(25mg/50mg daily) vs placebo	200/40	67.5 ± 1.81	67.3 ± 2.0	1.07 ± 0.12	1.1 ± 0.14	1 year	BMD percent change, bone markers, adverse event	Low risk
Nabhan et al. (25)	RCT	Egypt	IMN(20mg daily) vs alendronate (70mg weekly)	30/30	54.7 ± 6.51	53.07 ± 6.69	0.213 ± 0.05	0.215 ± 0.05	1 year	BMD change, adverse event	Low risk
Duhan et al. (16)	RCT	India	IMN(40mg daily) vs alendronate (70mg weekly)	45/45	71 ± 5.0	71 ± 5.1	0.67 ± 0.097	0.68 ± 0.067	9 months	BMD change, adverse event	High risk

NG, nitroglycerin; ISMO, short-acting isosorbide mononitrate; ISMN, long-acting isosorbide mononitrate; IMN, isosorbide mononitrate; T, treatment; C, control; BMD percent change: (BMD at follow-up – BMD at baseline)/BMD at the baseline ×100; BMD change: BMD at follow-up – BMD at baseline.

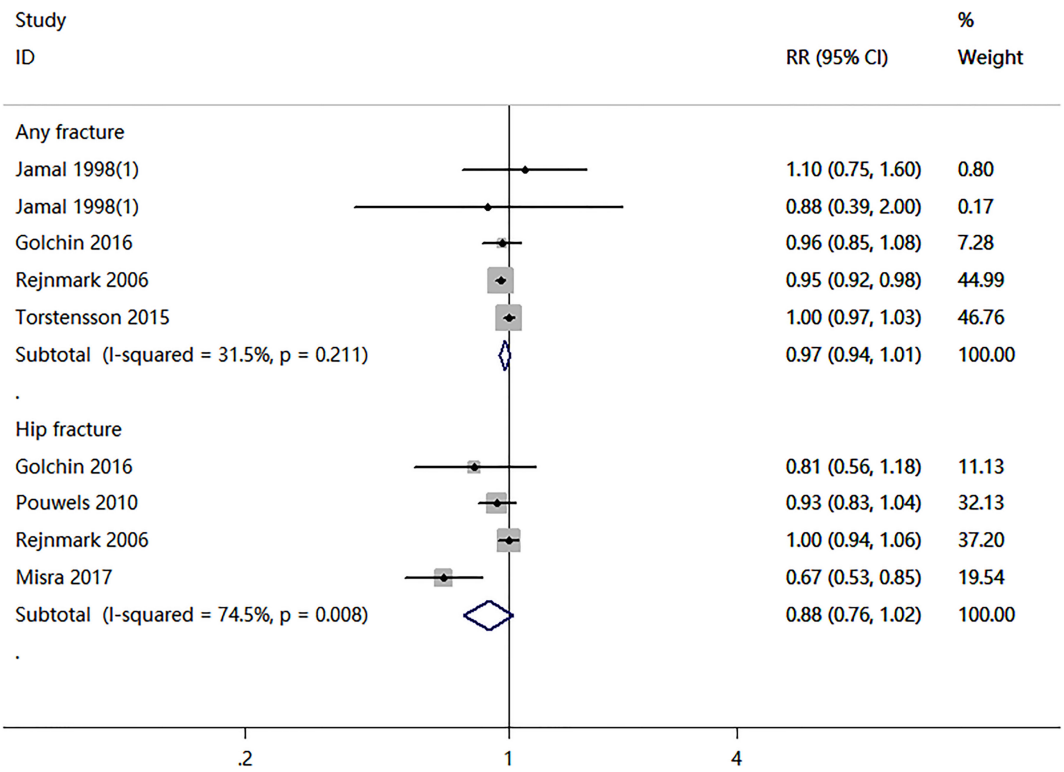

FIGURE 2 | Meta-analysis results of nitrates use for the risk of any fracture and hip fracture.

hip fracture risk of European people with nitrates was 0.89 (95% CI: 0.76–1.05, I^2 = 81.7%).

Sensitivity Analysis and Publication Bias

The results of the sensitivity analysis demonstrated the stability of outcomes in meta-analyses(**Supplementary Figures 1, 2**). No indication of publication bias was found for studies that reported any fracture risk (Begg P = 1.000; Egger P = 0.983) and hip fracture (Begg P = 0.139; Egger P = 0.308) (**Supplementary Figures 3, 4**).

DISCUSSION

In this meta-analysis of 10 studies, we found that nitrates use was not associated with a reduced risk of any fracture or hip fracture in observational studies. The results of four randomized controlled trials on the effects of nitrates on BMD were inconsistent. There were no statistically significant differences in BMD percent change at any sites in these two RCTs compared with a placebo (14, 15). In contrast, nitrates and alendronate had similar effects in increasing bone BMD in another two RCTs (16, 25).

NO is a short-lived free radical that regulates a variety of physiological processes, including bone remodeling (26). In the acid environment of the stomach, NO can be created nonenzymatically from nitrites. Organic nitrates (nitroglycerin, isosorbide mononitrate, isosorbide dinitrate) can operate as NO donors (27). Intermediate dosages of NO have been demonstrated to improve skeletal health in several studies. However, the benefits of NO supplements on bone mass have been controversial. Numerous *in vivo* animal studies have demonstrated that NO donors help to decrease bone resorption while also improving bone growth (10, 28, 29). NO appears to have a biphasic effect on bone-forming cells, promoting bone growth at low doses while inhibiting bone formation at higher concentrations (30). Because nitroglycerin has a somewhat narrow therapeutic window for osteoporosis treatment, the proper dose must be employed to get positive BMD results (15). Continuous exposure to nitrates may promote tachyphylaxis in bone, just as it does with angina symptom management. Once-daily treatment of nitroglycerin ointment enhanced BMD in ovariectimized rats, but more frequent application had little effect (31). Based on this potential for tachyphylaxis, randomized controlled trials using once-daily dosing of nitroglycerin ointment would not achieve satisfactory results for bone health. The most well-known study on nitrates found that nitroglycerin improved BMD by 6% to 7% at all sites over 24 months, with significant increases in markers of bone formation and decreases in markers of bone resorption, but the study was retracted five years later (32). Another observational study (33) reported that nitrate use was associated with increased BMD at the hip and

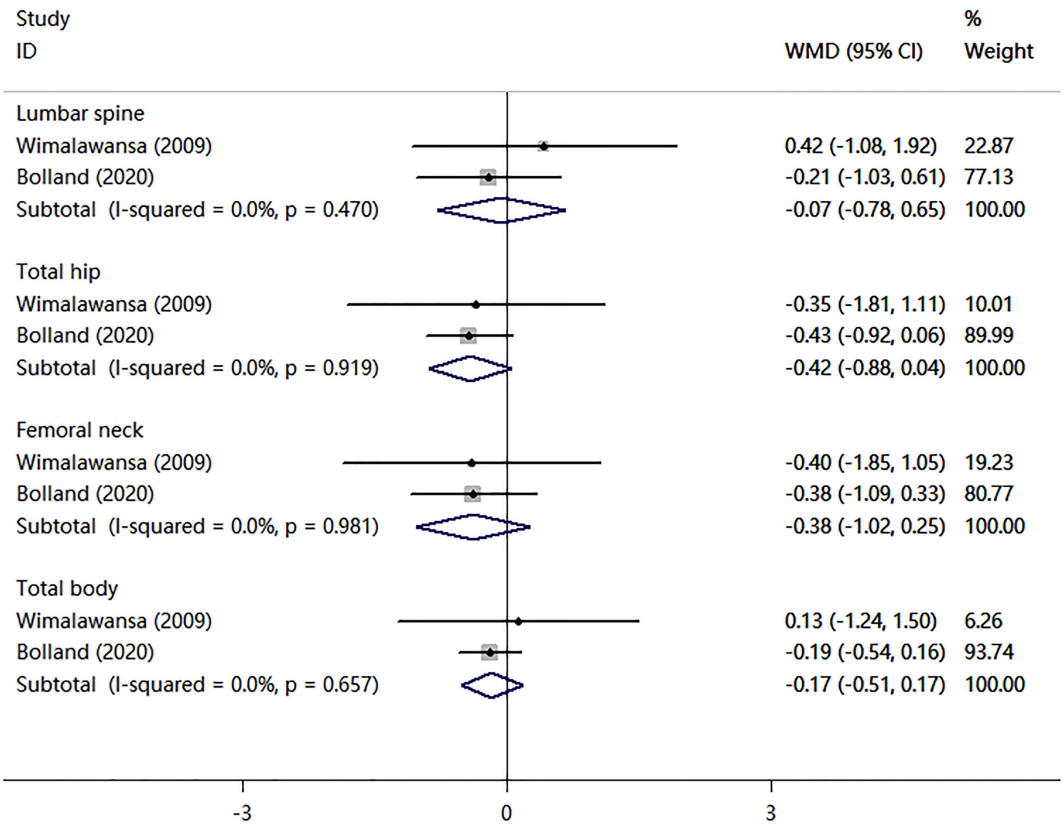

FIGURE 3 | Meta-analysis of the effects of nitrates on BMD compared with placebo.

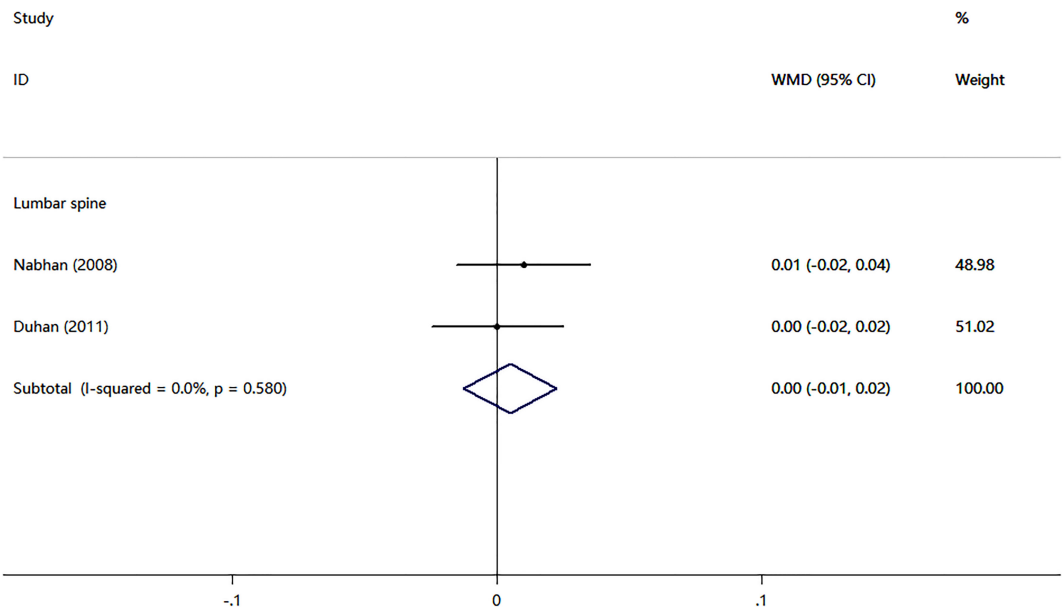

FIGURE 4 | Meta-analysis of the effects of nitrates on lumbar spine BMD compared with alendronate.

TABLE 3 | Risk of bias of randomized controlled trials evaluating the efficacy of nitrates for bone health.

Study, year	Sequence generation	Allocation concealment	Blinding of participants	Blinding of personnel	Blinding of outcome assessors	Incomplete Outcome data	Selective outcome reporting	Other sources of bias	Summary assessments of the risk of bias
Wimalawansa et al. (15)	Unclear risk	Unclear risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Unclear risk
Bolland et al. (14)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Nabhan et al. (25)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Duhan et al. (16)	Unclear risk	Unclear risk	High risk	High risk	Low risk	Low risk	Low risk	Low risk	High risk

TABLE 4 | Subgroup analysis of nitrates use and fracture risk.

Study	No of studies	RR with 95% CI	Heterogeneity		Study	No of studies	RR with 95% CI	Heterogeneity	
			I^2(%)	*P* value				I^2(%)	*P* value
Any fracture					**Hip fracture**				
All	5	0.97(0.94,1.01)	31.5	0.211	All	4	0.88(0.76,1.02)	74.5	0.008
Study design					Study design				
Cohort	4	1.00(0.97,1.03)	0.0	0.858	Cohort	2	0.71(0.58,0.86)	0.0	0.399
Case control	1	0.95(0.92,0.98)	–	–	Case control	2	0.98(0.92,1.04)	19.3	0.266
NOS score					NOS score				
9 point	2	0.97(0.93,1.03)	81.2	0.021	9 point	2	0.98(0.92,1.04)	19.3	0.266
8 point	3	0.97(0.87,1.09)	0.0	0.776	8 point	2	0.71(0.58,0.86)	0.0	0.399
Region					Region				
North America	3	0.97(0.87,1.09)	0.0	0.776	North America	1	0.81(0.56,1.18)	–	–
Europe	2	0.97(0.93,1.03)	81.2	0.021	Europe	3	0.89(0.76,1.05)	81.7%	0.004

spine in men and women. It was also retracted. Two articles about the results of nitrates and alendronate have similar effects in increasing bone BMD and should be carefully considered. More randomized control trials are needed to determine the effects of nitrates on bone health.

Our meta-analysis has several strengths. This meta-review was the first to review the efficacy of nitrates for bone health. In addition, it examined the associations stratified by the type of fracture, the study design, NOS score, and region. However, our meta-analysis has some limitations as well. First, due to the small number of RCT studies, the results of our meta-analysis of RCTs are highly heterogeneous. Second, we may have missed unpublished studies and those that were not in English, resulting in an overestimation of the efficacy of these treatments. Third, we were unable to conduct a meta-analysis on adverse events, because many studies failed to report different adverse events.

CONCLUSION

This meta-analysis of observational data found no association between nitrate use and fracture risk. The results of RCTs on the usage of nitrates and their effects on BMD are contradictory. Further well-designed trials confirming their benefit for bone health are required before it can be recommended for routine use.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

WL and GW designed the study and collected the data. WL drafted the manuscript. ZM contributed to the writing. GW provided critical feedback and contributed to the review of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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The Role of NPY in the Regulation of Bone Metabolism

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Bone diseases are the leading causes of disability and severely compromised quality of life. Neuropeptide Y (NPY) is a multifunctional neuropeptide that participates in various physiological and pathological processes and exists in both the nerve system and bone tissue. In bone tissue, it actively participates in bone metabolism and disease progression through its receptors. Previous studies have focused on the opposite effects of NPY on bone formation and resorption through paracrine modes. In this review, we present a brief overview of the progress made in this research field in recent times in order to provide reference for further understanding the regulatory mechanism of bone physiology and pathological metabolism.

Keywords: bone disease, NPY, bone formation, bone resorption, osteoporosis

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INTRODUCTION

The mammalian skeleton is a vital organ formed by several bone types, and it is also the place for hematopoiesis and mineral storage, with powerful self-repair ability and mineralized extracellular matrix. The traditional view of factors affecting bone metabolism such as endocrine, paracrine, and mechanical stimulation has long been discussed. Recent findings reported that bone tissue (including the periosteum, cortical and trabecular bone, bone marrow) was abundantly innervated by autonomic nerve terminals, which is one of the key factors regulating bone metabolism and remodeling through direct or indirect manner (1, 2), making the autonomic nerve system and bone metabolism closely linked.

When neuropeptide Y (NPY) was first discovered in 1983, the awareness of its function in energy balance, obesity, and bone metabolism has gradually increased (3, 4). As a 36-amino acid peptide belonging to the pancreatic polypeptide family, NPY is most abundantly produced and expressed in the nervous system (5). In the central nervous system, NPY is distributed in the amygdala, locus coeruleus, and cerebral cortex, with the highest expression level in the hypothalamus. It acts to coordinate signals from a wide variety of sources to participate in appetite, circadian rhythm, and energy utilization regulation (6, 7). In the periphery, NPY was found to be co-stored and co-released with neurotransmitter noradrenaline (NA) in postganglionic sympathetic nerves (8). Recent studies have reported that NPY and its receptors have also been identified in bone tissue, such as in osteoblasts, osteocytes, and adipocytes (2, 9, 10), indicating the potential role of NPY on bone remodeling in local sites. Moreover, it can also act as a mediator of the autonomic nervous system to mediate bone marrow mesenchymal cell (BMSC) differentiation fate by constructing a mouse model that lacks osteocyte-specific NPY (2). Even though various physiological conditions and

pathophysiological processes such as obesity (11), anxiety (12), food intake (13), chronic pain (14), neurodegenerative disorders (15), and bone disease (2) have been proven to require NPY to participate, its effect on bone metabolism is still poorly understood.

In this review, we focus on the effects of NPY on bone metabolism in some physiological and pathological states. The aims of this article are to review the regulatory effects and to achieve a comprehensive understanding of NPY on bone metabolism.

NEUROPEPTIDE Y AND ITS RECEPTORS

Bone remodeling involves mineralized bone removal by osteoclasts followed by bone matrix formation through osteoblasts that subsequently become mineralized (16). It is a key process for maintaining bone mass in a dynamic balance and continues throughout life. Previous studies have proven the vital role of NPY in the regulation of food intake and energy homeostasis, and its role in bone metabolism has gradually become a hot topic in recent years.

NPY is a highly conserved endogenous peptide and multifunctional neurotransmitter acting *via* five G-protein-coupled receptor subtypes named Y1R, Y2R, Y4R, Y5R, and Y6R, of which Y1R and Y2R modulate bone mass at differing sites and through different ways (2, 14, 17). The arcuate nucleus of the hypothalamus exhibited the greatest expression level of NPY, and Y2R is the most abundant subtype in the central nervous system (18), which is also peripherally found in the liver, intestine, spleen, muscle, and adipose tissue, suggesting Y2R may have local effects in these tissues (19). Y2 antagonist treatment resulted in reduced bone resorption level and greater bone mineral density in ovariectomized (OVX) mice (20). Hypothalamic Y2R knockout mice exhibited increased osteoblast activity, mineralization rate, and bone mass, indicating a catabolic role of Y2R in stimulating cortical and cancellous bone formation (**Table 1**) (28, 29).

Y1R has also been reported to be involved in many physiological activities, such as mitogenic activity, macrophage migration, and pulpal development (17, 21, 22). In bone tissue, Y1R is highly expressed in BMSCs, osteoblast, osteocyte, monocyte/macrophage, and osteoclast (2), prompting it to play a regulatory role in the local area. Y1R germline deletion resulted in elevated osteoblast activity and mineral apposition rate, together with increased formation of highly multinucleated osteoclasts and enhanced surface area, demonstrating a negative role of Y1R on bone mass maintenance (23, 24). Furthermore, the Y1R antagonist regulated gut microbiota and exhibited an anti-osteoporotic effect in OVX rats (25), revealing that Y1R may affect bone mass through multiple ways.

To date, little is known about the role of Y4R, Y5R, and Y6R in bone mass maintenance. Y4R was reported to mainly affect body weight, fat mass, energy expenditure, and anxiety-like and depression-related behavior (31, 32). Interestingly, male mice lacking both Y2R and Y4R displayed a synergistic effect in trabecular bone volume upregulation compared with Y2R knockout mice, but female double knockout mice did not show this bone phenotype, suggesting a synergy between Y2 and Y4 receptor pathways (33). Igura et al. reported that Y5R expression level in bone marrow cells declined with age and Y5R overexpression strengthened the proliferation effect induced by NPY, indicating that Y5R may take part in bone metabolism by affecting the self-renewal ability of bone marrow cells (34). Y6R, which is restricted to the suprachiasmatic nucleus (SCN) of the hypothalamus, is required for the maintenance of bone mass in mice. Mice lacking Y6R displayed reduced numbers of osteoblast precursors and increased osteoclast activity (37).

NPY AND BONE FORMATION

As seed cells in bone marrow, BMSCs are able to commit to osteogenic lineage and differentiate into mature osteoblasts. Intensive studies in recent years have demonstrated that a number of transcription factors are involved in this process. Among them, runt-related transcription factor 2 (runx2) and osterix are considered as master transcription factors in osteogenic differentiation and they control bone formation (39). Zhang et al. found that runx2 level and mineralized

TABLE 1 | Characterization, distribution, and functions of NPY receptors.

Receptor	Tissue distribution	Physiological functions on bone	Other functions	Ref.
Y1R	Hypothalamus, hippocampus, neocortex, thalamus, bone cells, pancreas, intestine	BMSC proliferation, osteogenic and adipogenic differentiation, macrophage migration, regulated gut microbiota, pulpal development	Vasoconstriction, anxiolysis, food intake, heart rate, anxiety	(17, 21–27)
Y2R	Hippocampus, hypothalamus, brain stem, articular cartilage, liver, intestine, spleen, muscle, and adipose tissue	Osteoblast activity and mineralization rate, cartilage homeostasis	Memory, circadian rhythm, angiogenesis, epilepsy	(10, 19, 20, 28–30)
Y4R	Total brain, heart, thoracic aorta, coronary artery, nasal mucosa, skeletal muscle, mesentery vasculature, stomach, ileum, and endometrium	Synergize with Y2R	Energy expenditure, anxiety-like and depression-related behavior, ion transportation, arterial pressure	(31–33)
Y5R	Hypothalamus, hippocampus	BMSC proliferation	Food intake, epilepsy, circadian rhythm	(34–36)
Y6R	Hypothalamus	Osteoblast precursor survival and Osteoclast activity	food intake	(37, 38)

nodules were decreased after NPY treatment in osteogenic differentiation of BMSCs, confirming that NPY inhibits osteogenesis by inhibiting runx2, and this effect may be achieved through Y1R (2). Germline deletion of Y1R and knockout of NPY produce anabolic responses in bone, with upregulated runx2 and osterix level, resulting in a generalized increase in bone mass owing to stimulated osteoblast activity and an increased bone formation rate (40, 41). Besides, dorsomedial nucleus NPY knockdown mice showed increased basal and obesity-induced decrease in bone mineral density (BMD) together with reduced activating transcription factor 4 (ATF4) expression level (42). Activator protein 1 (AP1) antagonists targeted to NPY neurons resulted in increased trabecular bone formation and mass (43). In glucocorticoid-induced osteoporotic skeleton, NPY expression and marrow adipogenesis were upregulated, together with increased post-translational modification of peroxisome proliferator-activated receptor gamma (PPARγ) (44).

Paradoxically, several studies have reported that NPY acts as a promoting factor in the process of bone formation and fracture repair. Liu et al. found that low doses of NPY stimulate BMSC osteogenic differentiation and mineralization while a high NPY concentration had the opposite effect (45). In patients with combined injuries, NPY levels were increased than in those with simple fractures, and further experiment demonstrated that NPY directly promotes BMSC osteogenic differentiation (46). Y1R antagonist-treated mice or Y1R-deficient mice exhibited a delay in fracture repair and cartilage removal, as evidenced by reduced calcified nodule area and decreased bone callus volume and strength (47, 48). Researchers recently used overexpression plasmids and small interfering RNA (siRNA) targeting NPY transfected into the MC3T3–E1 osteoblastic cell line and found that NPY overexpression markedly enhanced the osteogenic ability by an autocrine mechanism, together with the upregulation of osterix and runx2 level (49). Knockdown of the Y1R induced alkaline phosphatase (ALP) activity and mineralization together with upregulated mRNA expression of specific genes that characterize osteoblastic differentiation in MC3T3–E1 cells (50).

As an anxiolytic factor, NPY was reported to protect against chronic stress-induced bone loss specifically through Y2R, evidenced by increased bone mass and bone formation rate (51). Also, NPY can regulate bone formation through an indirect manner. Ma et al. found that NPY stimulated human osteoblast osteogenic activity by enhancing gap junction intercellular communication (52). The Y1R antagonist upregulated serum Ca^{2+} concentration, changed the gut microflora community composition, and improved bone mass in OVX rats (25). Although the studies mentioned above seem inconsistent, it is certain that bone formation is strongly influenced by NPY.

NPY AND BONE RESORPTION

Bone resorption was mediated by mature osteoclast, which is a tissue-specific multinuclear giant cell derived from hematopoietic stem cells through the myelomonocytic precursor cells/macrophage lineage. In brief, hematopoietic stem cells are committed to macrophage colony-forming units (CFU-M) in the presence of macrophage colony-stimulating factor (M-CSF). When the receptor activator of nuclear factor-kappa B ligand (RANKL) binds RANK on the surface of osteoclast precursors, osteoclastogenesis is immediately triggered. CFU-M is further differentiated into mononucleated osteoclasts and subsequently fused to multinucleated osteoclasts, then fully matured upon a cognate interaction with osteoblasts (53). Wu et al. reported that NPY greatly increased the amount of RAW264.7 cell (mouse leukemic monocyte macrophage cell line) migration at different concentrations, and this effect can be diminished by the Y1R antagonist and ERK1/2 inhibitor, which suggest that NPY promotes osteoclast migration through Y1R and ERK1/2 activation (22). NPY has also been shown to exhibit an inhibitory effect on isoprenaline-induced osteoclastogenesis by suppressing RANKL expression in mouse bone marrow cells (54). In addition, an *in-vitro* experiment confirmed that the regulator of osteoclastogenesis RANKL/OPG ratio was higher in NPY-treated BMSCs, and this effect can be reversed with Y1R antagonist treatment, making evidence that NPY may facilitate bone resorption through Y1R (55).

On the contrary, Park et al. found that NPY can mobilize hematopoietic stem/progenitor cells (HSPCs) from the bone marrow to the peripheral blood and ameliorated low bone density in an ovariectomy-induced osteoporosis mouse model by reducing osteoclast number (56). Seldeen et al. used an osteoporotic mouse model injected once daily with JNJ-31020028, a brain-penetrant Y2R small molecule antagonist. Then, primary bone cell cultures were isolated from the tibiae, and it was found that bone marrow cultures obtained from the Y2R antagonist-treated mice exhibited significantly more osteoclasts and greater areal coverage with *in-vitro* osteoclast differentiation induction, which means that central NPY inhibited osteoclastogenesis through Y2R (20).

In our study, osteoclast number and activity seem not be significantly influenced by bone-specific deficiency of NPY in young and aged mice (2). Matic et al. generated a mouse model where NPY was overexpressed specifically in mature osteoblasts and osteocytes and characterized the bone phenotype of 3-month-old mice. It was found that bone volume was reduced; however, bone formation rate and osteoclast activity were not significantly changed (57). The direct and indirect effects of NPY on bone resorption need further exploration.

OTHERS

In addition to participating in bone metabolism through affecting bone turnover, NPY may also affect bone mass through other ways. Blood vessels play an irreplaceable important role in the metabolic balance of bones. Several studies have confirmed that NPY-immunoreactive fibers were predominantly localized alongside with blood vessel walls in bone; moreover, Y1R, Y2R, and Y5R were confirmed to be expressed on endothelial cells (ECs), providing a material basis for the vasoregulatory role of NPY in addition to directly

regulating bone tissue cells (58). It has been observed that BMSC migration and VEGF expression were upregulated after NPY treatment (45) and increased levels of VEGF stimulate angiogenesis and osteoblastic differentiation of BMSCs (59). Besides, Y1R signaling disruption is responsible for enhancing the deposition and maturity of collagen and mineral hydroxyapatite layers in the skeletal muscle, and bone mechanical property was furthered improved (60) (**Figure 1**).

RELATIONSHIP OF NPY AND COMMON BONE DISEASE

Osteoporosis

Osteoporosis (OP) is a common skeletal disorder characterized by compromised bone mass and degraded bone microarchitecture, often resulting in fragility fractures and severely compromised quality of life in elderly people. Increasing age and postmenopausal state are proven to be associated with this condition. Zhang et al. reported that ovariectomy induced NPY upregulation in bone tissue after constructing a model of OP in adult female mouse. γ-Oryzanol (ORZ), a functional substance extracted from rice bran, alleviated the severity of postmenopausal and senile OP through the autonomic nervous system by inhibiting osteocytic-NPY secretion (2). In glucocorticoid-mediated bone loss, NPY mRNA expression and protein concentration were elevated, while BMD and bone microstructure were significantly reduced (44). Xie et al. reported that the OP group exhibited deteriorated bone microstructure and more microdamage than the osteoarthritis (OA) group, and they also measured NPY and Y1R expression levels in patients after constructing a postmenopausal osteoporotic rat model and found these to be both upregulated in OP groups. Y1R antagonist treatment *in vivo* for OVX rats could improve bone microstructure and decrease bone microdamage, and this may be achieved *via* the cAMP/PKA/CREB signaling pathway (10). Also, NPY is increased in the rat spinal cord after nerve injury in the model of peripheral nerve trauma (61). Above all, it is possible that NPY participates in the pathogenesis of osteoporosis. In detail, NPY plays a negative role in the process of osteoporosis.

Bone Fracture

Bone fracture healing is a multistep and overlapping process involving inflammation, osteogenesis, and angiogenesis (62). Among these processes, the formation of primary bone is a crucial one since it is the key process of fracture healing. Gu et al. focused on patients with traumatic brain injury–fracture-combined injuries and found that the NPY level was increased, accomplished with an increase of bone formation markers, indicating an active role of NPY in fracture healing (46). Sousa et al. generated germline ($Y1^{-/-}$) and osteoblastic-specific Y1R knockout mice to characterize whether Y1R plays a role in fracture healing. The fracture healing process was delayed in the global deletion of Y1R in mice, and this delay is independent from osteoblast-specific Y1R. In Y1R-specific deficient mice, delayed endochondral fracture healing seems to be the result of impaired inflammatory response and cartilage removal since Y1R is widely expressed in neuronal but also in non-neuronal cells, such as immune cells (47). However, Long et al. established an angular fracture rat model and found that regenerating NPY fibers were increased in the early stages and then reduced between 21 and 56 days on the concave side compared with the convex side, suggesting that NPY innervation appears to correlate with the loss of callus thickness in angular fractures (63). Based on the evidence mentioned above, the authors hypothesized that NPY plays an important role in fracture healing, and this role may not be achieved through Y1R. Further study is needed to clarify the underlying mechanism.

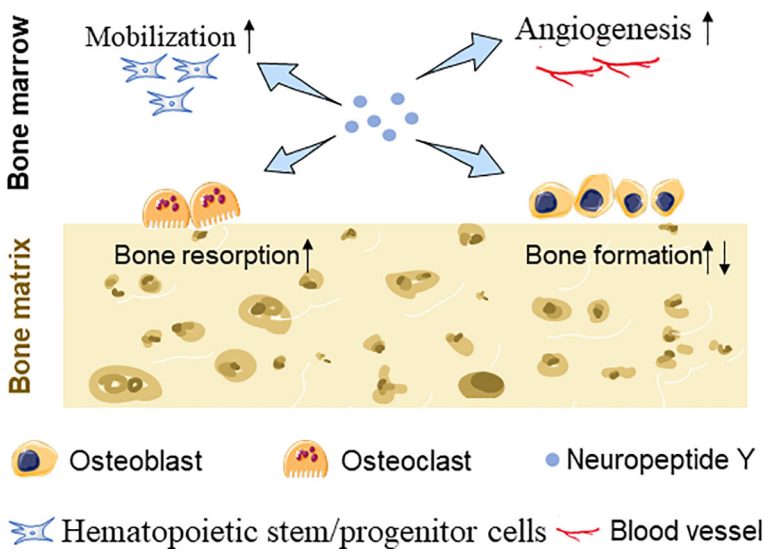

FIGURE 1 | Schematic diagram showing NPY-mediated BMSC mobilization, EC angiogenesis, and bone turnover changes.

Inflammation

NPY is produced not only by the central and peripheral nervous system but also by immune cells such as macrophages, B cells, neutrophils, and lymphocytes (64). It can cause the activation of immune cell response and induce the release of proinflammatory cytokines including TNF-α or interleukin-6, acting as a potent modulator of the immune responses during inflammation, infection, and autoimmunity (65–67). In animal models of systemic inflammation such as endotoxemia, the expression of NPY in the hypothalamus was slightly increased and positively correlated with the severity of inflammation (68, 69). A cross-sectional design of rheumatoid arthritis (RA) patients found that serum levels of NPY are significantly related to TNF-α levels and disease activity in RA independently of IL-6, TNF-α, or leptin levels (67). In patients with knee osteoarthritis, concentrations of NPY in synovial fluid were gradually upregulated with the severity of pain, suggesting a role for NPY as a putative regulator of joint homeostasis (66). This suggested that NPY plays a crucial role in both systematic and local sites, and often reflected the severity of inflammation.

Osteoarthritis

As the most common joint disease worldwide, OA is characterized by cartilage degradation, synovial inflammation, subchondral bone remodeling, and osteophyte formation and primarily identified as a non-inflammatory musculoskeletal degeneration (70). Several studies suggest the involvement of NPY in the pathogenesis of OA, and it has already been identified as the major peptide involved both in the generation of pain. NPY concentration in synovial fluid was significantly higher in OA patients compared with controls and positively correlated with pain intensity (66, 71). Kang et al. reported that NPY was overexpressed in human OA cartilage accompanied with increased Y2R expression. Stress stimulus resulted in the sympathetic release of NPY, which in turn promoted the upregulation of NPY and Y2R in articular cartilage and participated in chondrocyte hypertrophy together with cartilage matrix degradation (30). Hernanz et al. demonstrated a significant stimulatory activity of NPY on inflammatory factors such as IL-1β, IL-6, and TNF-α production by whole blood leukocytes from OA patients *in vitro*, which play critical roles in pain in the early stage of OA, indicating a positive effect of NPY in inflammation (72, 73).

Mood Disorders and Bone Abnormalities

Mood disorders such as chronic stress and depression often have adverse consequences on many organs, including the bone. In view of the negative effects of NPY signaling on bone metabolism mentioned above, NPY activity associated with chronic stress and depression would predict a deleterious influence on bone homeostasis. In multiple sclerosis (MS) patients, autonomic nervous system dysfunction and low BMD are intertwined with some mood disorders such as depression, fatigue, and migraine (74). Higher levels of depression were demonstrated in osteocalcin-deficient mice when compared with wild-type mice, giving evidence to bone signal back to the brain (75). Animal experiments also showed that antidepressants may exhibit clinical efficacy by increasing NPY expression levels (76). However, as a well-described anxiolytic factor, NPY was also reported to exhibit a stress-protective role specifically through Y2 receptors (51). The relationships between NPY and mood disorder and between NPY and bone mass maintenance are intriguing and need further investigations.

CONCLUSION

Previous studies have verified that NPY is widely present in the brain and bone tissue and strongly influences bone metabolism through direct and indirect manner. In addition to directly regulating bone formation and resorption, NPY may also participate in bone metabolism by affecting gut microbiota and blood vessel formation. Furthermore, NPY has also been reported to play an intermediary role in autonomic nerve regulation on bone metabolism. As a substance synthesized by multiple places, it will be a challenge to clearly clarify the role of NPY on bone turnover and elucidate the pathophysiology of common bone diseases mentioned above. Also, whether NPY derived from sympathetic nerve endings and osteocytes has different physiological effects remains to be explored. Even though previous studies have shown that NPY participates in bone metabolism, especially in the bone formation process and BMSC fate decision, the effect of NPY on osteoclastogenesis and mood disorder is not fully understood.

In spite of NPY being mostly expressed in the central nervous system, the role of NPY secreted by surrounding tissues, organs, and cell types in bone metabolism and cell signal transduction may be an important future research consideration. Future research on NPY and its receptors will be beneficial for new drug development and identifying new treatments for bone diseases.

AUTHOR CONTRIBUTIONS

YZ conceived and designed the manuscript. Q-CC wrote the paper. All authors contributed to the article and approved the submitted version.

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Differences in Hip Geometry Between Female Subjects With and Without Acute Hip Fracture: A Cross-Sectional Case-Control Study

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Background and Purpose: Although it is widely recognized that hip BMD is reduced in patients with hip fracture, the differences in geometrical parameters such as cortical volume and thickness between subjects with and without hip fracture are less well known.

Materials and Methods: Five hundred and sixty two community-dwelling elderly women with hip CT scans were included in this cross-sectional study, of whom 236 had an acute hip fracture. 326 age matched women without hip fracture served as controls. MIAF-Femur software was used for the measurement of the intact contralateral femur in patients with hip fracture and the left femur of the controls. Integral and cortical volumes (Vols) of the total hip (TH), femoral head (FH), femoral neck (FN), trochanter (TR) and intertrochanter (IT) were analyzed. In the FH and FN the volumes were further subdivided into superior anterior (SA) and posterior (SP) as well as inferior anterior (IA) and posterior (IP) quadrants. Cortical thickness (CortThick) was determined for all sub volumes of interest (VOIs) listed above.

Results: The average age of the control and fracture groups was 71.7 and 72.0 years, respectively. The fracture patients had significantly lower CortThick and Vol of all VOIs except for TRVol. In the fracture patients, cortical thickness and volume at the FN were significantly lower in all quadrants except for cortical volume of quadrant SA (p= 0.635). Hip fracture patients had smaller integral FN volume and cross-sectional area (CSA) before and after adjustment of age, height and weight. With respect to hip fracture discrimination, cortical volume performed poorer than cortical thickness across the whole proximal femur. The ratio of Cort/TrabMass (RCTM), a measure of the internal distribution of bone, performed better than cortical thickness in discriminating hip fracture risk. The highest area under curve (AUC) value of 0.805 was obtained for the model that included THCortThick, FHVol, THRCTM and FNCSA.

Conclusion: There were substantial differences in total and cortical volume as well as cortical thickness between fractured and unfractured women across the proximal femur. A combination of geometric variables resulted in similar discrimination power for hip fracture risk as aBMD.

Keywords: hip fracture, geometry, cortical thickness, volume, discrimination

INTRODUCTION

Hip fractures are amongst the most severe consequences of osteoporosis and are associated with high morbidity and mortality and a significant reduction in the patient's quality of life (1). Hip fracture patients have a mortality of 20% within the first year (2) and 10 to 20% of hip fracture individuals can no longer live independently (3). Hip fracture risk depends on the integrity of the proximal femur and the likelihood of experiencing forces that exceed bone strength (4). With aging, the geometrical integrity of the hip is compromised and the risk of falling increases, resulting in older individuals having an increasing risk of hip fracture. Thus, it is important to identify individuals at high risk of fracture. While areal bone mineral density (aBMD) derived from dual X-ray absorptiometry (DXA) is the routine method to evaluate osteoporosis, studies have consistently shown that it has only moderate capability to predict hip fractures (5–11).

The cortical bone of the proximal femur has become a focus of interest leading to the increased application of hip quantitative CT (QCT) in clinical trials (12). However, few studies have assessed the association of cortical bone with hip fractures, and some of these have only applied cross-sectional slice-based cortex measurements (i.e. one slice or the average of several slices) (6, 9, 13–15) instead of 3D segmented methods. Several studies have used femoral QCT to measure bone shape, volumetric BMD distribution and cortical bone thickness (CortThick) distribution (6, 11, 14, 16–18), concluding that smaller cross-sectional area, lower trabecular vBMD and thinner cortical thickness were all associated with increased hip fracture risk. However, parameters that characterize the strength of specific sub regions of bone compartments, such as bending and buckling, up to now were mostly limited to two-dimensional assessments derived from DXA hip structural analysis (HSA) (19–21). Further, DXA HSA variables are not independent of DXA aBMD (12). Assessment of femoral geometry by the QCT MIAF-Femur application (MIAF: medical image analysis framework) and volume-based structural parameters introduced by Engelke may allow for assessment of bone strength indicators in greater detail (22). MIAF-Femur software is based on 3D segmentation of the whole proximal femur, which also allows for assessment of the femoral head *in vivo* (23).

This cross-sectional case-control study aims to explore the associations of the geometrical parameters such as cortical volume and thickness with acute hip fractures. We also aim to assess differences in femoral head size between female participants with and without hip fracture.

MATERIALS AND METHODS

Participants

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), approved by the institutional review board of the principal investigator's hospital, and all participants provided their written informed consent. Five hundred and sixty two community-dwelling elderly women with hip CT scans, enrolled in the China Action on Spine and Hip Status (CASH) study, were included in the study. Two hundred and thirty six of the women had an acute hip fracture and were admitted to the Emergency Department of Orthopaedic Trauma at the Beijing Jishuitan Hospital between January 2012 and May 2016. CT scans were taken within 48 hours after fracture to minimize changes in vBMD and body composition. Inclusion and exclusion criteria of the hip fracture patients were described in detail previously (23, 24). In brief, only fully ambulatory, community-dwelling Chinese Han adults with a hip fracture resulting from low-energy trauma (falls from standing or sitting height) were included (24). Participants were excluded if they had prior or bilateral hip fractures or inability to stand or walk before their hip fracture.

Three hundred and twenty six age matched women served as controls. Exclusion criteria for the control subjects were inability to sit and stand independently or inability to walk with or without an assistive device (24). Further exclusion criteria for both groups were stroke, neurological disorders, rheumatic diseases, heart failure, severe chronic obstructive pulmonary disease and coagulation disorders, and other diseases that limited function.

QCT Scans

Spiral hip CT scans were performed for all participants using two Toshiba Aquilion scanners (Toshiba Medical Systems Division, Tokyo, Japan). A Mindways QCT calibration phantom (Mindways Software Inc., Austin, TX, USA) was scanned with each participant, and hip QCT scans were acquired in the supine position following the usual QCT procedures. Both hips were scanned from the top of the acetabulum to 3 cm below the lesser trochanter. The scan parameters were as follows: 120 kVp, 125 mAs, 1-mm thickness, 50-cm field of view (SFOV), and 512 × 512 matrix in standard reconstruction.

MIAF Measurements

CT images of the unfractured (hip fracture cohort) and left (control cohort) sides were analyzed by the MIAF-Femur application (Version 7.1.0MRH). The MIAF-Femur software provided standard volumes of interest (VOIs), namely the femoral head (FH), femoral neck (FN), trochanter (TR) and intertrochanter (IT) calculated relative to an anatomic coordinate system (ACS) with its origin centered at the smallest cross section of the femoral neck. The FN VOI had a height of 5 mm (**Figure 1**). The borders between VOIs were determined automatically based on anatomical landmarks and the ACS (23). Each VOI was separated into integral (Int), cortical (Cort), and trabecular (Trab) compartments for which bone mass (Mass) and volume (Vol) were determined. For the FH, however, only integral volume was measured. Cortical thickness (CortThick) of each VOI was also measured. Further, the FH and FN VOIs were each divided into four quadrants to assess the differential volume responses of their superior, inferior, posterior and anterior parts. The FN cross-sectional area (FNCSA) was calculated by the FN VOI Int volume/neck VOI height, The MIAF TH VOI was calculated as the sum of the FN, TR and IT VOIs (25). The details of measurements by MIAF-Femur have been described previously (20, 22). Precision and accuracy outcomes of MIAF-Femur have been reported earlier (20, 23). Further, to assess the internal distribution of bone, we proposed a

geometric measure of the ratio of Cort/TrabMass (cortical/trabecular bone mass) of femur VOIs, which represents the cortex instability. Since in the intertrochanteric VOI, cortical bone contributes to most of the bone mass of the whole VOI, we did not calculate the ratio of Cort/TrabMass for the intertrochanteric VOI.

Statistics

Continuous variables were reported as mean ± standard deviation (SD). The Shapiro-Wilk test was used to evaluate data for normality. Covariance Analysis (ANCOVA) was used to examine group differences for normally distributed variables. The Mann-Whitney test was used for non-normal variables. A generalized linear model (GLM) with adjustment for age, height and weight was used to compare differences in hip geometry and other variables between hip fracture patients and controls. Logistic regression was used to identify variables contributing to hip fractures based on the significantly different hip geometric parameters from GLM. We found that the ratios of cortical/trabecular mass of VOIs (total hip, neck and trochanter) and cortical thickness of neck, supero-anterior neck and intertrochanter were not normally distributed. Then we checked the log transformed data of these variables by P-P plots to see whether they were closer to being normally distributed. All variables were standardized to have a distribution with a mean of 0 and an SD of 1 to calculate odds ratios of fracture per SD decrease, similar to the analysis used in the EFFECT study papers (11, 26). The area under the receiver operating characteristic curve (AUC) was used as the performance characteristic. All statistical analyses were performed using IBM SPSS Statistics for Windows version 20.0 (IBM SPSS Inc., Chicago, IL). A p-value < 0.05 was considered statistically significant.

RESULTS

Participants' Characteristics

The average ages of the control and hip fracture groups were 71.7 and 72.0 years, respectively. The hip fracture patients had lower weight and higher height. More details of the characteristics of the two cohorts are shown in **Table 1**.

Cortical Volume and Thickness

The hip fracture cohort had significantly lower CortVol and CortThick in all VOIs except for TRVol. In the fracture cohort, the ratio of cortical to total bone mass was significantly higher for the TH, FN and TR VOIs. A closer inspection of the quadrants showed that at the FN, in the fracture patients, CortVol and CortThick were significantly lower in all quadrants except for CortVol of quadrant SA ($p = 0.635$). Details are summarized in **Table 1**.

Femoral Head and Neck Volume

Femoral head volume of the entire FH and the superior quadrants was higher ($p < 0.05$ for quadrants SP and SA) in the hip fracture cohort. However, the hip fracture patients had smaller integral femoral neck volume and cross-sectional area before and after adjustment for age, height and weight (**Table 1** and **Figure 2**).

Associations of Geometry Parameters With Hip Fracture

Table 2 shows the associations of cortical parameters with hip fractures after adjustment for age, height and weight. With respect to hip fracture discrimination, cortical volume was a poorer parameter than cortical thickness across the entire proximal femur. Amongst the cortical thickness and volume parameters, the parameter with the best discrimination was IT CortThick (odds ratio (OR) 2.10; CI 95% 1.70-2.60). The ratio of Cort/TrabMass, a measure of the internal distribution of bone, was superior to cortical thickness at discriminating hip fracture risk for the TH, FN, and TR VOIs (**Table 2**). The ratio of Cort/TrabMass of total hip (THRCTM) had the best discrimination amongst all the geometric variables (OR 2.57; CI 95% 1.94-3.40). Association with fracture was also determined for five selected models (Models 1–5) combining different geometric parameters. The highest AUC value of 0.805 was obtained for Model 1 (THCortThick + FHVol + THRCTM + FNCSA), and AUC values for Models 2-5 were all lower (AUC values: 0.735 to 0.703) (**Figure 3**). We repeated the GLM analysis using log

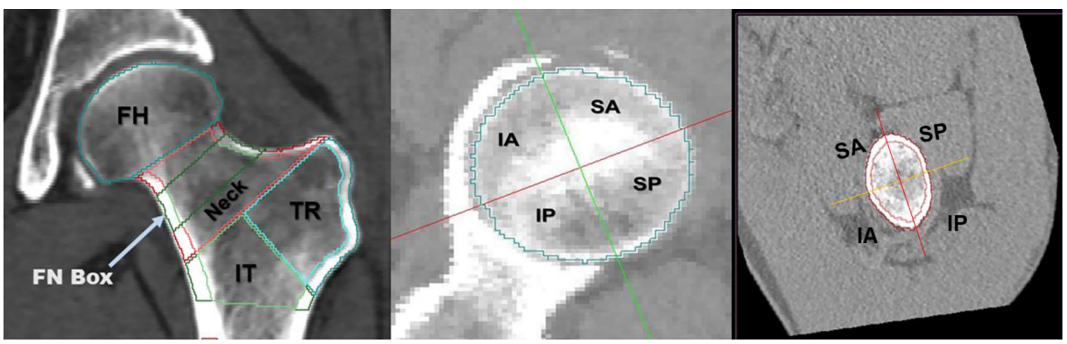

FIGURE 1 | Volumes of interest (VOIs) measured at the proximal femur by MIAF-Femur (left). Axial view along with the neck axis showing anatomic quadrants of femoral head (middle) and femoral neck (right). FH, femoral head; FN, femoral neck; TR, trochanter; IT, intertrochanter; SA, supero-anterior; IA, infero-anterior; IP, infero-posterior; SP, supero-posterior.

TABLE 1 | Characteristics of participants.

Variable/VOI	SubVOI	Controls (N=326)	Hip Fractures (N=236)	P
Age (years)		71.7 ± 7.4	72.0 ± 8.5	0.334
Height (cm)		155.30 ± 18.2	157.6 ± 15.5	0.044
Weight (kg)		60.5 ± 11.7	57.1 ± 16.7	0.014
Total Femur				
	THCortVol (cm^3)	16.1 ± 2.7	15.2 ± 2.6	<0.001
	THCortThick (mm)	1.9 ± 0.2	1.8 ± 0.2	<0.001
	RTHCTM	3.0 ± 1.2 (1.3*)	4.2 ± 2.4 (2.0*)	0.006
Femoral Head				
	HeadVol (cm^3)	35.7 ± 5.5	37.9 ± 5.9	<0.001
	HeadVol_IP (cm^3)	9.4 ± 1.7	9.5 ± 1.8	0.594
	HeadVol_IA (cm^3)	9.9 ± 1.7	10.1 ± 1.9	0.175
	HeadVol_SP (cm^3)	8.1 ± 1.6	8.9 ± 1.8	<0.001
	HeadVol_SA (cm^3)	8.4 ± 1.6	9.4 ± 2	<0.001
Femoral Neck				
	FNboxVol (cm^3)	3.7 ± 1.4	3.2 ± 0.5	<0.001
	FNCSA (cm^2)	7.4 ± 2.8	6.4 ± 0.9	<0.001
	FNCortVol (cm^3)	4.5 ± 1.1	4.2 ± 1.1	<0.001
	RFNCTM	3.2 ± 1.8 (1.7*)	4.3 ± 3.3 (2.3*)	<0.001
	FNCortVol_IP (cm^3)	1.3 ± 0.4	1.2 ± 0.4	<0.001
	FNCortVol_SP (cm^3)	1 ± 0.3	0.9 ± 0.2	<0.001
	FNCortVol_IA (cm^3)	1.2 ± 0.3	1.1 ± 0.3	<0.001
	FNCortVol_SA (cm^3)	1 ± 0.3	1 ± 0.3	0.635
	FNCortThick (mm)	1.8 ± 0.3 (0.3*)	1.7 ± 0.3 (0.3*)	<0.001
	FNCortThick_IP (mm)	2.2 ± 0.4	2 ± 0.4	<0.001
	FNCortThick_SP (mm)	1.8 ± 0.3	1.7 ± 0.3	<0.001
	FNCortThick_IA (mm)	1.7 ± 0.3	1.5 ± 0.3	<0.001
	FNCortThick_SA (mm)	1.6 ± 0.3 (0.3*)	1.5 ± 0.4 (0.4*)	<0.001
Trochanter				
	TRCortVol (cm^3)	6.2 ± 1.2	6.1 ± 1.1	0.163
	TRCortThick (mm)	1.9 ± 0.3	1.7 ± 0.2	<0.001
	RTRCTM	2.9 ± 1.3 (1.4*)	3.9 ± 1.8 (1.8*)	<0.001
Intertrochanter				
	ITCortVol (cm^3)	5.3 ± 1.5	4.9 ± 1.3	0.001
	ITCortThick (mm)	2.1 ± 0.3 (0.4*)	1.9 ± 0.2 (0.3*)	<0.001

TH, total hip; VOI, volume of interest; Vol, volume; Cort, cortical; Thick, thickness; CortThick, cortical thickness; HeadVol, femoral head volume; RTHCTM, ratio of total hip cortical/trabecular mass; RFNCTM, ratio of femoral neck cortical/trabecular mass; RTRCTM, ratio of trochanter cortical/trabecular mass; FNCSA, femoral neck cross-sectional area; TR Trochanter; IT, intertrachanter; SA, Supero-anterior; IA, Infero-anterior; IP, Infero-posterior; SP, Supero-posterior.

P values represent the comparison outcomes of Covariance Analysis (ANCOVA) for normally distributed variables and the Mann-Whitney test for non-normal variables.

**Refers to the interquartile range (IQR) for the non-normal variables.*

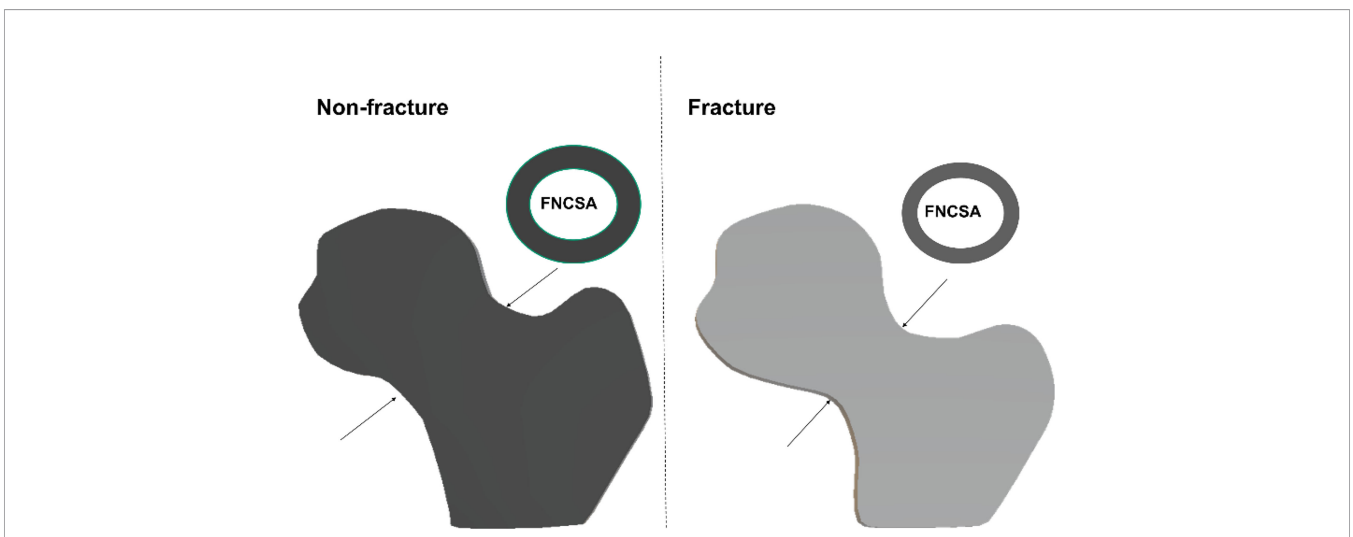

FIGURE 2 | Simple conceptual impression of femoral head volume and femoral neck cross-sectional area (FNCSA) indicates hip fracture subjects with larger head volume [adjusted odd ratio (OR) 1.57; 95% CI 1.30-1.90] and smaller FNCSA (adjusted OR 1.68; 95% CI 1.35-2.10) after adjustment of age, height and weight.

transformed variables (ratios of cortical/trabecular mass of VOIs (total hip, femoral neck and trochanter) and cortical thickness of femoral neck, SA_FN and IT) and confirmed that there were still statistically significant differences between hip fracture patients and controls.

DISCUSSION

Based on the analysis of 562 participants enrolled in the CASH cross-sectional case–control cohort, our study shows structural differences between elderly women with and without hip fractures, and a combination of selected geometry variables resulted in equivalent discrimination power to the aBMD model reported previously (11, 24). Our study outcomes also confirm observations of previous studies that the addition of bone volume did not significantly improve hip fracture discrimination. However, inclusion of the FH volume may allow improved prediction of hip fracture propensity.

An interesting finding of this study was that elderly women with hip fracture had larger FH but smaller FN size compared to controls. The femoral head connects continuously with the femoral neck. Thus, the head directly participates in the weight-bearing transfer to the femoral neck and the femoral neck and the trochanter are affected by the stresses and strains in the femoral head (23). Therefore, with respect to hip fracture risk prediction, the traditional DXA regions such as the FN, TR and IT may not be fully adequate to capture the risk of hip fracture. However, up to now, only two studies have reported the relationship between bone deterioration of the femoral head and hip fractures. In the European Femur Fracture Study (EFFECT) the femoral head BMD was associated with hip fracture but there was no difference in femoral head volume between participants with and without hip fracture (11). In the other QCT study, loss of FH vBMD was also found to be related to hip fracture (27). Our findings demonstrated that femoral head volume discriminated hip fracture risk with an AUC value of 0.67 after adjusting for age, height and weight, and inclusion of the FH volume improved the power of the model (**Figure 3**). Associations between geometric features of the proximal femur and hip fracture have been extensively investigated. For example, the strength of the femur is associated with the shape and size of its cross sections, the lengths of its neck and shaft, the neck–shaft angle, etc. (12). Differences in geometry of the proximal femur between women with and without hip fracture (larger head but a smaller neck in fractured subjects) identified by our study offer a new view of the femur strength and may prove useful in the construction of finite element models.

In agreement with three previous QCT studies (11, 13, 26), our results confirmed that with respect to hip fracture discrimination, cortical volume is an inferior parameter compared to cortical thickness. Previous studies have shown the power of cortical bone in resisting fracture and in hip fracture risk prediction (10, 13, 14, 26, 28–31), although the accurate measurement of cortical bone is still challenging due to the partial volume effect (25). One BMD combined with one geometry variable, for example TR vBMD with one structural parameter (e.g. FN cortical thickness), would be the preferred method of discriminating hip fracture risk using hip QCT (12, 26). The ratio of cortical/trabecular bone mass, a measure of the internal distribution of bone, is a superior parameter to cortical thickness in discriminating hip fracture risk across the entire proximal femur.

The combination of selected geometry variables in this study resulted in a similar AUC value (0.805) as the use of aBMD alone (AUC 0.796 or 0.804) reported previously in case-control studies

TABLE 2 | Associations of cortical volume and thickness with hip fracture.

Cortical Variables	Unadj.OR	95%CI		Adj.OR	95%CI	
THCortVol	1.44	1.20	1.74	1.39	1.15	1.67
THCortThick	2.00	1.63	2.45	1.93	1.57	2.37
FNCortVol	1.39	1.17	1.66	1.37	1.15	1.64
FNCortVol_IP	1.50	1.26	1.80	1.47	1.22	1.77
FNCortVol_SP	1.43	1.20	1.70	1.42	1.19	1.71
FNCortVol_IA	1.44	1.21	1.72	1.43	1.19	1.72
FNCortThick	1.77	1.45	2.15	1.71	1.40	2.09
FNCortThick_IP	1.58	1.31	1.89	1.49	1.24	1.80
FNCortThick_SP	1.62	1.34	1.95	1.59	1.31	1.93
FNCortThick_IA	1.76	1.44	2.14	1.75	1.43	2.15
FNCortThick_SA	1.40	1.17	1.69	1.39	1.16	1.68
TRCortVol	1.13	0.95	1.34	1.08	0.90	1.29
TRCortThick	1.60	1.32	1.93	1.56	1.29	1.89
ITCortVol	1.36	1.13	1.62	1.32	1.10	1.58
ITCortThick	2.18	1.77	2.70	2.10	1.70	2.60
RTHCTM	2.48	1.90	3.22	2.57	1.94	3.40
RFNCTM	1.71	1.35	2.17	1.70	1.34	2.16
RTRCTM	2.07	1.67	2.55	2.08	1.67	2.60

Adjusted for age, height and weight. Unadj., unadjusted; Adj., adjusted; OR, odd ratio; TH, total hip; VOI, volume of interest; Vol, volume; Cort, cortical; Thick, thickness; CortThick, cortical thickness; FN, Femoral neck; HeadVol, femoral head volume; Int, integral; RTHCTM, ratio of total hip cortical/trabecular mass; RFNCTM, ratio of femoral neck cortical/trabecular mass; RTRCTM, ratio of trochanter cortical/trabecular mass; FNCSA, femoral neck cross-sectional area; TR, Trochanter; IT, intertrachanter; SA, Supero-anterior; IA, Infero-anterior; IP, Infero-posterior; SP, Supero-posterior.

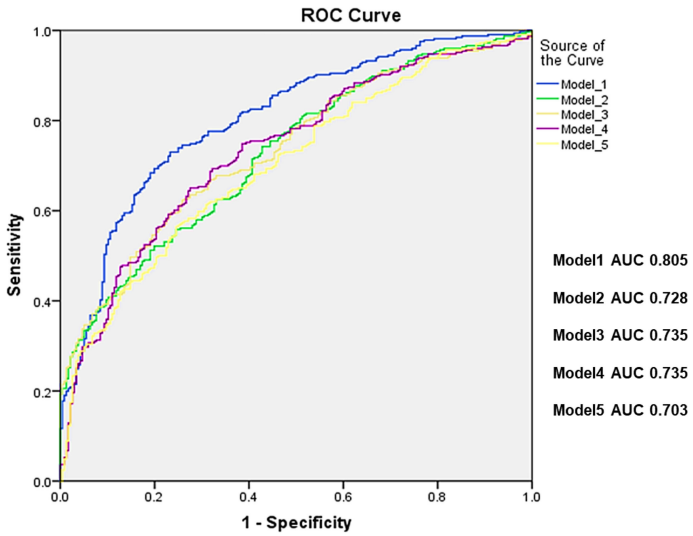

FIGURE 3 | Receiver operating characteristic curves (ROC) for standard models alone (Model 1: THCortThick +HeadVol+THRCTM +FNCSA, Model 2: THCortThick +HeadVol+FNCSA, Model 3: ITCortThick+HeadVol+FNCSA, Model 4: ITCortThick+HeadVol, and model 5: ITCortThick+FNCSA. All 5 models were adjusted for age, height and weight, respectively, and the p values for all models were <0.001. TH, total hip; CortThick, cortical thickness; HeadVol, femoral head volume; RTHCTM, ratio of total hip Cortical/Trabecular Mass; FNCSA, femoral neck cross-sectional area; IT, intertrachanter.

(11, 24). Further, the AUC values of the combination of selected geometry variables in this study were similar to those reported for reference aBMD in prospective studies, ranging from 0.70 to 0.86 (32–38). Although AUC and OR results varied amongst these studies of different datasets, evidence is accumulating for a slight improvement in hip fracture risk assessment. The resulting five best-subset models for discrimination of hip fractures are ordered according to the BIC information criterion of the best-subset procedure, which combines number of variables and goodness of fit of the binary regression model (26). Similar to an earlier study (12), the combination of BMD measures and geometric parameters improved association with hip fracture but results have to be validated in prospective cohort studies. Unfortunately, the radiation dose of QCT scans hampers the application in osteoporosis screening and frailty hip fracture risk assessments. The integration of QCT-based geometry evaluations may trigger a paradigm shift in hip fracture prediction, namely, under certain circumstances, such geometry parameters could be derived from clinical routine CT images and used as predictors of hip fracture risk.

Our study has several limitations. First, due to the cross-sectional design, the analysis was limited to the evaluation of associations with hip fracture instead of prediction. Second, our results were confined to Chinese women, although our findings are consistent with a few Caucasian studies (11, 39). Third, we did not include comparisons with BMD measurements but only focused on geometric parameters. Fourth, we only studied the intact contralateral femur of the hip fracture patients by taking advantage of the anatomical similarity with the fractured side (40) despite the fact that some subjects hips can be surprisingly asymmetric.

In conclusion, there are substantial differences in total and cortical volume as well as cortical thickness between women with and without hip fractures across the entire proximal femur. The combination of geometric variables resulted in similar discrimination power for hip fracture risk as aBMD alone.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Beijing Jishuitan Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XW and XC had full access to the data, take responsibility for the content, and guarantee the integrity and accuracy of the work undertaken. LW, MY, XW, KE, and XC designed the study. LW and YL led the analysis with input from MY, YG, SZ, KE, and GB. YL and YS did the literature search. YS, YG, and SZ collected the data. YL did the measurements. All authors contributed to data interpretation. LW and YL wrote the manuscript and all authors reviewed the manuscript.

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Development and Validation of the Nomograms for Predicting Overall Survival and Cancer-Specific Survival in Patients With Synovial Sarcoma

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Background: The study aimed to build and validate practical nomograms to predict overall survival (OS) and cancer-specific survival (CSS) for patients with synovial sarcoma (SyS).

Methods: A total of 893 eligible patients confirmed to have SyS between 2007 and 2015 were selected from the Surveillance, Epidemiology, and End Results (SEER) database. Patients were randomly divided into the training cohort (n = 448) and validation cohort (n = 445). Clinically independent prognostic and important factors were determined according to the Akaike information criterion in multivariate Cox regression models when developing the nomograms with the training cohort. The predictive accuracy of nomograms was bootstrapped validated internally and externally with the concordance index (C-index) and calibration curve. Decision curve analysis (DCA) was performed to compare the clinical usefulness between nomograms and American Joint Commission on Cancer (AJCC) staging system.

Results: Two nomograms shared common indicators including age, insurance status, tumor site, tumor size, SEER stage, surgery, and radiation, while marital status and tumor site were only included into the OS nomogram. The C-index of nomograms for predicting OS and CSS was 0.819 (0.873–0.764) and 0.821 (0.876–0.766), respectively, suggesting satisfactory predictive performance. Internal and external calibration curves exhibited optimal agreement between the nomogram prediction and the actual survival. Additionally, DCA demonstrated that our nomograms had obvious superiority over the AJCC staging system with more clinical net benefits.

Conclusions: Two nomograms predicting 3- and 5-year OS and CSS of SyS patients were successfully constructed and validated for the first time, with higher predictive accuracy and clinical values than the AJCC staging system regarding OS and CSS.

Keywords: synovial sarcoma, nomogram, overall survival, cancer-specific survival, decision curve analysis

INTRODUCTION

Synovial sarcoma (SyS) is a rare malignancy that most commonly occurs in adolescents and young adults, accounting for about 6%–9% of the soft tissue sarcomas (1). SySs often originate in para-articular regions of the extremity, hardly arising within the joint (2). SySs have always been considered high-grade with particular molecular mechanism and poor prognosis (3). Due to its lower incidence, most analyses of clinical characteristics and outcome for this disease are mainly from retrospective reviews in a single center with few prospective studies available, leading to a poor understanding of this tumor. Furthermore, there still lacks a consensus of local and systemic management for SyS among clinicians, although there are multimodal approaches including surgical resection, radiotherapy, and adjuvant chemotherapy.

Because of the rarity of this tumor, to date, there is no perfect model for survival outcome prediction. Tumor-node-metastasis (TNM) staging system of the American Joint Commission on Cancer (AJCC) has long been a generally accepted formula for predicting prognosis of malignancies and represents the gold standard classification method for SyS (4). Nevertheless, a growing number of studies have demonstrated that several other factors such as age, race, tumor site and size, and non-biological factors also have an obvious impact on the prognosis of SyS patients. Additionally, the current AJCC staging system roughly divided patients into various groups but fails to evaluate the individualized survival based on patients' demographic and clinical characteristics. Therefore, there is an urgent need to construct a novel staging system considering both patients' status and tumor characteristics.

Prognostic nomograms are graphic and quantitative models with high precision and forecasting ability, and they have been developed in clinical practice to evaluate survival for several cancers (5–8). Compared with the AJCC staging system, nomograms can more accurately estimate survival for individual patients by integrating important prognostic variables (9). However, due to the small sample of SyS patients in each single center, no nomograms that predict overall survival (OS) or cancer-specific survival (CSS) have been developed for SyS so far.

The Surveillance, Epidemiology, and End Results (SEER) database collects the demographics, clinicopathological, and survival data of various cancer patients from population-based cancer registries in the USA, providing a favorable source to investigate rare tumors (10). In this study, we aimed to establish and validate the first comprehensive and practical SyS-targeting nomograms for OS and CSS prediction based on the SEER database. Subsequently, we comprehensively compared the performance of nomograms with that of the current AJCC staging system.

MATERIALS AND METHODS

Patients

Patients diagnosed with SyS between 2007 and 2015 were identified from the SEER database and included in our study. The inclusion criteria were as follows: 1) International Classification of Diseases for Oncology third edition (ICD-O-3) histology code for SyS was not otherwise specified (9040/3), spindle cell (9041/3), epithelioid cell (9042/3), and biphasic (9043/3); 2) SyS was confirmed as the first and only primary malignancy by histology; 3) Patients were older than age 18 years; 4) Clinical and pathologic features were complete and detailed; 5) The follow-up was active with known outcomes. Patients whose diagnostic information could only be derived from a death certificate or autopsy report, as well as those who died within 1 month since initial diagnosis, were excluded. All the included patients were randomly allocated to the training cohort (n = 448, 50%) and validation cohort (n = 445, 50%). Institutional review board approval was not required in our study, since the SEER database is publicly available for researchers worldwide. Our accession ID to the SEER database was 10165-Nov 2017.

Study Variables

Age, sex, race, marital status, insurance status, tumor size, pathology, histologic grade, SEER stage, chemotherapy, radiotherapy, surgery, survival months, vital status, and causes of death for each patient were extracted from the SEER database. The races included white, black, and others (American Indian/AK Native, Asian/Pacific Islander). Marital status was described as married or unmarried, while insurance status was described as Any Medicaid, insured, or uninsured. Tumor size was a continuous variable and converted to categorical variable according to optimal cutoffs, which were determined by X-tile program, a favorable software to determine optimum cut point value (tumor size, ≤6 cm, 6–10 cm, >10 cm). The tumor primary site was described as head and neck, trunk, thorax and pleura, extremities, or others. Cancer stages recorded according to the 6th AJCC stages were regrouped according to the 7th edition. OS and CSS were determined as the primary endpoints of our study. Survival time (in months) was calculated as the interval from diagnosis to death from any cause (OS) or death from SyS (CSS).

Statistical Analysis

Construction of the Nomograms

The training cohort was used to build the nomograms. The univariate Cox regression analysis was used to determine factors associated with survival. Then, variables significantly associated with survival in univariate analysis were subsequently subjected to the multivariable Cox regression analysis. Finally, using the minimum value of Akaike information criterion (AIC), the backward stepwise process was used to stop rule for the multivariable Cox regression analysis and select the independent prognostic factors that strikingly contributed to patients' survival for the constructions of the nomograms, and those factors were integrated to construct the nomograms for 3- and 5-year OS and CSS.

Validation of the Nomograms

The validations of the nomograms were conducted both internally (training cohort) and externally (validation cohort) using C-index and calibration curve. To minimize the overfitting

bias, the nomograms were subjected to 1,000 bootstrap resamples in both validations. Predictive performance was examined using the concordance index (C-index), which was analogous to the area under the curve (AUC) but more suited to censored data (11). The value of the C-index fluctuates between 0.5 (no discrimination) and 1 (perfect discrimination), and a higher C-index value means a better prognostic model (12). Calibration curves were plotted to represent the calibration between the nomogram prediction and the actual outcome. In a perfectly calibrated nomogram, the prediction would fall on a 45-degree diagonal of the calibration curve.

Decision Curve Analysis

Decision curve analysis (DCA), a new algorithm, was performed to assess the clinical usefulness of nomograms that predict survival (13). The best nomogram would exhibit higher net clinical benefits throughout a wide range of threshold probabilities. In our study, DCA was used to compare the clinical value of the nomogram with AJCC staging system in the training and validation cohort, respectively.

All statistical analyses were performed by R software (version 3.3.0). The R packages used in our study included *rms*, *cmprsk*, *rcorrcens*, and *DecisionCurve*. All statistical tests were two-sided, and P value <0.05 was statistically significant.

RESULTS

Patient Characteristics

A total of 893 eligible SyS patients diagnosed between 2007 and 2015 in the SEER database were included in our analysis. The flowchart of the patient selection process was shown in **Figure 1**. A total of 448 and 445 of those patients were randomly allocated to the training cohort and the validation cohort, respectively. Among all the patients, the median age was 41 years with a wide range of 18–93 years. The majority of SyS patients were white (79.1%) and insured (73.3%). The most frequent tumor site was the extremities (50.1%), followed by trunk (28.9%), head and neck (13.9%), and other sites (13.2%). Regarding tumor size, ≤6 cm (43.3%) was the most frequent. Based on SEER staging, most patients (58.5%) were at SEER regional stage, 25.8% at distant stage, and 15.8% at localized stage. More than half (60.1%) of SyS patients had undergone radiotherapy, and 84.3% had received surgery. The results of a descriptive analysis about the demographic and clinicopathological characteristics were summarized in **Table 1**.

Prognostic Nomograms for Overall Survival and Cancer-Specific Survival

In the univariate analysis, age, marital status, insurance status, pathology type, tumor site, tumor size, surgery, radiotherapy,

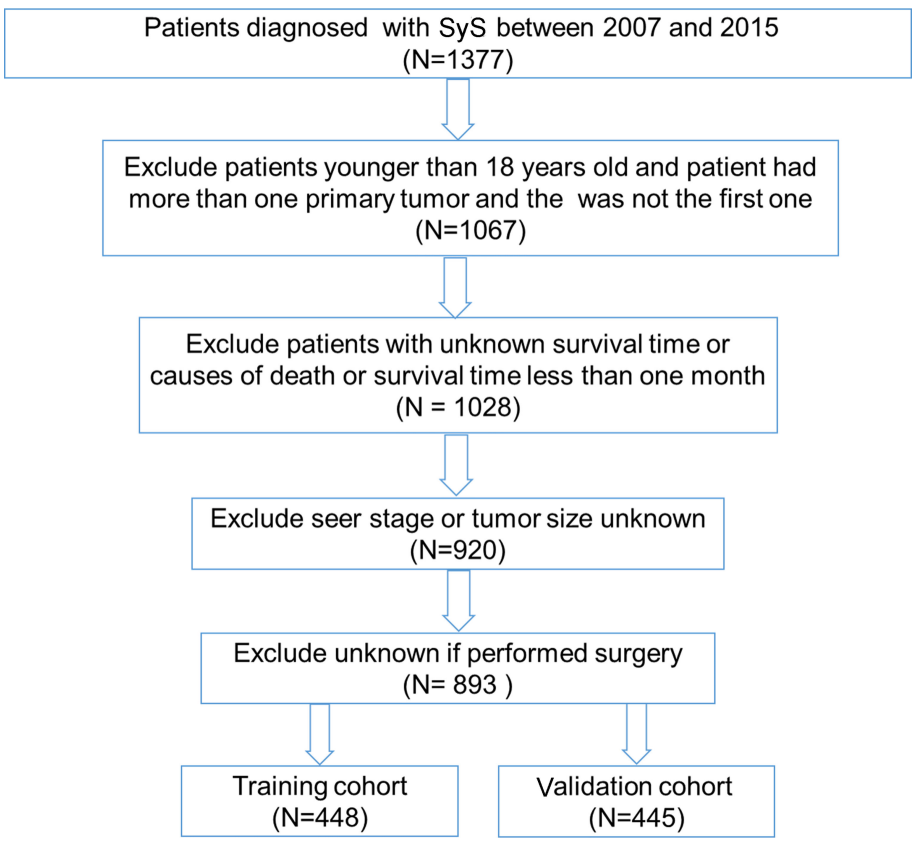

FIGURE 1 | Flowchart of the synovial sarcoma (SyS) patient selection process in our study.

TABLE 1 | Patient characteristics in the training and validation cohorts.

Characteristics	Total N (%)	Training cohort N (%)	Validation cohort N (%)
	893 (100%)	448 (50%)	445 (50%)
Age (median, range)	41 (18–93)	39.0 (18–93)	42.0 (18–93)
Sex			
Female	404 (45.2)	205 (45.8)	199 (44.7)
Male	489 (54.8)	243 (54.2)	246 (55.3)
Race			
Black	90 (10.1)	38 (8.5)	52 (11.7)
White	706 (79.1)	358 (79.9)	348 (78.2)
Others	97 (10.9)	52 (11.6)	45 (10.1)
Marital status			
Married	448 (50.2)	222 (49.6)	226 (50.8)
Unmarried	445 (49.8)	226 (50.4)	219 (49.2)
Insurance status			
Any Medicaid	160 (17.9)	72 (16.1)	88 (19.8)
Insured	655 (73.3)	341 (76.1)	314 (70.6)
Uninsured	78 (8.7)	35 (7.8)	43 (9.7)
Tumor site			
Head and neck	124 (13.9)	65 (14.5)	59 (13.3)
Trunk	130 (14.6)	71 (15.8)	59 (13.3)
Thorax and pleura	74 (8.3)	37 (8.3)	37 (8.3)
Extremities	447 (50.1)	219 (48.9)	228 (51.2)
Other	118 (13.2)	56 (12.5)	62 (13.9)
Tumor size			
≤6 cm	388 (43.4)	195 (43.5)	193 (43.4)
6–10 cm	260 (29.1)	125 (27.9)	135 (30.3)
>10 cm	245 (27.4)	128 (28.6)	117 (26.3)
Pathology			
Biphasic cell	169 (18.9)	96 (21.4)	73 (16.4)
Epithelioid cell	66 (7.4)	32 (7.1)	34 (7.6)
Spindle cell	278 (31.1)	143 (31.9)	135 (30.3)
NOS	380 (42.6)	177 (39.5)	203 (45.6)
Grade			
I	42 (4.7)	18 (4.0)	24 (5.4)
II	120 (13.4)	57 (12.7)	63 (14.2)
III	259 (29.0)	128 (28.6)	131 (29.4)
IV	169 (18.9)	89 (19.9)	80 (18.0)
Unknown	303 (33.9)	156 (34.8)	147 (33.0)
SEER stage			
Localized	141 (15.8)	253 (56.5)	193 (43.4)
Regional	522 (58.5)	120 (26.8)	135 (30.3)
Distant	230 (25.8)	75 (16.7)	117 (26.3)
Chemotherapy			
Not done	444 (49.7)	228 (50.9)	76 (17.1)
Done	449 (50.3)	220 (49.1)	369 (82.9)
Radiotherapy			
Not done	320 (39.9)	183 (40.8)	172 (38.9)
Done	537 (60.1)	265 (59.2)	272 (61.1)
Surgery			
Not done	140 (15.7)	64 (14.3)	216 (48.5)
Done	753 (84.3)	384 (85.7)	229 (51.5)

Others, American Indian/Alaska Native/Asian/Pacific Islander; NOS, not otherwise specified.

and SEER stage were found to be significantly associated with both OS and CSS (**Table 2**). In the subsequent multivariate Cox regression, at first, all these significant factors were subjected to the Cox regression model. In order to pick out the independent prognostic factors that strikingly contributed to patients' survival and could be admitted into the nomograms, we could take the minimum value of AIC to do the variable selection. As shown in **Table 3**, key factors for predicting OS were identified, including age, marital status, insurance status, tumor site, tumor size, SEER stage, surgery, and radiotherapy. These factors were incorporated into the nomogram for predicting the 3- and 5-year OS (**Figure 2A**). As for CSS, marital status and tumor site were ruled out from the selection (**Table 4**). Therefore, a second nomogram for predicting 3- and 5-year CSS was created using the remaining variables (**Figure 2B**).

Nomogram Internal and External Validation

Regarding internal validation, the C-index for the nomograms to estimate OS and CSS in the training cohort was 0.819 (0.873–0.764) and 0.821 (0.876–0.766), respectively. As for external validation, the C-index for the nomograms to predict CSS and OS was 0.816 (0.865–0.767) and 0.831 (0.889–0.772), respectively. The results of C-index all demonstrated that our nomograms were suitable for SyS patients. The calibration curves of OS and CSS nomograms in the training and validation cohorts were shown in **Figures 3** and **4**, respectively, revealing optimal consistency between the prediction by our nomograms and actual survival.

Additionally, we made a comprehensive comparison between SyS nomograms for predicting OS/CSS and the current 7th AJCC staging system. In the training cohort, our nomograms yielded minimum AIC values along with maximal log-likelihoods and C-indexes for both OS and CSS compared with the AJCC stages (**Table 5**), with all between-group *P* values <0.001. Similar distinction was also observed in the validation cohort. The results indicated that our nomograms had more accurate and robust predicting power than the traditional AJCC staging system.

Decision Curve Analysis

After addressing the model accuracy, DCA was performed to render clinical usefulness to the nomograms using the training cohort and generalize it to the validation cohort. The nomogram had high potential for clinical application in predicting CSS and OS of SyS patients because of their wide and practical range of threshold probability through total survival of 3 or 5 years in both cohorts. When further comparing with the current AJCC staging system, our nomograms still had superiority over the AJCC staging system for the fact that more clinical net benefits were obtained in a rather wide range of threshold probabilities when using the nomograms than those when using the AJCC stages (**Figures 5A–D**).

DISCUSSION

Due to its rarity, an accurate assessment of the prognosis for SyS remains challenging. Our knowledge of SyS is restricted to small single-center or multicenter analysis, resulting in uncertainty for the prognostic factors and optimal treatment. The SEER database provides a large sample size for researchers to identify survival-associated factors and has a greater statistical power when studying rare tumors. Herein, using the SEER database,

TABLE 2 | Univariate Cox regression analysis for OS and CSS of the SyS patients in the training cohort.

Characteristics	OS		CSS	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age at diagnosis	1.026 (1.016–1.036)	<0.001	1.023 (1.013–1.033)	<0.001
Sex				
Female	Reference		Reference	
Male	1.236 (0.896–1.704)	0.197	1.293 (0.923–1.812)	0.135
Race	0.4	0.8		
Black			Reference	
White	0.938 (0.483–1.822)	0.852	0.690 (0.408–1.167)	0.167
Others	0.938 (0.484–1.822)	0.209	1.002 (0.511–1.965)	0.996
Marital status				
Married	Reference		Reference	
Unmarried	1.16 (1.06–1.27)	0.002	1.411 (1.05–1.895)	0.022
Insurance status				
Any Medicaid	Reference		Reference	
Insured	0.496 (0.341–0.724)	<0.001	0.502 (0.339–0.743)	<0.001
Uninsured	0.607 (0.323–1.143)	0.122	0.559 (0.283–1.103)	0.093
Tumor site				
Head and neck	Reference		Reference	
Trunk	1.183 (0.692–2.021)	0.539	1.508 (0.846–2.687)	0.163
Thorax and pleura	2.210 (1.212–4.031)	0.009	2.658 (1.393–5.073)	0.003
Extremities	0.843 (0.527–1.349)	0.478	0.986 (0.586–1.662)	0.9604
Other	0.200 (0.081–0.491)	<0.001	0.214 (0.0793–0.576)	0.002
Tumor size				
≤6 cm	Reference		Reference	
6–10 cm	2.259 (1.440–3.545)	<0.001	2.549 (1.571–4.134)	<0.001
>10 cm	5.008 (3.336–7.518)	<0.001	5.706 (3.681–8.847)	<0.001
Pathology				
Biphasic cell	Reference		Reference	
Epithelioid cell	1.890 (1.026–3.483)	0.041	1.758 (0.922–3.357)	0.086
Spindle cell	0.878 (0.551–1.397)	0.582	0.840 (0.517–1.366)	0.015
NOS	1.761 (1.132–2.739)	0.012	1.759 (1.114–2.780)	0.482
Grade				
I	Reference		Reference	
II	0.434 (0.163–1.158)	0.095	0.478 (0.166–1.377)	0.172
III	0.923 (0.396–2.156)	0.853	1.062 (0.422–2.669)	0.899
IV	0.859 (0.363–2.034)	0.729	0.899 (0.351–2.306)	0.826
Unknown	0.672 (0.287–1.570)	0.358	0.739 (0.293–1.866)	0.523
SEER stage				
Localized	Reference		Reference	
Regional	1.875 (1.266–2.776)	0.002	1.895 (1.248–2.878)	0.003
Distant	6.918 (4.706–10.171)	<0.001	7.671 (5.141–11.448)	<0.001
Chemotherapy				
Not done	Reference		Reference	
Done	1.329 (0.966–1.828)	0.080	1.328 (0.952–1.854)	0.095
Radiotherapy				
Not done	Reference		Reference	
Done	0.6204 (0.451–0.852)	0.003	0.637 (0.456–0.888)	0.008
Surgery				
Not done	Reference		Reference	
Done	0.221 (0.154–0.317)	<0.001	0.212 (0.146–0.307)	<0.001

Others, American Indian/Alaska Native/Asian/Pacific Islander; NOS, not otherwise specified; OS, overall survival; CSS, cancer-specific survival; Sys, synovial sarcoma; HR, hazard ratio.

we established the first two novel comprehensive and convenient nomograms for estimating the 3- and 5-year OS and CSS of patients diagnosed with SyS. Our nomograms exhibited satisfactory accuracy and discriminative performance in both internal and external validation. In addition, the variables in our nomograms can be easily obtained from routine clinical practice. With these nomograms, we can identify patients with different prognoses, thus facilitating individualized treatment and follow-up schedule for this rare tumor.

The nomogram has shown a wide application prospect in modern medical decision-making. It provides graphical depiction of statistical model that combines multiple parameters to calculate the probability of survival (7, 14). A number of cancer nomograms have been constructed and showed higher prediction accuracy than the current AJCC staging system, such as prostate, breast, soft tissue sarcoma, and other cancers (15), and thus it has been accepted as an alternative or even a novel staging system (16–18). To our

TABLE 3 | Multivariate Cox regression analysis for OS of the SyS patients in the training cohort.

Characteristics	Full model		AIC-based model	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age at diagnosis	1.030 (1.019–1.041)	<0.001	1.033 (1.022–1.044)	<0.001
Marital status				
Married	Reference		Reference	
Unmarried	1.535 (1.092–2.159)	0.014	1.552 (1.114–2.164)	0.009
Insurance status				
Any Medicaid	Reference		Reference	
Insured	0.474 (0.244–0.921)	0.027	0.484 (0.252–0.933)	0.030
Uninsured	0.783 (0.509–1.204)	0.266	0.771 (0.505–1.177)	0.227
Tumor site				
Head and neck	Reference		Not selected	
Trunk	1.296 (0.866–1.937)	0.207	1.218 (0.818–1.814)	0.329
Lung and pleura	1.991 (1.276–3.104)	0.002	2.078 (1.339–3.224)	0.001
Extremities	0.775 (0.550–1.094)	0.147	0.782 (0.555–1.103)	0.161
Other	0.348 (0.197–0.614)	<0.001	0.327 (0.186–0.577)	<0.001
Tumor size				
≤6 cm	Reference		Reference	
6–10 cm	1.891 (1.137–3.144)	0.014	1.55 (1.35–1.77)	<0.001
>10 cm	3.735 (2.289–6.094)	<0.001	2.17 (1.89–2.47)	<0.001
Pathology			Not selected	
Biphasic cell	Reference			
Epithelioid cell	1.205 (0.622–2.334)	0.581	—	—
Spindle cell	0.718 (0.444–1.163)	0.466	—	—
NOS	1.195 (0.739–1.931)	0.178	—	—
SEER stage				
Localized	Reference		Reference	
Regional	1.088 (0.716–1.653)	0.694	1.148 (0.757–1.739)	0.514
Distant	4.734 (3.046–7.356)	<0.001	5.063 (3.289–7.792)	<0.001
Radiotherapy				
Not done	Reference		Reference	
Done	0.684 (0.484–0.968)	0.032	0.616 (0.439–0.862)	0.004
Surgery				
Not done	Reference		Reference	
Done	0.426 (0.274–0.662)	<0.001	0.366 (0.242–0.556)	<0.001

Others, American Indian/Alaska Native/Asian/Pacific Islander; NOS, not otherwise specified; AIC, Akaike information criterion; CI, confidence interval; OS, overall survival; SyS, synovial sarcoma; HR, hazard ratio.

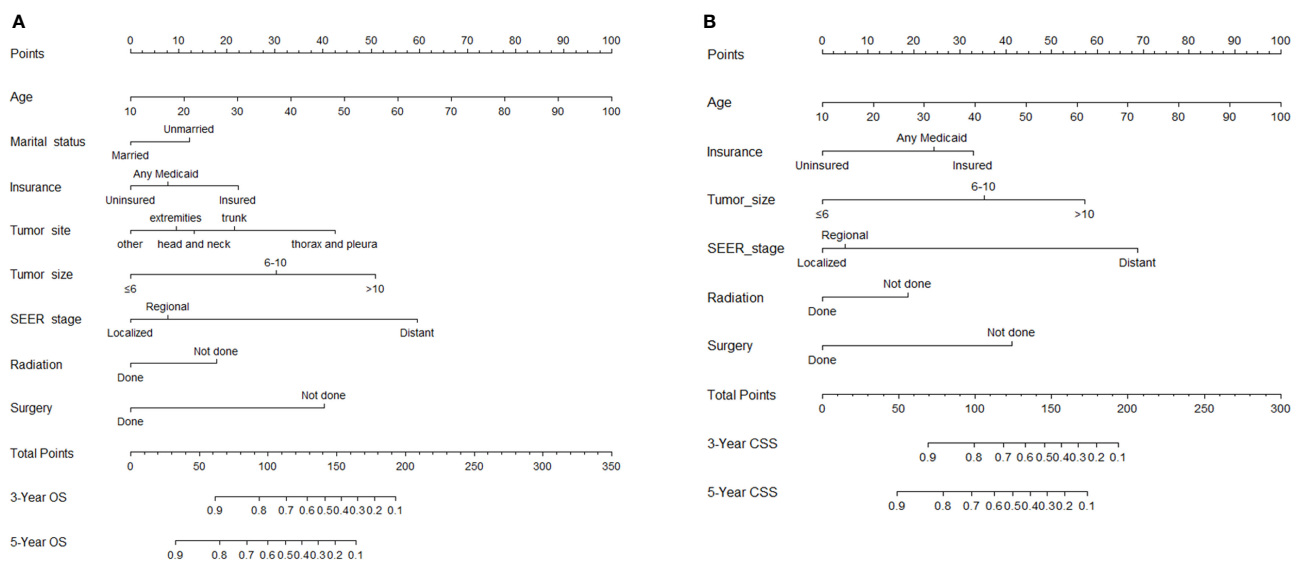

FIGURE 2 | Nomogram for predicting 3- and 5-year overall survival (OS) **(A)** and cancer-specific survival (CSS) **(B)** for synovial sarcoma (SyS) patients.

TABLE 4 | Multivariate Cox regression analysis for CSS of the SyS patients in the training cohort.

Variables	Full model		AIC-based model	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age at diagnosis	1.030 (1.019–1.041)	<0.001	1.0278 (1.017–1.039)	<0.001
Marital status			Not selected	
Married	Reference		—	—
Unmarried	1.046 (0.716–1.528)	0.817	—	—
Insurance status				
Any Medicaid	Reference		Reference	
Insured	0.406 (0.197–0.836)	0.015	0.442 (0.219–0.889)	0.0221
Uninsured	0.824 (0.526–1.291)	0.398	0.808 (0.528–1.235)	0.324
Tumor site			Not selected	
Head and neck	Reference			
Trunk	1.516 (0.816–2.816)	0.188	—	—
Lung and pleura	2.127 (1.068–4.236)	0.0316	—	—
Extremities	0.928 (0.529–1.629)	0.795	—	—
Other	0.659 (0.224–1.93)	0.448	—	—
Tumor size				
≤6 cm	Reference		Reference	
6–10cm	2.199 (1.269–3.809)	0.005	2.404 (1.455–3.972)	<0.001
>10 cm	4.376 (2.583–7.413)	<0.001	4.138 (2.575–6.649)	<0.001
Pathology			Not selected	
Biphasic cell	Reference		—	—
Epithelioid cell	1.173 (0.577–2.385)	0.659	—	—
Spindle cell	0.691 (0.411–1.162)	0.163	—	—
NOS	1.249 (0.751–2.079)	0.390	—	—
SEER stage				
Localized	Reference		Reference	
Regional	0.992 (0.629–1.566)	0.973	1.132 (0.729–1.757)	0.578
Distant	4.738 (2.973–7.549)	<0.001	5.503 (3.523–8.597)	<0.001
Radiotherapy				
Not done	Reference		Reference	
Done	0.686 (0.475–0.991)	0.044	0.629 (0.443–0.894)	0.010
Surgery				
Not done	Reference		Reference	
Done	0.387 (0.246–0.608)	<0.001	0.359 (0.235–0.549)	<0.001

Others, American Indian/Alaska Native/Asian/Pacific Islander; NOS, not otherwise specified; AIC, Akaike information criterion; CI, confidence interval; CSS, cancer-specific survival; SyS, synovial sarcoma; HR, hazard ratio.

knowledge, however, the established nomogram in our study represents the first OS and CSS nomograms for SyS that applied to the general population. Besides, higher predictive accuracy does not mean better clinical practicality. Hence, in order to overcome the limitations of the previous nomograms for other tumors, we introduced DCA in this study, and the results showed that our nomograms obtained better clinical validity and practicality with more clinical net benefits.

Recently, the impact of non-biological factors on human disease has been attached with more emphasis (19, 20). Hence, insurance and marital status were incorporated into our nomogram, which was not mentioned in all the previously reported nomograms for soft tissue sarcoma. In our analysis, we found that the insured patients had better survival OS and CSS compared with those uninsured ones. Recent studies reported that uninsured status was related to decreased diagnosis rates and increased conservative treatment for cancer patients (21), thus impairing patients' survival. At present, the management for SyS has become prolonged, multidisciplinary, and high priced. In fact, uninsured patients usually suffer a relatively vulnerable social support network with which to tackle the challenges from SyS treatment and ultimately faced reduced access to health services and delayed admission to hospital. Just as we know, marriage is an important part of human social life, which could influence patients' emotion, immunological function, nutrition behavior, and fit of therapy (22). And in our analysis, marital status was demonstrated to be an independent prognostic factor for OS. This result has been confirmed in various kinds of cancers (23–25). The married patients tend to enjoy good psychological state, healthy lifestyles, and sound social support networks (26), and this could contribute to their survival advantages to a large extent. Taken together, we strongly recommend integration of non-biological factors into the prognosis prediction system for cancer patients.

Generally speaking, our study has several advantages in the following aspects. First, no prognostic nomogram has been established for SyS patients before. We established the first two nomograms for these patients and made the individualized prediction of prognosis become possible. Furthermore, our nomogram showed better discriminating power in predicting

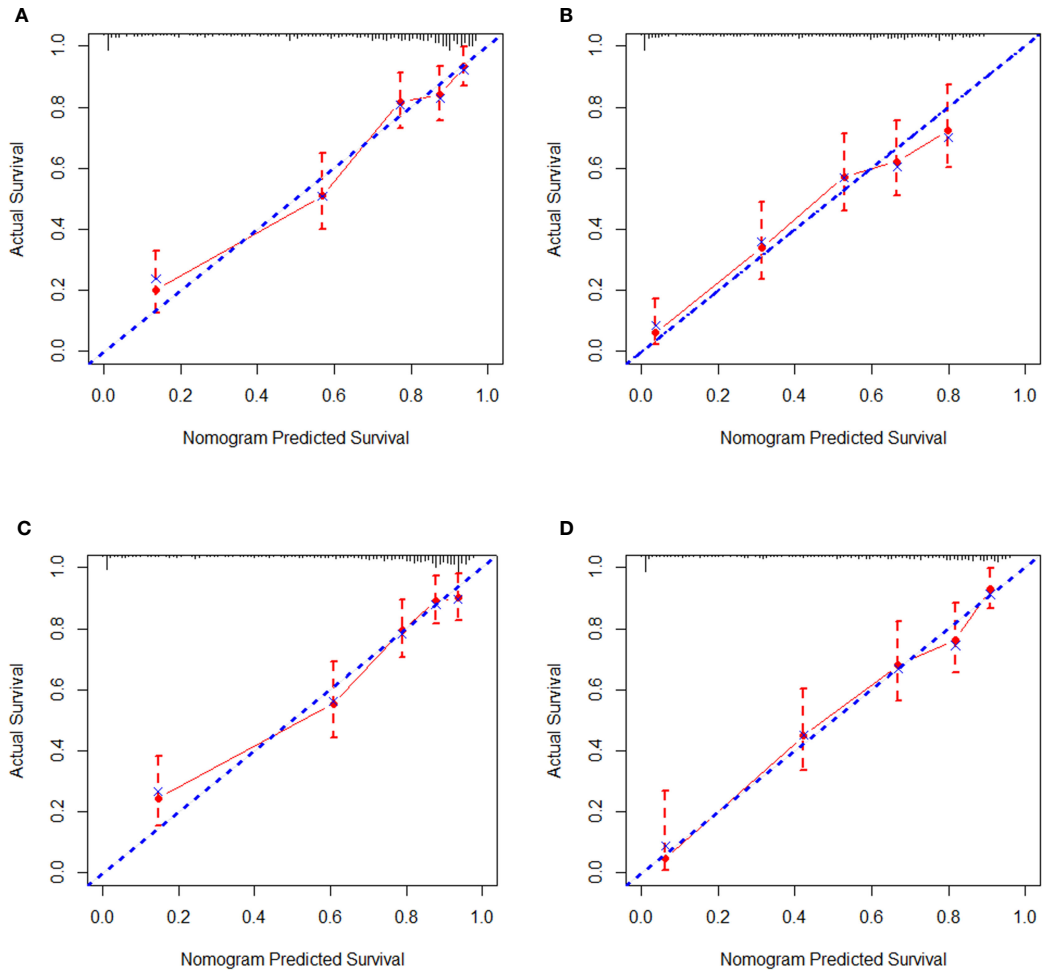

FIGURE 3 | Internal calibration curves in the training cohort. **(A)** The 3-year and **(B)** 5-year overall survival (OS) nomogram calibration curves. **(C)** The 3-year and **(D)** 5-year cancer-specific survival (CSS) nomogram calibration curves.

OS and CSS than the SEER and 7th edition AJCC staging system did. Second, our nomograms were based on a larger-scale population than the SEER database, which provided rich and detailed data. Actually, sufficient samples incorporated are necessary for the accuracy of nomograms. Third, simplicity and user-friendliness were a strength of our nomogram. We used the AIC to minimize the number of parameters used in the nomograms, and these parameters were easily available and measurable for clinicians. Fourth, as we mentioned above, it was the first to reveal that non-biological factors including marital status and insurance status were independent prognostic factors for SyS patients and were incorporated into our nomograms for OS and CSS prediction. Last but not least, DCA, a novel method for analyzing clinical usefulness, was introduced in our nomograms and showed that the new nomograms had wider clinical applicability than the current AJCC staging system.

Inevitably, our study had several limitations that should be noted. The nomograms were established using retrospective data from the SEER database, which may introduce several unavoidable biases, such as treatment selection bias and missing data. Second, the several important prognostic factors of soft tissue sarcoma that were determined in previous studies, such as performance status score, comorbidity, the usage of mammalian target of rapamycin (mTOR) inhibitors or anti-angiogenic agents, and the detailed information of chemotherapy and surgery, were not taken into consideration in our study, since they were unavailable in the SEER database. Third, there was no other independent database available to validate our nomograms externally, hence we used the same retrospective dataset to establish and validate the nomograms. As we know, external validation with independent data was required to evaluate whether it was applicable for another patient groups. And to further refine our nomograms, prospective validation with independent patients was warranted.

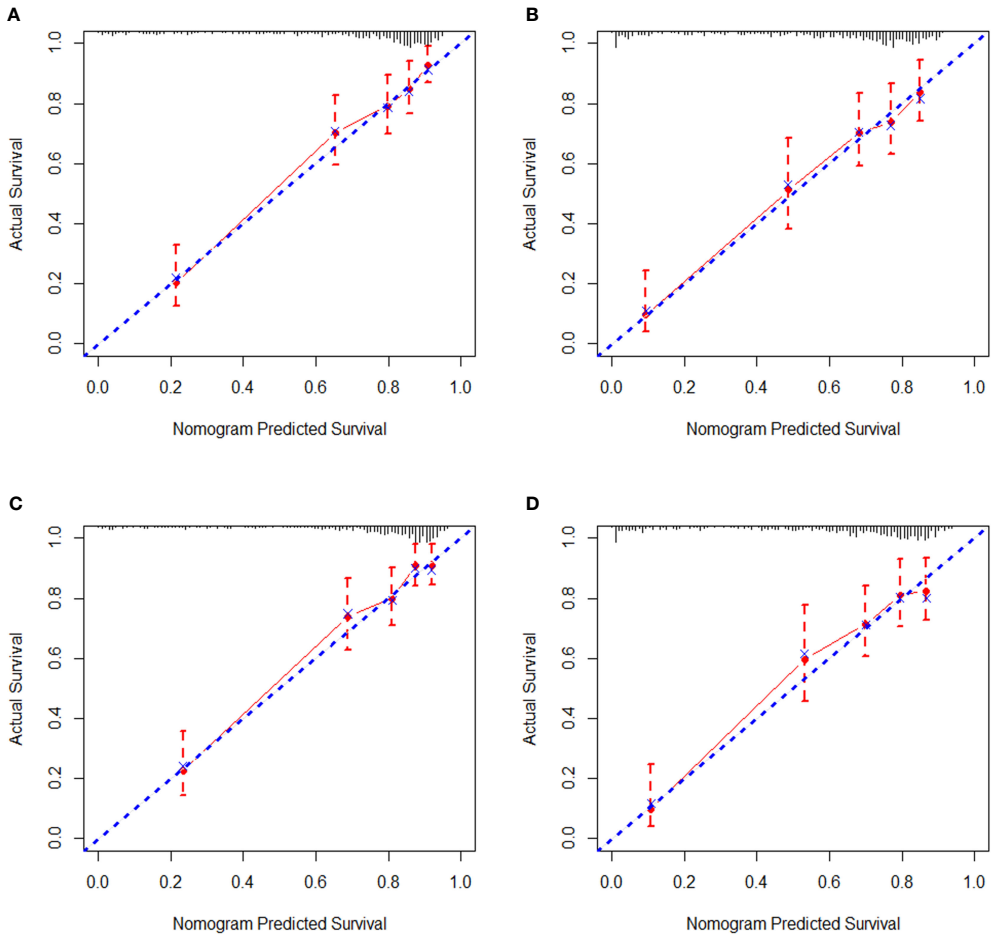

FIGURE 4 | External calibration curves in the validation cohort. **(A)** The 3-year and **(B)** 5-year overall survival (OS) nomogram calibration curves. **(C)** The 3-year and **(D)** 5-year cancer-specific survival (CSS) nomogram calibration curves.

TABLE 5 | The comprehensive comparison between our nomograms and the current 7th AJCC staging system.

	Nomogram	AJCC system	*P*
Training cohort, OS			
AIC	1,321.357	1,362.406	—
Log-likelihood	-620.6	-677.2	<0.001
C-index (95% CI)	0.819 (0.873–0.764)	0.715 (0.765–0.664)	<0.001
Training cohort, CSS			
AIC	1,175.849	1,233.787	—
Log-likelihood	-575.55	-612.89	<0.001
C-index (95% CI)	0.821 (0.876–0.766)	0.726 (0.781–0.671)	<0.001
Validation cohort, OS			
AIC	1,212.145	1,259.111	
Log-likelihood	-593.0	-625.56	<0.001
C-index (95% CI)	0.816 (0.865–0.767)	0.731 (0.784–0.678)	<0.001
Validation cohort, CSS			
AIC	1,095.952	1,137.772	—
Log-likelihood	-534.98	-564.89	<0.001
C-index (95% CI)	0.831 (0.889–0.772)	0.744 (0.801–0.687)	<0.001

OS, overall survival; CSS, cancer-specific survival; AIC, Akaike information criterion; CI, confidence interval; AJCC, American Joint Commission on Cancer.

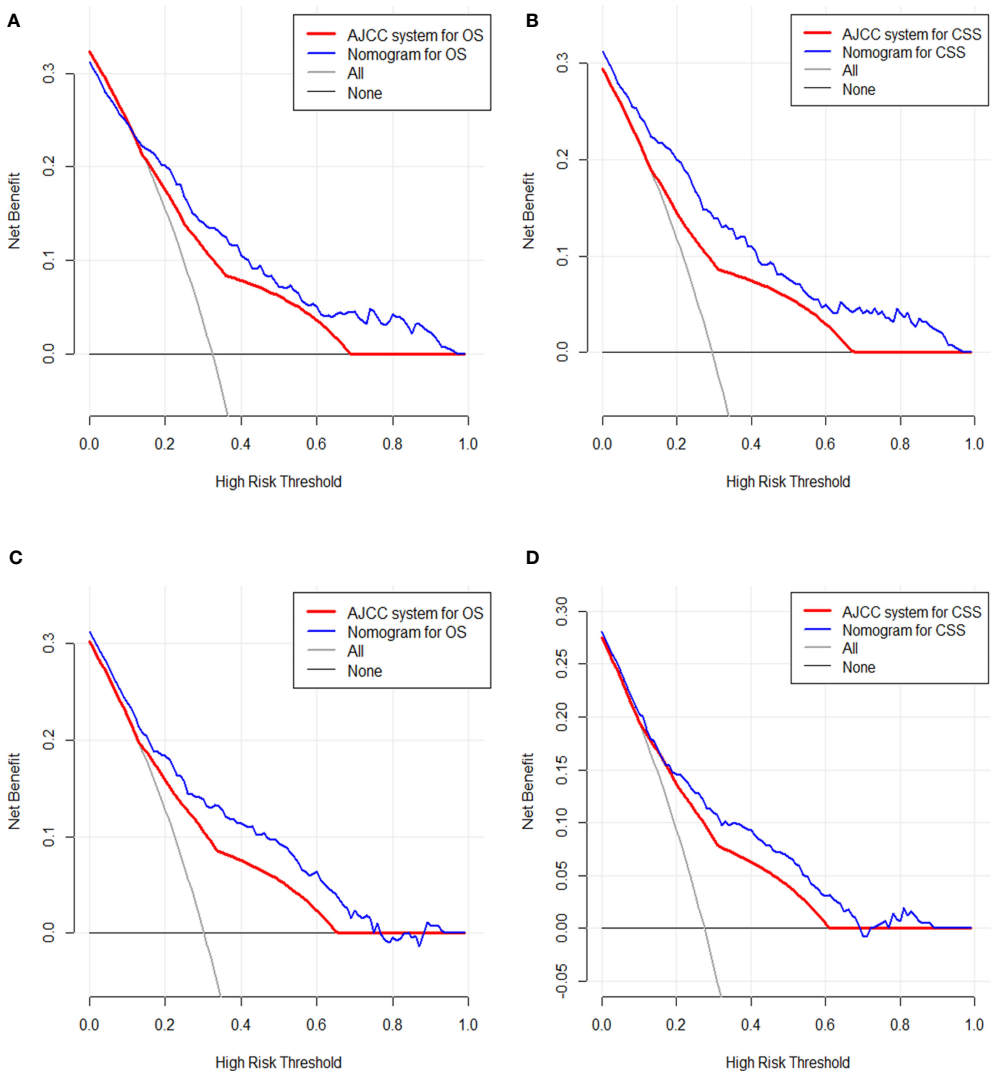

FIGURE 5 | Decision curve analysis of the clinical utility between the nomograms and American Joint Commission on Cancer (AJCC) staging system regarding the overall survival (OS) **(A)** and cancer-specific survival (CSS) **(B)** in the training cohort and OS **(C)** and CSS **(D)** in the validation cohort.

In conclusion, for patients with SyS, we developed and validated the first two nomograms that estimated 3- and 5-year OS and CSS by using population-based data. These nomograms showed more accurate predictive performance and clinical usefulness than the AJCC staging system for predicting CSS and OS. However, performing further external valuation with other independent patients is still warranted.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

ETHICS STATEMENT

The experiments were approved by the Ethics Committee of Zhongshan Hospital, Fudan University.

AUTHOR CONTRIBUTIONS

ZW and YZ: conceptualization, investigation, methodology, project administration, writing—review, editing, and supervision. ZS and LC: data curation, formal analysis, investigation, methodology, and writing—review. LL and WL: methodology, validation, writing—review, and editing. All authors have read and approved the article.

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MicroRNAs in Serum Exosomes as Circulating Biomarkers for Postmenopausal Osteoporosis

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Postmenopausal osteoporosis (PMOP) is the most common skeletal disease in postmenopausal women and has become a global public health issue. Emerging evidence demonstrated the important relationship between microRNAs and PMOP. However, miRNAs have not yet been reported in PMOP. Hence, the present study aimed to investigate the differences in miRNA expression profiles in PMOP with fragility fractures to identify the key circulating miRNAs in serum exosomes and to validate these molecules as potential biomarkers. Postmenopausal women with osteoporotic fracture and normal bone mass were enrolled. Serum exosomes were isolated by traditional differential ultracentrifugation from participants. Isolated exosomes were identified by electron microscopy, western blotting and nanoparticle-tracking analysis and then examined for exosomal small RNA sequencing. The expression of miRNAs was compared by sRNA deep sequencing and bioinformatics analysis. Three miRNAs (mir-324-3p, mir-766-3p and mir-1247-5p) were found to be associated with BMD of L1-L4, FN (femur neck) and TH (total hip), while mir-330-5p and mir-3124-5p were associated with BMD of FN and TH. Furthermore, mir-330-5p was found to promote the ALP activity of hBMSCs, while mir-3124-5p showed the opposite result. The results showed that serum exosomal miRNAs were differentially expressed in postmenopausal osteoporosis patients with fragility fractures. Our study provides the first evidence that exosomal miRNA profiling revealed aberrant circulating miRNA in postmenopausal osteoporosis. Mir-324-3p, mir-766-3p, mir-1247-5p, mir-330-5p and mir-3124-5p, which were associated with bone mineral density (BMD), may serve as candidate diagnostic biomarkers as well as potentially contribute to pathophysiology of PMOP.

Keywords: exosome, miRNAs, postmenopausal osteoporosis, circulating biomarker, fragility fracture

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INTRODUCTION

Osteoporosis (OP) is a systemic bone disorder characterized by an imbalance between bone formation and resorption, which leads to a reduction in bone mass (1). In women, postmenopausal osteoporosis (PMOP) is characterized by low bone mass and consequent fragility fractures, which have impaired quality of life and increased mortality in the population (2, 3). Although the

measurement of BMD by dual energy X-ray absorptiometry (DXA) has been regarded as the "gold standard" and current approaches for predicting fractures are largely based on the measurement of BMD, BMD is associated with only 30–50% patients with major fragility fractures (4). In addition, the change in bone mass by DXA is gradual, and a period of 1 or 2 years is usually necessary to identify significant changes, which is inadequate to monitor bone loss (5). It is urgent to find a more accurate way to diagnose OP and predict fracture risk.

In recent years, miRNAs have attracted extensive attention for their roles in many biological processes, such as cell proliferation, differentiation, apoptosis and migration (6–9). It is known that miRNAs in serum may be associated with biological processes and play an important role in the progression of diseases. Regarding bone metabolism, some researches have shown that miRNAs were associated with bone metabolic disorders (10) and several miRNAs were proved to regulate the osteogenic differentiation of BMSCs by mediating β-catenin-dependent migration (11) or contribute to the regulation of Smad5 (12) and Runx2 (13). Additionally, it was revealed that miRNAs might be biomarkers with diagnostic and prognostic potential in cancer and other diseases (14). However, the complexity and inherent heterogeneity of miRNAs in the circulation make it difficult to develop biomarkers and let alone evaluate the prognosis of diseases.

Exosomes are cell-derived spherical lipid bilayer vesicles (EVs) with a diameter around 40-160nm, widely present in various body fluids, carrying proteins, mRNAs and miRNAs that can be transferred from donor to recipient cells *via* target cell membrane fusion. After release, exosomes are taken up by neighboring or distant cells, and the miRNAs contained within modulate such processes as interfering with the microenvironment, facilitating proliferation, differentiation, senenscence and apoptosis. Exosome also regulate epigenetic processes by delivering miRNAs and regulate the biological function of recipient cells in bone regeneration. A number of researches have confirmed that bone marrow mesenchymal stem cells-derived exosomes could improve bone mass by promoting osteogenesis or inhibiting osteoclastogenesis through mir-196a (15), mir-150-3p (16), mir-181a (17), mir-218 (18), and mir-29a (19). More importantly, serum exosomal miRNAs may affect bone metabolism, and can be good biomarkers based on their stability under various storage conditions. Ruchun Dai et al. reported that, serum exosomes highly expressing mir-19b-3p improved the osteogenic differentiation ability by decreasing the expression of PTEN protein (20). MiRNAs in exosomes were stable enough under different storage conditions even at 4°C for a short time (21), suggesting that serum exosome miRNA panel could be as a noninvasive biomarker for the assessment of bone loss and the detection of fragility fractures. However, there are few studies on serum exosome miRNAs in osteoporosis.

In this study we tested the potential role of these molecules as biomarkers in diagnosis and prognosis of osteoporosis and fragility fracture. We compared the serum exosomal miRNAs between osteoporosis with fragility fractures and normal BMD without fracture in postmenopausal women, to dissect the links between serum exosomal miRNAs and severe osteoporosis. The study of miRNA signatures will provide a deeper understanding of bone turnover mechanism to further identify potential diagnostic biomarkers of fragility fracture and assess fracture risk.

MATERIALS AND METHODS

Patient Serum Samples

A total of 577 postmenopausal women aged 65-75 years from two communities were enrolled and history of fragility fractures were collected, BMD of the lumbar, vertebra and hip were detected by DXA. Hip fractures and spine fractures were verified by review of medical records and imaging examinations. According to the National Osteoporosis Foundation, fragility fractures are fractures resulting from any fall from a standing height or less (22). All the participants were divided into two groups: subjects of control group (CON) had no fracture history with T-score of BMD of any site >-1.0, while subjects of severe osteoporosis group (SOP) suffered from fragility fractures in the vertebral spine and/or hip with BMD T-score of any site ≤-2.5. Serum levels of calcium, phosphorus, 25-hydroxyvitamin D(25(OH)D), parathyroid hormone (PTH), Procollagen 1 N-Terminal Propeptide (P1NP) and β-carboxy-terminal collagen crosslinks (β-CTX) were obtained to rule out secondary OP. Participants using insulin, sex hormones, glucocorticoids, anti-osteoporosis drugs such as bisphosphonates, estrogen and progesterone replacement, selective estrogen receptor modulators (SERMs), parathyroid gland hormones or other drugs affecting bone metabolism, or those who suffered from diabetes, severe cardiopulmonary disease, liver and kidney disease, endocrine and metabolic diseases, autoimmune diseases, malignant tumors and hyperlipemia were excluded from the study.

We implemented the following exclusion criteria: participants without consent form (n = 52), those not finishing the DXA scan (n = 37), BMD or history of fragility fracture not meeting the requirement of the study (n = 298), and those who had medical condition excluded from the study (n = 156). Finally, there were 18 participants remained in CON, and16 participants remained in SOP. This study was approved by the Medical Ethics Committee of Huadong Hospital (2019K055) and informed consent was obtained from all participants. The participants' information was listed in **Table 1**.

Serum Exosomes Isolation

For this study, blood from 34 participants were sampled on weekday mornings between June 2017 and December 2017. A 10 ml tube of whole blood was collected by the trained nurse following standard procedures using a serum separator tube (367820, BD) from each participants. Serum samples were allowed to clot for 30 minutes at room temperature, and then centrifuged at approximately 1000g for 10 minutes. 3 ml peripheral serum from each participant was collected, and exosomes were isolated from serum by traditional differential ultracentrifugation in four steps. At first, serum was diluted with

TABLE 1 | Characteristics of the participants in this study.

Characteristics	Control, n = 18	SOP, n = 16	*p*-value
Age (year)	67.28 ± 5.2	67.0 ± 3.2	0.86
Height (cm)	157.53 ± 5.8	153.0 ± 6.8	0.04
Weight (kg)	59.62 ± 6.7	53.51 ± 7.4	0.02
BMI (kg/m^2)	24.02 ± 2.4	22.92 ± 3.6	0.31
Fracture of vertebra (%)	0	68.75	0.000
Fracture of hip (%)	0	43.75	0.000
Serum creatinine (umol/L)	61.8 ± 12.9	56.93 ± 8.9	0.27
AKP (U/L)	74.6 ± 17.8	80.23 ± 25.1	0.52
Serum calcium (umol/L)	2.41 ± 0.06	2.38 ± 0.11	0.53
Serum phosphorus (umol/L)	1.28 ± 0.23	1.19 ± 0.13	0.25
25(OH)D_3 (ng/ml)	30.33 ± 15.11	28.79 ± 14.59	0.82
PTH(pg/ml)	31.8 ± 9.0	45.3 ± 20.2	0.14
β-CTX(pg/ml)	482.48 ± 209.58	476.79 ± 286.96	0.97
P1NP(ng/ml)	61.2 ± 12.4	61.1 ± 13.3	0.96
BMD of LS (g/cm^2)	0.884 ± 0.13	0.603 ± 0.06	0.000
BMD of FN (g/cm^2)	0.705 ± 0.11	0.511 ± 0.10	0.000
BMD of TH (g/cm^2)	0.752 ± 0.21	0.612 ± 0.16	0.04

LS, lumbar spine; FN, femoral neck; TH, total hip.

sterile phosphate-buffered saline to 50ml, centrifugation at 3000×g for 30 min was performed, then supernatant was centrifuged at 12,000×g for 45min followed by ultracentrifugation for 2h at 120,000 ×g in 4°C. The exosome pellet was re-suspended in 100ul lysis buffer or sterile PBS, depending on subsequent experiments.

Transmission Electron Microscopy (TEM)

The suspension was mixed with an equal volume of 4% paraformaldehyde, and 25ul of the solution was taken up to the loaded copper mesh, dried at room temperature for 20 minutes, and the liquid on the filter screen was blotted from one side with a filter paper, and 30ul of phosphotungstic acid solution was added, stained for 5 min at room temperature, and then was blotted with a filter paper and dried at room temperature. The exosomes were photographed under a transmission electron microscope.

Western Blot Analysis

Exosomes were lysed in RIPA buffer with 1% phenylmethylsulfonyl fluoride (PMSF) and placed on ice for 10 minutes. Protein was quantified by using BCA protein quantitative kit (Sangon Biotech, Shanghai) according to the instruction. The concentration was adjusted by appropriate amount of radioimmunoprecipitation assay (RIPA) buffer, and sodium dodecyl sulfate (SDS) loading buffer of 1/4 volume was added. Protein samples were loaded, separated on 10% SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride (PVDF) membranes, followed by blocking for 1 hour in 5% non-fat skimmed milk in tris buffered saline with tween(TBST) solution. After blocking, membranes were incubated with primary antibodies against TSG101 (1:1000 dilution, ab125011, Abcam) and CD63 (1:1000 dilution, ab216130, Abcam) respectively overnight at 4°C. Membranes were then washed using TBST for three times and incubated in secondary antibody for 1hour in room temperature. At last, membranes were washed and developed by Tanon3500 gel imaging and photographing system (Tanon Science & Technology Co, Ltd.)

Nanoparticle Tracking Analysis (NTA)

Isolated pallets were analyzed by the Nanosight NS300 System (Malvern Instruments, UK) configured with a 488 nm laser and a high sensitivity scientific CMOS camera to determine the size and quantity of particles. The exosome samples were diluted (1:300) in particle-free PBS to an acceptable concentration, according to the manufacturer recommendations. Samples were analyzed under constant flow conditions (flow rate=50) at 25°C. For bootstrapped samples, 30 s × 60 s successive videos were captured with a camera level of 16. Data were analyzed using NTA 3.1.54 software with a detection threshold of 5. For the validation cohort, 15 s × 60 s videos were captured with a camera level of 16 and a detection threshold of 10. Laser-irradiated nanoparticles are captured for 60 seconds and particle were analyzed by NTA software.

MiRNA Library Construction and Sequencing

Serum exosomes were isolated, prepared and sent to BGI-Wuhan (Wuhan, China) for miRNA library construction and next-generation sequencing. For each sample, clean reads were obtained *via* removing the low quality readsand aligned with the human genome. Clean reads were further mapped to sRNA in the GenBank and Rfam to analyze their distribution and annotate small RNA sequences. After sequencing by an Illumina sequencer, image analysis, and base identification, the raw reads after quality control were harvested. Clean reads were aligned against known miRNA precursors and mature miRNAs in the miRBase to identify conserved miRNAs. We filtered out all the samples with library size (total uniquely mapped reads) <50,000 reads. We calculated miRNAs normalized counts by using Variance stabilization normalization (VSN). The resulting VSN counts were corrected for various cohorts along with the removal of the unwanted variances by using the R (v 3.2.2) package RUVSeq (v 1.14.0). We filtered out miRNAs that had a VSN read count less than 0.5 in the 95% of control and PMOP samples, respectively. Fold-change (FC) > 2($|\log_2 FC|>1$) and FDR < 0.05 were the criteria for differential expression.

MiRNA Target Prediction and Relevant Signaling Pathway

Targets of miRNA were predicted by using Targetscan (http://www.targetscan.org) and Gene Ontology (GO) enrichment analysis of all predicted target genes was performed using DAVID online tool (https://david.ncifcrf.gov/summary.jsp). The relevant signaling pathways were analyzed using the MirPath in DIANA (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/page&view=software).

Cell Cultures, Transfection and Osteogenic Differentiation

Bone Mesenchymal Stem Cells (BMSCs) were purchased from CyagenBioscience Inc and cultured in human bone marrow

mesenchymal stem cell basal medium with 10% fetal bovine serum, penicillin-streptomycin and glutamine at 37°C with 5% CO_2. Cells were transfected with 20nM microRNA mimics on day 0 and cultured in human mesenchymal stem cell osteogenic differentiation basal medium with 10% fetal bovine serum, 1% glutamine, 1%ascorbate, 0.2%β-Glycerophosphate and 0.01% dexamethasone from day1 to day7 to induce osteogenic differentiation. Mediums were changed every 2 days.

Alkaline Phosphatase (ALP) Activity Assay

ALP activity was examined by using Alkaline Phosphatase Assay Kit (ab83369, Abcam) in bone mesenchymal stem cells on day 7 after transfecting with related miRNA mimics or vehicles. 5mg pNPP was dissolved in solution with 0.1 M glycine, pH 10.4, 1 mM $MgCl_2$ and 1 mM $ZnCl_2$. Cell culture medium was discarded and 100ul pNPP solution was added 15 minutes. The absorbance was examined at 405 nm.

Statistical Analysis

Numerical data was presented as the mean ± standard deviation. Significant differences between two groups were determined by Student's t test. Differences between multiple groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Statistical analysis was performed using SPSS and correlations were analyzed using Spearman data. $p<0.05$ was considered statistically significant. Statistical significance is displayed as *P < 0.05, **P < 0.01, and ***P < 0.001.

RESULTS

Characterization of Participants

18 participants were in control group who had a normal bone mass and16 participants were in SOP group who suffered from vertebral fracture (68.75%) and/or hip fracture (43.75%). BMI, age, biochemical markers and bone metabolism markers, 25 (OH)D, PTH, and BMD between the two groups were shown in **Table 1**. No statistical differences in the age were observed between the two groups. However, the mean values of height and weight were lower in SOP than those in CON ($p<0.05$). In addition, the SOP group have significantly lower BMD at all measured sites (lumbar spine, femoral neck and total hip) compared to CON group.

Serum Exosome Characterization

Isolated exosomes from participants were identified by a combination of TEM, western blotting and NTA. In **Figure 1** results on exosome isolation were only shown for control group as representative for the feasibility of the method to isolate exosomes. According to TEM results, we observed that isolated particles were approximately 80 nm in diameter and appeared to be round vesicles (**Figure 1A**). To further confirm the identity of the isolated pellets as exosomes, we performed the NTA measurements and observed that the size of isolated particles was112.8 ± 2.0nm in diameter (**Figure 1B**). In detail, the size of exosome was 112.8 ± 2.0nm in CON group and 175.7 ± 5.1nm in SOP group (**Supplementary Figures 1A, B**). The concentration of exosome was 6.07e+008 ± 3.90e+007 particles/ml in CON group and 6.52e+008 ± 1.57e+007 particles/ml in SOP group (**Supplementary Figures 1C, D**). Western blotting identified increased exosome-enriched protein markers CD63 and TSG101 in isolated particle samples, compared to serum samples after exosome-isolation procedure (**Figure 1C**).

Serum Exosome-Associated miRNAs Profile

DEGseq was used to identify differentially expressed microRNAs between CON group and SOP group by second-generation sequencing. According to Volcano Plots, there were statistically significant regulated miRNAs between CON and SOP (**Figure 2A**). We further filtered and analyzed the differentially expressed miRNAs in serum exosome with the miRBase database to obtain all known miRNA counts, and unknown miRNAs were excluded. Compared to control group, 169miRNAs were significantly upregulated (p-value<0.05 and $\log_2$FC [$\log_2$FC]>1) and 70miRNAs were downregulated in SOP group (**Figure 2B** and **Supplementary Table 1**).

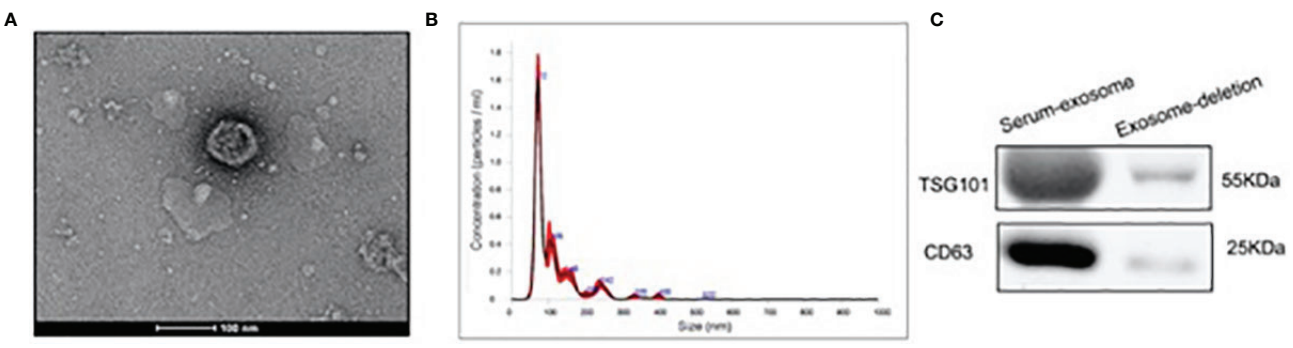

FIGURE 1 | Characterization and identification of serum exosome by ultracentrifugation. **(A)** Exosomes were analyzed by electron microscopy (Scale bar=100 nm). **(B)** Size distribution of exosomes were analyzed by the Nanoparticle tracking analysis. **(C)** Western blotting was applied to detect the exosomal markers TSG101 and CD63 in serum sample after exosome isolation and exosomes isolated from serum sample.

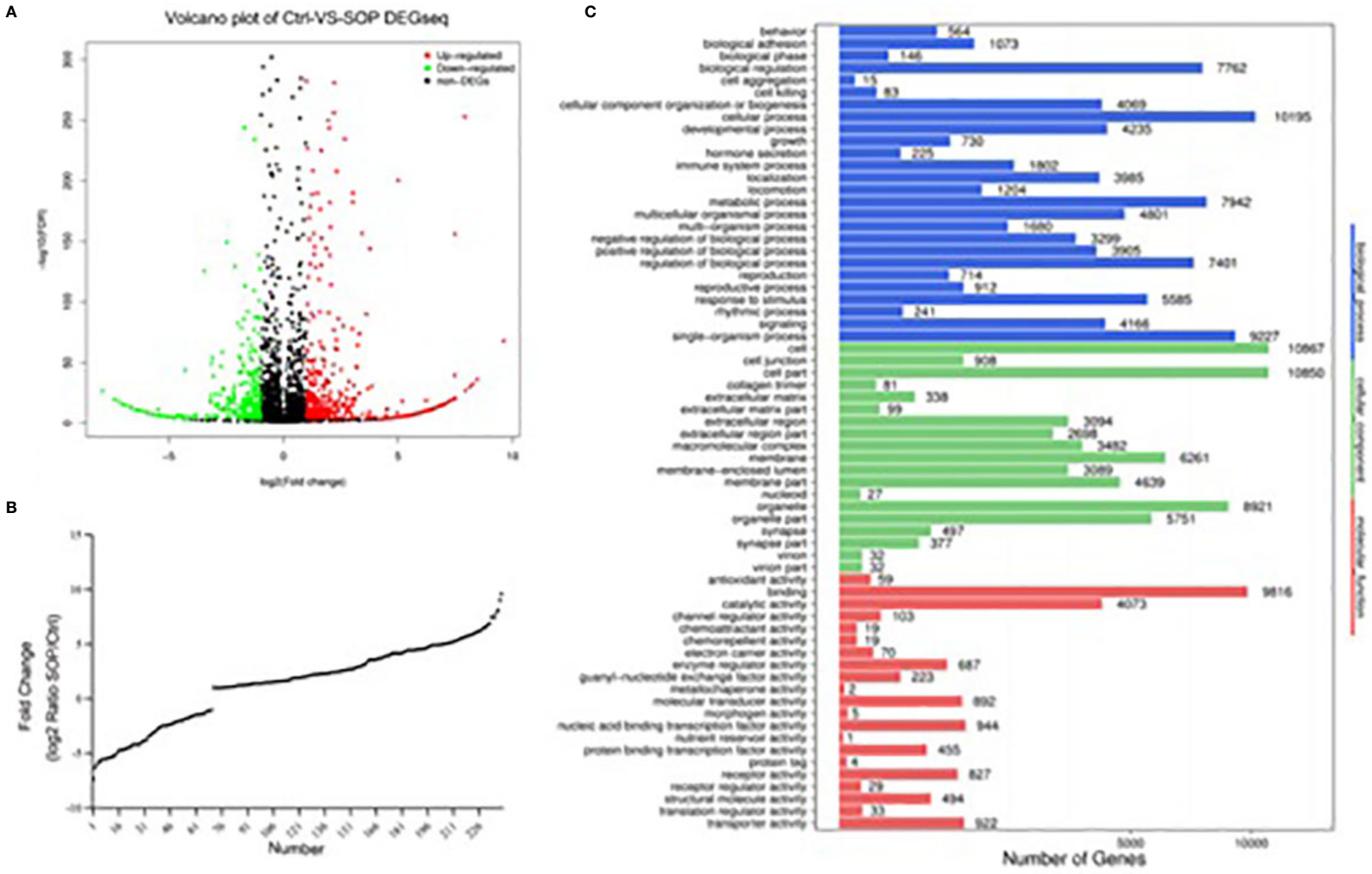

FIGURE 2 | Differentially expressed miRNAs in serum exosomes between SOP and control library. **(A)** Volcano plot was applied to show differentially expressed exosomalmiRNAs in severe osteoporosis group and control group. **(B)** Deletion of unknown miRNA and diverseregulatedmiRNAs were analyzed with miRbase database. X axis shows the number of differently expressed known miRNAs, and the Y axis shows the fold change of SOP/Ctrl. **(C)** Classification of potential target genes for differently expressed known miRNAs by GO analysis. X axis shows the number of target genes, and the Y axis shows the GO terms of biological process, cellular component and molecular function.

Gene Ontology Enrichment Analysis

To greater determine the role of differentially expressed miRNAs in pathological process of PMOP, we input predicted target genes of these known miRNAs to DAVID for GO functional analysis to understand the functional distribution characteristics. The items of biological process(BP), cellular component(CC) and molecular function(MF) terms were presented in **Figure 2C**. The top 3 significant terms from the analysis showed that in the BP category, the diverse miRNAs were involved in cellular process, single-organism process and metabolic process. For the CC category, the different miRNAs were correlated with cell, cell part and organelle. For the MF category, the diverse miRNAs were enriched in binding, catalytic activity and nucleic acid binding transcription factor activity.

Signaling Pathway Analysis of Target Genes

We further analyzed and investigated the potential function of differently expressed top 10miRNAs with online bioinformatics data analysis tools TargetScan and DIANA. Target genes of upregulated miRNAs were mainly involved in rheumatoid arthritis, maturity onset diabetes of the young, glycosphingolipid biosynthesis-globo series, N-glycan biosynthesis, glycosphingolipid biosynthesis-lacto and neolacto series (**Figure 3A**). Target genes of downregulated top 10miRNAs were mainly involved in proteoglycans in cancer, adrenergic signaling in cardiomyocytes, arrhythmogenic right ventricular cardiomyopathy (ARVC), and mucin type O-glycan biosynthesis (**Figure 3B**).

Correlation Analysis of miRNAs With Bone Mineral Density

In this study, we analyzed the correlation between miRNA profiling that was filtered with FC>2(|log2FC|>1) and p-value<0.05 and BMD so as not to miss miRNAs that may have impact on the bone metabolism.To greater confirm the core exosomal miRNAs involved in the progression of PMOP, correlations between known miRNAs and BMD of lumbar L1-L4, FN, and TH were analyzed and the results were shown in **Table 2**. Five miRNAs have been found associated with BMD of 2 or 3 areas of the bone: mir-324-3p [log_2FC=-1.54,P<0.0001], mir-766-3p [log_2FC=-1.3,P<0.0001], mir-1247-5p [log_2FC=2.34,P=0.0029], mir-330-5p [log_2FC=-5.84, P=0.002], mir-3124-3p [log_2FC=5.72,P<0.0001].Three miRNAs were related to the BMD of L1-L4,FN and TH (mir-324-3pand mir-766-3p were positively correlated, while mir-1247-5p was negatively correlated). In addition, two miRNAs were associated

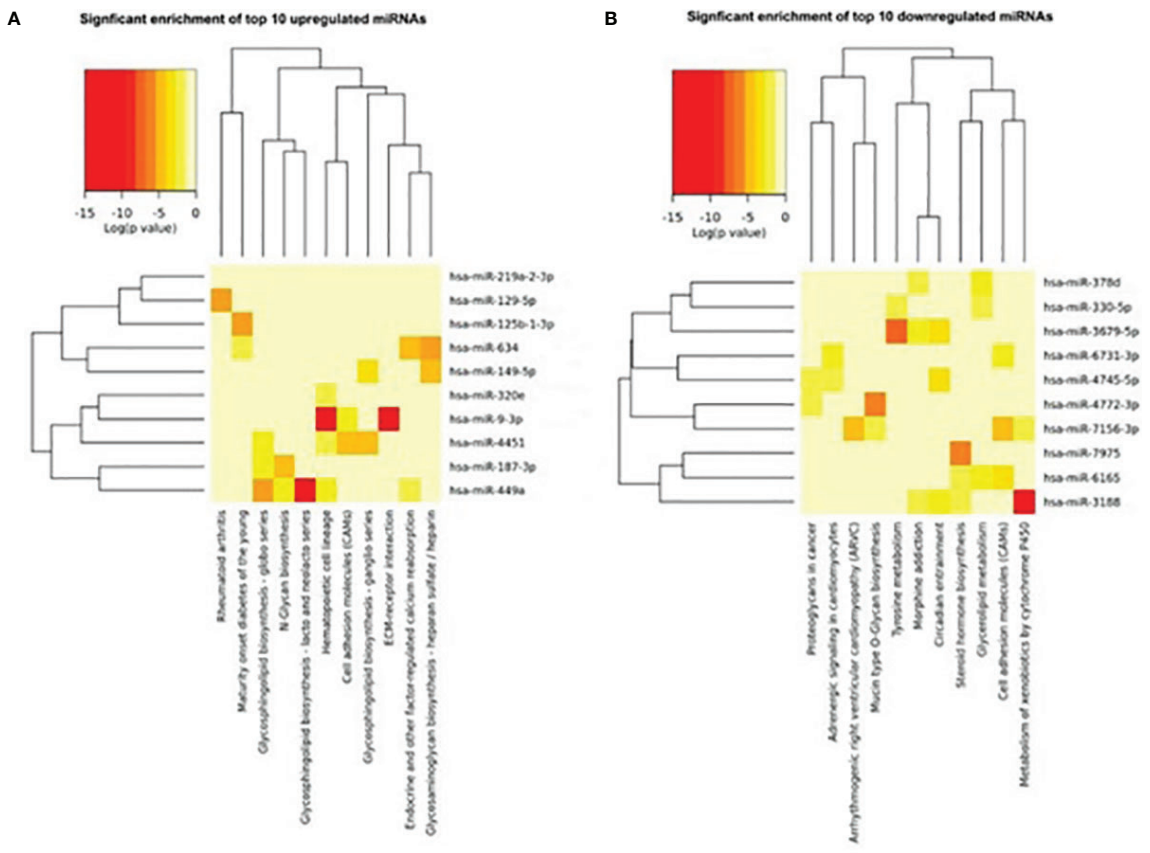

FIGURE 3 | Heat map of signaling pathway enrichment for target genes of top 10 differentially expressed miRNAs. Data of top 10 upregulated **(A)** and downregulated **(B)** miRNAs were analyzed by online bioinformatics tool DIANA. Each row and column represent a miRNA and pathway respectively. The red color shades represent high relative levels and yellow shades represent lower relative levels.

TABLE 2 | Differently expressed miRNAs in serum exosomes related to BMD.

miRNA-name	L1-L4		FN		TH	
	R	*p*	R	*p*	R	*p*
hsa-mir-324-3p	0.511	0.001	0.403	0.009	0.353	0.020
hsa-mir-766-3p	0.408	0.008	0.451	0.004	0.372	0.015
hsa-mir-1247-5p	-0.365	0.017	-0.341	0.024	-0.348	0.022
hsa-mir-330-5p	0.268	0.006	0.355	0.020	0.338	0.025
hsa-mir-3124-3p	-0.205	0.122	-0.339	0.025	-0.298	0.044

LS, lumbar spine; FN, femoral neck; TH, total hip.

with BMD of FN and TH (mir-330-5p was positively correlated, whilemir-3124-3p was negatively correlated). Signaling pathway enrichment for target genes of these five miRNAs were investigated and the results showed that Wnt signaling pathway was the most enrichment pathway relating to bone metabolism and osteogenic differentiation as shown in **Table 3**. Hence, we further analyzed the potential role of these five miRNA candidates in Wnt signaling pathway and online bioinformatics tools as TargetScan and DAVID was applied to help to predict target genes that may be involved in Wnt signaling pathway (**Figure 4**). Wnt family members, frizzled class receptors and dishevelled segment polarity proteins in Wnt signaling pathway were found for more than 3 times as target genes of those 5 miRNAs (**Table 4**)

MiRNA Candidates Relating to BMD Could Regulate ALP Activity in hBMSCs

Signaling pathway enrichment results showed that 5 miRNA candidates relating to BMD were also involved in regulating pluripotency of stem cells. To further confirm the function of these five miRNA candidates on bone turnover imbalance, ALP activity were detected in bone mesenchymal stem cells by transfecting with miRNA mimics or vehicles. ALP activity results showed that mir-330-5psuppressed ALP activity and inhibited the osteogenic differentiation of BMSCs, while mir-3124-3p showed the opposite result (**Figure 5**). In aggregate, these observations suggest that these differentially expressed miRNAs may be involved in the progression of PMOP and have potential to be novel diagnostic biomarkers of PMOP.

TABLE 3 | Signaling pathway enrichment for target genes of miRNAs related to BMD.

Term	*p*-Value	Fold Enrichment
Regulation of actin cytoskeleton	1.37E-04	2.790967
Wnt signaling pathway	0.004861	2.682394
Estrogen signaling pathway	0.040353	2.492729
Ras signaling pathway	9.60E-04	2.456883
Regulating pluripotency of stem cells	0.014823	2.423734
Rap1 signaling pathway	0.003138	2.350288
Hippo signaling pathway	0.023872	2.247171
VEGF signaling pathway	0.027434	2.174344
Long-term depression	0.040105	2.148561
Calcium signaling pathway	0.029788	2.067991
PI3K-Akt signaling pathway	0.003639	1.967089
Cytokine-cytokine receptor interaction	6.07E-04	1.867280
Vascular smooth muscle contraction	0.036487	1.853134
MAPK signaling pathway	0.003362	1.721263
Endocytosis	0.039149	1.611420

DISCUSSION

Osteoporosis and fracture have been strongly associated with women in post-menopausal age. Although clinical and basic research is constantly progressing, patients are still facing delayed diagnosis and fragility fractures, which indicate that exploration of circulating biomarkers is needed to provide a convenient and noninvasive diagnosis. MiRNAs are regarded as promising biomarkers to evaluate disease progression and miRNAs in the serum of OP patients has been investigated, and the patterns of circulating miRNAs are likely to be diagnostic predictors of OP (23). However, few data about miRNAs, let alone circulating exosomal miRNAs, are available in PMOP with fragility fractures. Hence, we presented, for the first time, the serum exosomal miRNAs expression profiles in postmenopausal women, and compared the differences between women suffered from severe osteoporosis and those with normal BMD and discovered exosome miRNAs with promising diagnostic values. In the DEGseq, among miRNAs with fold change >2 and p-value<0.5, 169miRNAs were found to be significantly upregulated, while 70miRNAs were downregulated in SOP group, compared to CON group. GO functional analysis was applied to describe the items of BP, CC and MF terms that provided significant clues to studying molecular functions in the progression of osteoprosis.

PMOP with fragility fractures is a complex biological process that involves complicated signaling pathways. In this study, we focused on miRNAs associated with BMD and related molecular mechanisms to gain insight into the link between PMOP with fragility fractures and miRNAs. We found five exosomal miRNAs (mir-324-3p, mir-766-3p, mir-1247-5p, mir-330-5p and mir-3124-5p) were related to BMD. Moreover, predicted target genes of these five miRNAs were highly associated with Wnt signaling pathway. Wnt signaling pathway is well known for its role in regulating self-renewal and differentiation in stem cells and bone metabolism (24–27). Wnt family members, frizzled class receptors and dishevelled segment polarity proteins in Wnt signaling pathway were notable as target genes of those 5 miRNAs that may provide rewarding points for further research. Among them, mir-324-3p, mir-766-3p and mir-1247-5p were found to be associated with BMD of the lumbar spine, femoral neck and hip sites, while mir-330-5p and mir-3124-5p were found to be associated with BMD of the hip. Previous studies (28) proved that mir-324-3p was expressed at low levels in low-traumatic fractures, indicating that in elderly individuals, low expression of mir-324-3p may result in fractures by reducing bone density. Mir-766-3p could reduce the protein expression of Wnt3a (29) and NF-κB (30), which play important roles in OP. In breast tumors, mir-1247-5p promotes tumor growth *via* the Dishevelled1 (DVL1)/Wnt/β-catenin signaling pathway (31), which promotes the differentiation of skeletal cells and accelerates bone regeneration (32). However, not for all the five miRNAs found associated with BMD, the function in bone metabolism has been validated with *in vitro* study. The only two miRNAs found involved in ALP activity are mir 330-5p and mir- 3124 with the highest fold change (mir-330-5p [log_2FC=-5.84], mir-3124-5p [log_2FC=5.72]). Mir-330-5p suppress ALP activity and inhibit the osteogenic differentiation of BMSCs, and mir-3124-5p significantly promoted osteogenic differentiation of BMSCs. Mir-330-5p was reported to be

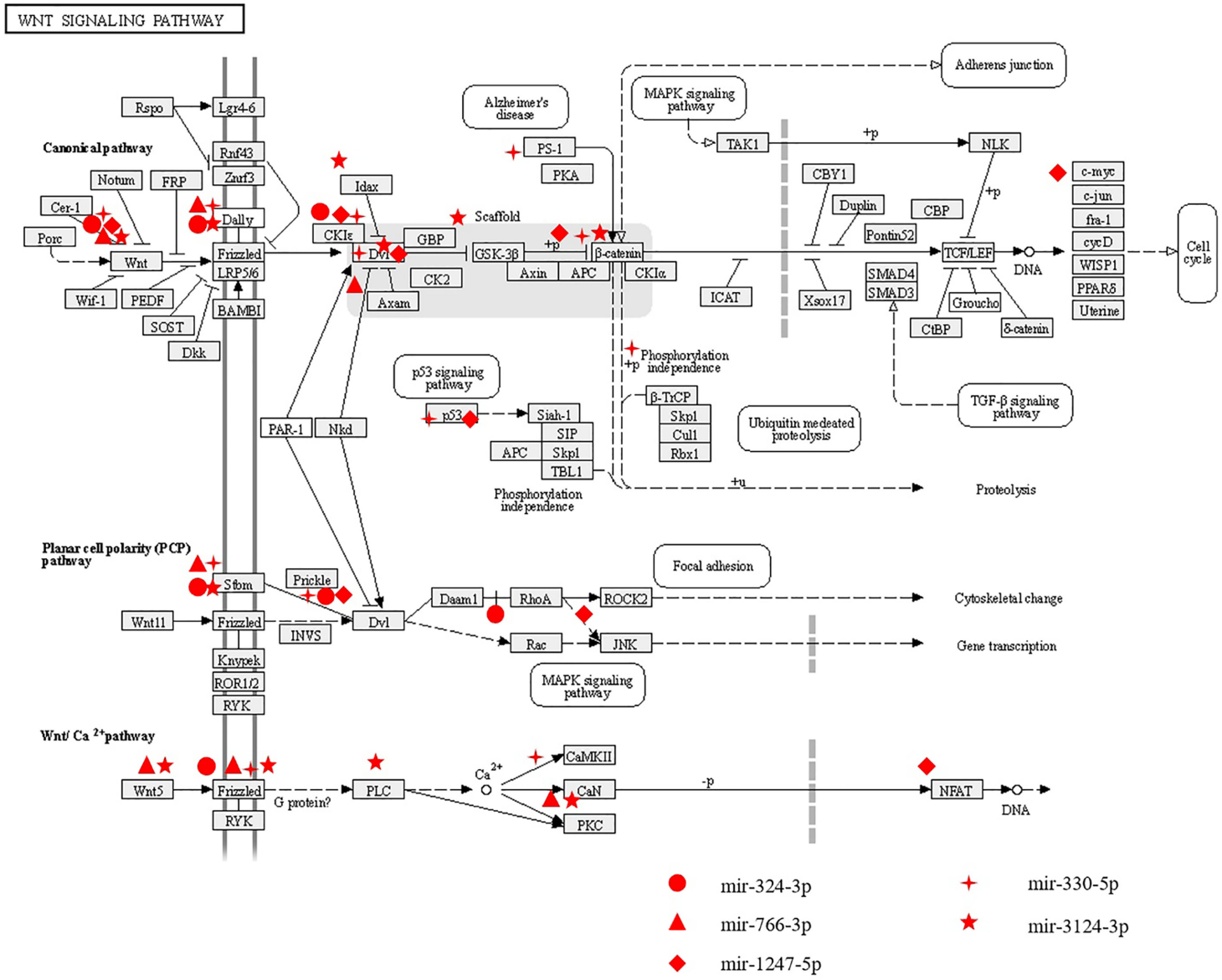

FIGURE 4 | The potential roles of miRNAs related to BMD in Wnt signaling pathway. Five differently expressed miRNAs (including mir-324-3p, mir-776-3p, mir-1247-5p,mir-330-5p and mir-3124-3p) associated with BMD were predicted to play roles in Wnt signaling pathway through regulating their potential target genes.

TABLE 4 | Predicted target genes of miRNAs involved in Wnt signaling pathway.

mir-324-3p	mir-776-3p	mir-1247-5p	mir-330-5p	mir-3124-3p
WNT8B	WNT10A	WNT9B	WNT2B	WNT10B
FZD2	FZD10	DVL3	FZD4	LRP6
CSNK1E	SENP2	CSNK2A2	DVL3	CXXC4
DVL1	VANGL1	APC2	PRKACA	CSNK2A1
RAC3	WNT5B	MYCBP2	APC2	GSK3B
	PRKCA	RND1	BTRC	CSNK1A1L
	NFATC2		DVL1	FZD1
			PPP3CB	WNT5A
				PLCB1
				PRKCA

Wnt, Wnt family member; FZD, frizzled class receptor; CSNK1E, casein kinase 1 epsilon; DVL, dishevelled segment polarity protein; RAC3, rho family, small GTP binding protein Rac3; SENP2, SUMO1/sentrin/SMT3 specific peptidase 2; VANGL1, VANGL planar cell polarity protein 1; PRKCA, protein kinase C alpha; NFATC2, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2; CSNK2A, casein kinase 2, alpha prime polypeptide; APC2, adenomatosis polyposis coli 2; MYCBP2, MYC binding protein 2, E3 ubiquitin protein ligase; RND1, Rho family GTPase 1; BTRC, beta-transducin repeat containing E3 ubiquitin protein ligase; PPP3CB, protein phosphatase 3 catalytic subunit beta; LRP6:LDL receptor related protein 6; CXXC4:CXXC finger protein 4; GSK3B, glycogen synthase kinase 3 beta; CSNK1A1L, casein kinase 1 alpha 1 like; PLCB1, phospholipase C beta 1.

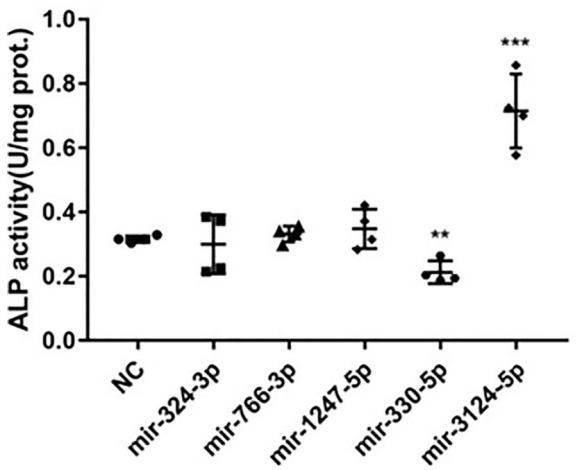

FIGURE 5 | The function of miRNAs related to BMD in regulating ALP activity in hBMSCs. Five differently expressed miRNAs mimics (including mir-324-3p, mir-776-3p, mir-1247-5p,mir-330-5p and mir-3124-3p) associated with BMD were transfected into hBMSCs to upregulate correspondent miRNAs expression. ALP activity was examined on day 7 after transfection assay. Error bars represent SD of three independent experiments; $^{**}p < 0.01$, $^{***}p < 0.001$.

upregulated in senescent MSCs compared with young MSCs (33). MSCs are known to have self-renewal and multi-differentiation abilities, and a reduction in osteogenic differentiation of MSCs leads to loss of bone mass and contributes to increased risk of fracture. Knockdown of mir-330-5p facilitates osteogenesis through the biglycan-induced bone morphogenetic protein (BMP)/Smad pathway and further to influence the progression of OP (34). On the other hand, mir-330-5p was found to silence SPRY2 expression and further influence the progression of tumors *via* Mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) signaling (35), which is a regulator of osteoclastogenesis and plays an important role in bone loss (36). In our study, mir-330-5p in exosomes was positively correlated to the BMD of FN and TH and *in vitro* suppress ALP activity and inhibit the osteogenesis. These results indicate that for those people with relatively high bone mass, the expression of mir-330-5p may be elevated to suppress the osteogenic differentiation of MSCs to maintain the balance of bone metabolism. There were few studies on mir-3124-5p before, however, in this study we found mir-3124-5p was negatively related to BMD *in vivo*, and significantly promoted osteogenesis *in vitro*. We consider that in patients with low bone mass, the expression of mir-3124-5p may be upregulated compensatively to promote the osteogenic differentiation of MSCs to prevent bone loss. These results suggested that the exosomal miRNAs candidates associated with BMD might play complex regulatory roles in the progression of PMOP by networking with cell signaling pathways, and mir-330-5p and mir-3124-5p in circulating exosomes could not only be biomarkers but also functional molecules in the progression of PMOP. However, more studies are needed to clarify the molecular mechanisms of miRNAs in circulating exosomes in PMOP with fragility fractures.

In conclusion, this study provided the first information on differential serum exosomes miRNA expression profiling between severe osteoporosis and normal BMD in postmenopausal women using second-generation sequencing. mir-324-3p, mir-766-3p, mir-1247-5p,mir-330-5p and mir-3124-5p were found to be associated with BMD, but only miR-330 and miR-3124 had been confirmed its role in bone metabolism *in vitro*, which may serve as circulating biomarkers as well as therapeutic targets and treatment options for PMOP. However, in order to apply these miRNA profiles in clinical practice, further studies on prospectively collected datasets are needed to validate these findings, and more reliable and reproducible analysis model are required in following studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. They can be found on Figshare via the DOI 10.6084/m9.figshare.17086307.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Huadong Hospital (2019K055). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QC designed the study. HS and QC drafted the manuscript. QC, XJ, and HS contributed to its refinement. QC and HS recruited the patients and collected the data. HS, XJ, and CX performed the statistical analysis. QC, HS, and XJ interpreted the analytical data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.819056/full#supplementary-material

Supplementary Figure 1 | Size distribution and concentration difference of exosomes in two groups were analyzed by the Nanoparticle tracking analysis.

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Relationship Between Non-Alcoholic Fatty Liver Disease and Degree of Hepatic Steatosis and Bone Mineral Density

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Background: The liver and bones are both active endocrine organs that carry out several metabolic functions. However, the link between non-alcoholic fatty liver disease (NAFLD) and bone mineral density (BMD) is still controversial. The goal of this study was to discover if there was a link between non-alcoholic fatty liver disease and bone mineral density in US persons aged 20 to 59 years of different genders and races.

Methods: Using data from the National Health and Nutrition Examination Survey (NHANES) 2017–2018, multivariate logistic regression models were utilized to investigate the association between NAFLD and lumbar BMD. Fitted smoothing curves and generalized additive models were also used.

Results: The analysis included a total of 1980 adults. After controlling for various variables, we discovered that NAFLD was negatively linked with lumbar BMD. The favorable connection of NAFLD with lumbar BMD was maintained in subgroup analyses stratified by sex, race and age in men, other race and aged 20-29 years. The relationship between NAFLD and lumbar BMD in blacks and people aged 40-49 years was a U-shaped curve with the inflection point: at 236dB/m and 262dB/m. Furthermore, we discovered that liver advanced fibrosis and liver cirrhosis were independently connected with higher BMD, while no significant differences were detected in severe liver steatosis and BMD.

Conclusions: Our study found an independently unfavorable relationship between NAFLD and BMD in persons aged 20 to 59. We also discovered a positive link between BMD and advanced fibrosis and cirrhosis. More research is needed to back up the findings of this study and to look into the underlying issues.

Keywords: bone mineral density, osteoporosis, NHANES, non-alcoholic fatty liver disease, cross-sectional study, hepatic steatosis

Abbreviations: NAFLD, Non-alcoholic fatty liver disease; BMD, bone mineral density; NHANES, National Health and Nutrition Examination Survey; VCTE, vibration controlled and transient elastography; CAP, controlled attenuation parameter; HBV, hepatitis B virus; HCV, hepatitis C virus; BMI, body mass index.

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BACKGROUND

Osteoporosis is a long-term disorder marked by reduced bone mineral density (BMD) that affects a huge number of people (1). According to the International Osteoporosis Foundation, more than 30 percent of women and more than 20 percent of men over the age of 50 have osteoporosis or osteopenia, putting them at risk for osteoporotic fractures (2). Simultaneously, the prevalence of osteoporosis continues to climb as the population ages and expands (3). Apart from genetics, age, and gender, other variables that affect bone metabolisms, such as food intake and lifestyle, have lately received a lot of attention (4–6). Meanwhile, scientists are working to discover novel ways to prevent and treat osteoporosis.

NAFLD (non-alcoholic fatty liver disease) is the most common chronic liver disease and one of the leading causes of severe liver disease across the world. In the absence of severe alcohol consumption or secondary reasons, NAFLD is characterized as excessive fat infiltration into the liver. Currently, the prevalence in Asia is about one out of four people with NAFLD, which is comparable to many Western countries (7). In addition, a physically inactive lifestyle and a rising trend of metabolic diseases such as hypertension, type 2 diabetes, dyslipidemia and obesity are associated with the prevalence and development of NAFLD (8).

Both the bone and the liver are active endocrine organs with a variety of metabolic functions (9, 10). A growing body of research implies a relationship between NAFLD and low BMD (11–14). According to various studies, patients with NAFLD are more likely to have low BMD and an increased risk of osteoporotic fractures, and the underlying mechanism is convoluted and unknown (11). The occurrence of significant liver fibrosis as determined by vibration controlled and transient elastography (VCTE) was connected to poor BMD in NAFLD in a small number of studies (15). The link between low BMD and NAFLD has only been studied in a few large-scale longitudinal investigations. Furthermore, the mechanism underlying this is unknown, but Circulating molecules, insulin resistance, TNF-α and vitamin D insufficiency appear to be potential linkages (16). As a result, we assessed the connection of NAFLD with BMD in adults in this study using a comprehensive fraction of individuals aged 20 to 59 from the National Health and Nutrition Examination Survey (NHANES).

MATERIALS AND METHODS

Data Source and Study Population

The NHANES is a major, continuing cross-sectional survey in the United States that aims to give objective statistics on health issues and address emerging public health concerns among the general public. The NHANES datasets were utilized for this investigation from 2017 to 2018. The participants in the research had to be between the ages of 20 and 59. Among the 1980 eligible adults, we excluded 3306 individuals with missing Median CAP data, 2686 with missing BMD data, 752 participants with significant alcohol consumption, 889 individuals younger than 20 years, 14 hepatitis B antigen-positive and 29 hepatitis C antibody-positive or hepatitis C RNA-positive samples, and 72 individuals with cancer diagnoses. Finally, 1980 people were enrolled in the study. Finally, 1980 people were enrolled in the study (**Figure 1**).

Ethics Statement

The National Center for Health Statistics Research Ethics Review Board authorized the protocols for the NHANES and got signed informed consent. After anonymization, the NHANES data is available to the public. This enables academics to transform data into a study-able format. We agree to follow the study's data usage guidelines to guarantee that data is only utilized for statistical analysis and that all experiments are carried out in compliance with applicable standards and regulations.

Study Variables

Clinicians use VCTE as a noninvasive approach to determine the prevalence and severity of NAFLD in clinical practice, and it has been found to be trustworthy. NHANES staff used FibroScan® model 502 V2 Touch equipped to conduct VCTE evaluations on participants throughout the 2017-2018 period. According to a recent landmark study, controlled attenuation parameter values, which also be called CAP, ≥274 dB/m was considered suggestive of NAFLD status since had 90% sensitivity in detecting all degrees of liver steatosis (17). Dual-energy X-ray absorptiometry was performed using a Hologic QDR 4500A device and Apex software version 3.2 by qualified radiology technologists to assess lumbar BMD. Covariates in multivariate models may cause the correlations between urinary caffeine and caffeine metabolites and lumbar BMD to be muddled. Age, gender, race, body mass index, poverty to income ratio, education, diabetes status, waist circumference, Glycated hemoglobin, Total cholesterol, Triglyceride, LDL- cholesterol, HDL- cholesterol, ALT, ALP, GGT, AST, Serum creatinine, Serum iron, Lumbar bone mineral density, CAP and LSM were all covariates in this study. The NHANES website (https://www.cdc.gov/nchs/nhanes/) has a thorough explanation of how these variables are calculated.

Statistical Analysis

We used R (http://www.r-project.org) and EmpowerStats (http://www.empowerstats.com) for all statistical analyses, with statistical significance set at $P < 0.05$. Because the goal of NHANES is to produce data that is representative of the civilian noninstitutionalized population in the United States, all estimates were calculated using sample weights in accordance with NCHS's analytical guidelines. Model 1 had no variables adjusted, model 2 had age, gender, and race adjusted, and model 3 had all of the covariates listed in **Table 1** adjusted. There were also subgroup analyses performed. A weighted generalized additive model and smooth curve fitting were employed to deal with non-linearity.

RESULTS

Baseline Characteristics

The demographic and laboratory data of the participants (1210 Non-NAFLD, 281 NAFLD and 489 Severe steatosis) are presented in **Table 1**. Compared to Non-NAFLD participants,

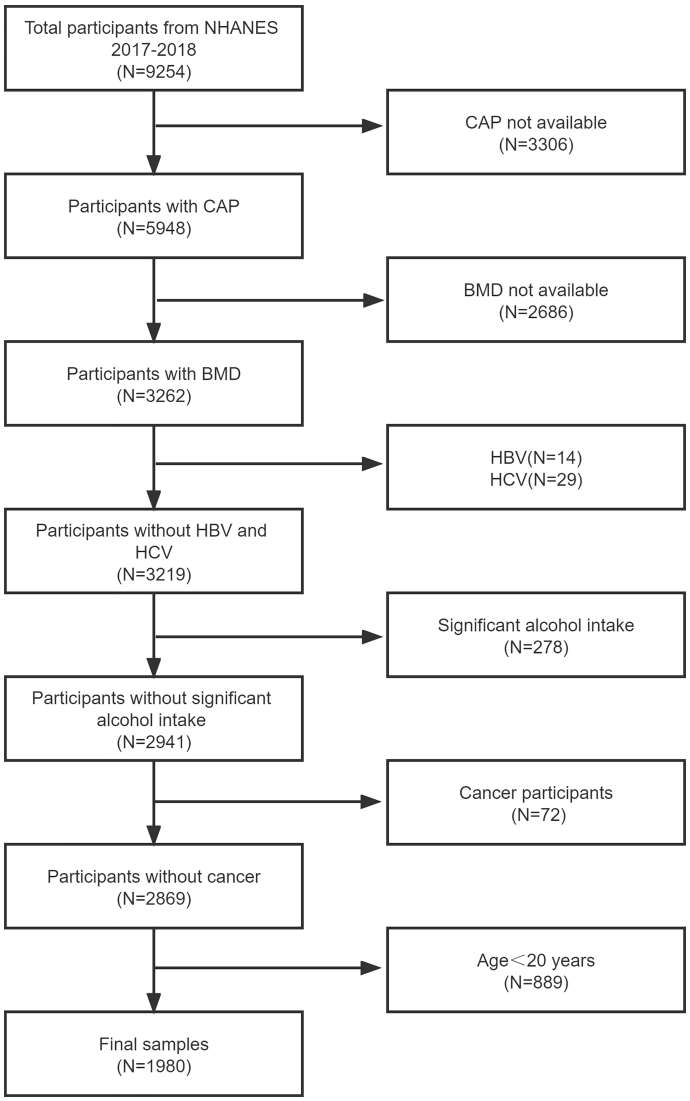

FIGURE 1 | Flow chart of participants selection. NHANES, National Health and Nutrition Examination Survey; CAP, controlled attenuation parameter; BMD, bone mineral density; hepatitis B virus, HBV; hepatitis C virus, HCV.

NAFLD participants and Severe steatosis participants were more likely to be male, Mexican American, and diabetic populations. Participants with NAFLD and Severe steatosis had significantly higher BMI, waist circumference and higher wrist fractured rate, and significantly higher levels of Glycated hemoglobin, Total cholesterol, Triglyceride, LDL- cholesterol, ALT, AST, ALP, GGT, CAP, and LSM, while HDL- cholesterol, and Serum iron, and Lumbar bone mineral density were lower. The weighted characteristics of the study population based on LSM are shown in **Table S1**.

Relationship Between NAFLD and BMD

The findings of the multivariate regression analysis are shown in **Table 2** and **Figure 2**. NAFLD was negatively linked with lumbar BMD in the unadjusted model [-0.022 (-0.035, -0.008)]. However, this significant correlation becomes insignificant after adjusting for the covariates in Model 2[-0.012 (-0.026, 0.001)] and Model 3[-0.013 (-0.049, 0.023)]. With the point of inflection discovered by two-piecewise linear regression model, at 367(dB/m) (**Table 5**).

Subgroup Analysis

After adjusting for covariates, the results of subgroup analysis, smooth curve fittings and generalized additive models showed that the association among NAFLD and BMD was mainly present in males, other race and participants aged 20 to 29. Detailed information on the subgroup analysis is shown in **Tables 2**–**4**.

TABLE 1 | Weighted characteristics of the study population based on CAP.

	Non-NAFLD (CAP<274, n = 1210)	NAFLD (274≤CAP<302, n = 281)	Severe steatosis (CAP≥302, n = 489)	*P* value
Age (years)	36.835 ± 11.891	40.665 ± 11.886	41.183 ± 10.901	<0.00001
Gender (%)				<0.00001
Male	44.600	53.731	61.260	
Female	55.400	46.269	38.740	
Race/Ethnicity (%)				<0.00001
Non-Hispanic White	57.645	48.903	56.806	
Non- Hispanic Black	13.689	11.455	9.606	
Mexican American	7.567	15.343	14.303	
Other Race	21.099	24.300	19.285	
Diabetes (%)				<0.00001
Yes	1.735	5.032	11.909	
No	98.265	94.968	88.091	
Moderate activities				0.09520
Yes	52.879	49.436	49.004	
No	47.121	50.564	50.996	
Smoke at least 100 cigarettes				0.30105
Yes	32.692	33.267	33.097	
No	67.308	66.733	66.903	
Broken or fractured a hip				0.54585
Yes	0.327	1.328	0.368	
No	99.673	98.672	99.632	
Broken or fractured a wrist				0.03451
Yes	17.919	11.184	8.346	
No	82.081	88.816	91.654	
Broken or fractured spine				0.73514
Yes	4.079	3.601	1.686	
No	95.921	96.399	98.314	
Ever taken prednisone or cortisone daily				0.39680
Yes	10.170	8.125	5.263	
No	89.830	91.875	94.737	
Income to poverty ratio	3.063 ± 1.659	3.069 ± 1.601	2.995 ± 1.591	0.74727
BMI (Kg/m2)	26.484 ± 5.634	30.883 ± 5.703	34.975 ± 7.195	<0.00001
Waist circumference (cm)	90.845 ± 14.221	102.653 ± 12.657	112.635 ± 15.418	<0.00001
Laboratory features				
HbA1c (%)	5.327 ± 0.552	5.624 ± 0.907	5.842 ± 1.061	<0.00001
Total cholesterol (mmol/L)	4.753 ± 0.953	4.965 ± 0.965	5.049 ± 0.935	<0.00001
Triglyceride(mmol/L)	1.015 ± 0.658	1.647 ± 1.629	1.921 ± 1.709	<0.00001
LDL- cholesterol(mmol/L)	2.787 ± 0.853	2.976 ± 0.909	3.055 ± 0.833	0.00022
HDL- cholesterol(mmol/L)	1.467 ± 0.382	1.270 ± 0.367	1.190 ± 0.305	<0.00001
ALT (IU/L)	19.855 ± 13.137	26.308 ± 17.980	31.708 ± 22.117	<0.00001
AST (IU/L)	21.072 ± 11.305	21.365 ± 8.724	24.567 ± 13.545	<0.00001
ALP(IU/L)	71.010 ± 22.289	75.605 ± 19.544	78.627 ± 21.957	<0.00001
GGT (IU/L)	23.071 ± 26.912	31.363 ± 30.740	37.902 ± 34.435	<0.00001
Serum creatinine (umol/L)	75.129 ± 17.899	75.437 ± 20.164	75.237 ± 18.139	0.77658
Serum iron(umol/L)	17.028 ± 7.614	15.618 ± 5.559	15.196 ± 5.879	<0.00001
CAP (dB/m)	217.329 ± 36.446	287.384 ± 7.872	341.624 ± 30.199	<0.00001
LSM (kPa)	4.676 ± 1.981	6.121 ± 6.987	7.500 ± 8.264	<0.00001
Lumbar bone mineral density (g/cm^2)	1.058 ± 0.151	1.034 ± 0.137	1.039 ± 0.150	0.00935

Mean+SD for continuous variables: P value was calculated by weighted linear regression model.
% for Categorical variables: P value was calculated by weighted chi-square test.

For males, NAFLD exhibited a significant inverse association with BMD in Model 1[-0.029 (-0.048, -0.009)], but not in Model 2[-0.020 (-0.040, 0.000)] and Model3[-0.004 (-0.060, 0.052)]. In addition, the nonlinear relationship was characterized by smooth curve fittings and generalized additive models (**Table 2** and **Figure 3**).

For other race, the adverse association as same as males in Model 1[-0.024 (-0.046, -0.003)], but not in Model 2[-0.012 (-0.026, 0.001)] and Model3[-0.013 (-0.049, 0.023)]. Of note, when stratified by race, we found a U-shape relationship between NAFLD and BMD in blacks (**Table 3** and **Figure 4**). With the point of inflection discovered by two-piecewise linear regression model, at 236(dB/m) (**Table 5**).

For people aged 20-29, there is a significant negative association with NAFLD and BMD in Model 1[-0.064 (-0.089, -0.038)], Model 2[-0.050 (-0.076, -0.025)] but not in Model3 [-0.058 (-0.119, 0.003)]. Furthermore, we found a U-shape relationship between NAFLD and BMD in people aged 40-49

TABLE 2 | Association between NAFLD and lumbar bone mineral density (g/cm2) stratified by gender.

	Model 1:β(95% CI), *p*	Model 2:β(95% CI), *p*	Model 3:β(95% CI), *p*
Non-NAFLD	Reference	Reference	Reference
NAFLD	-0.022 (-0.035, -0.008) 0.00206	-0.012 (-0.026, 0.001) 0.07913	-0.039 (-0.081, 0.003) 0.07250
Males			
Non-NAFLD	Reference	Reference	Reference
NAFLD	-0.029 (-0.048, -0.009) 0.00495	-0.020 (-0.040, 0.000) 0.05423	-0.021 (-0.050, 0.062) 0.08322
Females			
Non-NAFLD	Reference	Reference	Reference
NAFLD	-0.014 (-0.033, 0.005) 0.15842	-0.003 (-0.022, 0.016) 0.72778	-0.017 (-0.052, 0.039) 0.06461

Model 1: No covariates were adjusted. Model 2: Age, gender, race were adjusted.
Model 3: Age, gender, race, body mass index, poverty to income ratio, education, smoking behavior, Moderate activities, Diabetes status, Waist circumference, HbA1c (%), Total cholesterol, Triglyceride, LDL- cholesterol, HDL- cholesterol, ALT, ALP, GGT, AST, Serum creatinine, Serum iron, Lumbar bone mineral density, CAP and LSM were adjusted.
**In the subgroup analysis stratified by gender or race, the model is not adjusted for the stratification variable itself.*

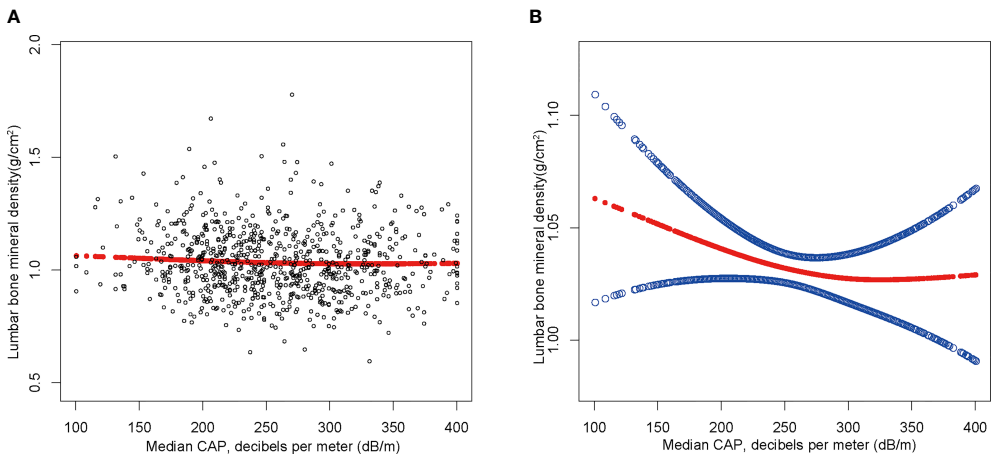

FIGURE 2 | The association between NAFLD and lumbar bone mineral density. **(A)** Each black point represents a sample. **(B)** The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% of confidence interval from the fit. Age, gender, race, body mass index, poverty to income ratio, education, diabetes status, waist circumference, Glycated hemoglobin, Total cholesterol, Triglyceride, LDL- cholesterol, HDL- cholesterol, ALT, ALP, GGT, AST, Serum creatinine, Serum iron, Lumbar bone mineral density, CAP and LSM were adjusted.

TABLE 3 | Association between NAFLD and lumbar bone mineral density (g/cm2) stratified by race.

Race/Ethnicity (%)	Model 1:β(95% CI), *p*	Model 2:β(95% CI), *p*	Model 3:β(95% CI), *p*
Non-Hispanic White			
Non-NAFLD	Reference	Reference	Reference
NAFLD	-0.013 (-0.039, 0.013) 0.32101	-0.008 (-0.035, 0.018) 0.54638	-0.016 (-0.082, 0.042) 0.51292
Non- Hispanic Black			
Non-NAFLD	Reference	Reference	Reference
NAFLD	-0.005 (-0.039, 0.030) 0.77792	0.003 (-0.033, 0.038) 0.88626	0.030 (-0.078, 0.118) 0.46515
Mexican American			
Non-NAFLD	Reference	Reference	Reference
NAFLD	-0.021 (-0.049, 0.006) 0.12916	-0.021 (-0.049, 0.007) 0.14525	-0.041 (-0.128, 0.011) 0.31224
Other Race			
Non-NAFLD	Reference	Reference	Reference
NAFLD	-0.024 (-0.046, -0.003) 0.02556	-0.012 (-0.026, 0.001) 0.07913	-0.011 (-0.090, 0.068) 0.78868

Model 1: No covariates were adjusted. Model 2: Age, gender, race were adjusted.
Model 3: Age, gender, race, body mass index, poverty to income ratio, education, smoking behavior, Moderate activities, Diabetes status, Waist circumference, HbA1c (%), Total cholesterol, Triglyceride, LDL- cholesterol, HDL- cholesterol, ALT, ALP, GGT, AST, Serum creatinine, Serum iron, Lumbar bone mineral density, CAP and LSM were adjusted.
**In the subgroup analysis stratified by gender or race, the model is not adjusted for the stratification variable itself.*

TABLE 4 | Association between NAFLD and lumbar bone mineral density (g/cm2) stratified by age.

Age	Model 1:β(95% CI), *p*	Model 2:β(95% CI), *p*	Model 3:β(95% CI), *p*
Age (20-29)			
Non-NAFLD	Reference	Reference	Reference
NAFLD	-0.064 (-0.089, -0.038) <0.00001	-0.050 (-0.076, -0.025) 0.00013	-0.051 (-0.139, 0.011) 0.05521
Age (30-39)			
Non-NAFLD	Reference	Reference	Reference
NAFLD	-0.017 (-0.041, 0.007) 0.17505	-0.010 (-0.034, 0.015) 0.44259	-0.028 (-0.078, 0.032) 0.63545
Age (40-49)			
Non-NAFLD	Reference	Reference	Reference
NAFLD	-0.008 (-0.037, 0.020) 0.56467	-0.001 (-0.029, 0.027) 0.94431	0.020 (-0.055, 0.109) 0.62401
Age (50-59)			
Non-NAFLD	Reference	Reference	Reference
NAFLD	0.011 (-0.020, 0.042) 0.49400	0.004 (-0.028, 0.035) 0.82339	0.029 (-0.048, 0.107) 0.45933

Model 1: No covariates were adjusted. Model 2: Age, gender, race were adjusted.
Model 3: Age, gender, race, body mass index, poverty to income ratio, education, smoking behavior, Moderate activities, Diabetes status, Waist circumference, HbA1c (%), Total cholesterol, Triglyceride, LDL- cholesterol, HDL- cholesterol, ALT, ALP, GGT, AST, Serum creatinine, Serum iron, Lumbar bone mineral density, CAP and LSM were adjusted.
**In the subgroup analysis stratified by gender or race, the model is not adjusted for the stratification variable itself.*

TABLE 5 | Threshold effect analysis of NAFLD on lumbar bone mineral density using two-piecewise linear regression model.

Lumbar bone mineral density	Adjusted β(95%CI) *P* value
NAFLD	
Inflection point	367
CAP<236(dB/m)	-0.000 (-0.000, 0.000) 0.7497
CAP>236(dB/m)	0.001 (-0.001, 0.003) 0.3573
Log likelihood ratio	0.342
Non-Hispanic black	
Inflection point	236
CAP<236(dB/m)	-0.005 (-0.008, -0.001) 0.0101
CAP>236(dB/m)	-0.000 (-0.001, 0.001) 0.8620
Log likelihood ratio	0.009
Aged 40-49	
Inflection point	262
CAP<262(dB/m)	0.000 (-0.000, 0.001) 0.8448
CAP>262(dB/m)	-0.004 (-0.006, -0.001) 0.0015
Log likelihood ratio	0.028

Age, gender, race, body mass index, poverty to income ratio, education, smoking behavior, Moderate activities, Diabetes status, Waist circumference, HbA1c (%), Total cholesterol, Triglyceride, LDL- cholesterol, HDL- cholesterol, ALT, ALP, GGT, AST, Serum creatinine, Serum iron, Lumbar bone mineral density, CAP and LSM were adjusted.

years, when stratified by age in **Table 4** and **Figure 5**. With the point of inflection identified using a two-piecewise linear regression model, at 262(dB/m) (**Table 5**).

Relationship Between Degree of Hepatic Steatosis and BMD

We further investigated the connection among degree of hepatic steatosis and BMD in adults with NAFLD, we found a significant positive association between advanced liver fibrosis and BMD in Model1[-0.064 (-0.089, -0.038)], Model2[-0.064 (-0.089, -0.038)] but not in Model3[-0.064 (-0.089, -0.038)]. And there is a significant positive association between liver cirrhosis and BMD in Model1[0.067 (0.021, 0.112)], Model2[0.068 (0.024, 0.112)] and Model3[0.153 (0.032, 0.274)]. However, no significant differences were found in severe liver steatosis with BMD as well as Significant liver fibrosis with BMD. **Table 6** provide more details on the subgroup analysis.

DISCUSSION

In this study of individuals aged 20-59 years, we demonstrated the negative association between NAFLD and BMD. In addition, on subgroup analysis, however, we discovered a U-shaped relationship among other studies of NAFLD and BMD in other races and people aged 20-29. Moreover, based on the non-invasive fibrosis markers, we found a positive correlation between BMD and Advanced fibrosis and Cirrhosis.

Clinical studies on the relationship between NAFLD and BMD in adults are still inconclusive. And the majority of these epidemiological studies are centered on Asian and menopausal female populations, with only a handful focusing on European and American males. There was no notable change in BMD among patients with NAFLD and controls, according to a recent meta-analysis of five cross-sectional studies (18). NAFLD was likewise linked to self-reported osteoporotic fractures in the other meta-analysis, but not to poor BMD (14). Other studies, on the other hand, refuted this conclusion. The findings of cohort research involving 4318 Chinese with NAFLD and 17,272 Chinese without NAFLD revealed that NAFLD may enhance the risk of new-onset osteoporosis (19). A Korean cross-sectional study of 3739 premenopausal women discovered a negative link between NAFLD and BMD (12). Other Korean and Chinese cross-sectional investigations

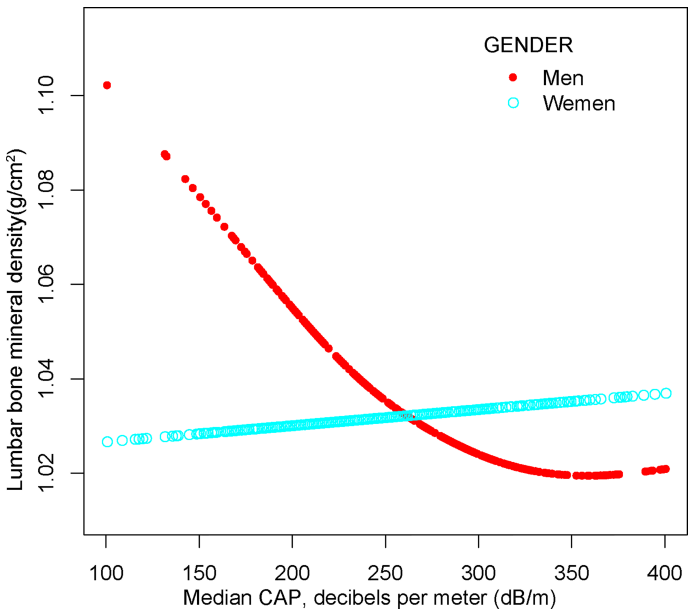

FIGURE 3 | The association between NAFLD and lumbar bone mineral density stratified by gender. Age, gender, race, body mass index, poverty to income ratio, education, diabetes status, waist circumference, Glycated hemoglobin, Total cholesterol, Triglyceride, LDL- cholesterol, HDL- cholesterol, ALT, ALP, GGT, AST, Serum creatinine, Serum iron, Lumbar bone mineral density, CAP and LSM were adjusted.

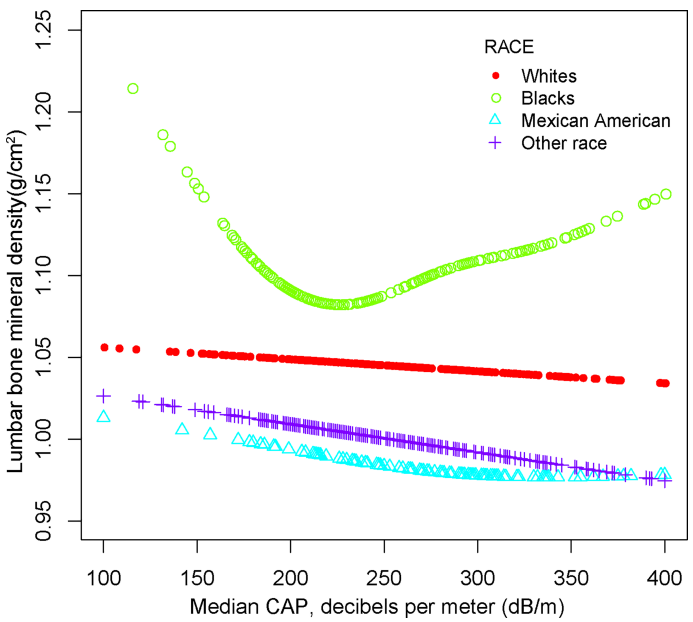

FIGURE 4 | The association between NAFLD and lumbar bone mineral density stratified by race. Age, gender, race, body mass index, poverty to income ratio, education, diabetes status, waist circumference, Glycated hemoglobin, Total cholesterol, Triglyceride, LDL- cholesterol, HDL- cholesterol, ALT, ALP, GGT, AST, Serum creatinine, Serum iron, Lumbar bone mineral density, CAP and LSM were adjusted.

backed up the same conclusion (20–24), as well as a cohort study from America (25). NAFLD was strongly connected to an increased risk of low BMD in men but not in women, and in other race but not in whites, blacks, or Mexican Americans, according to our findings. According to previous studies, NAFLD is a hermaphroditic dimorphic condition that is more frequent in males and postmenopausal women, whereas inadequate bone mineral density is more frequent in

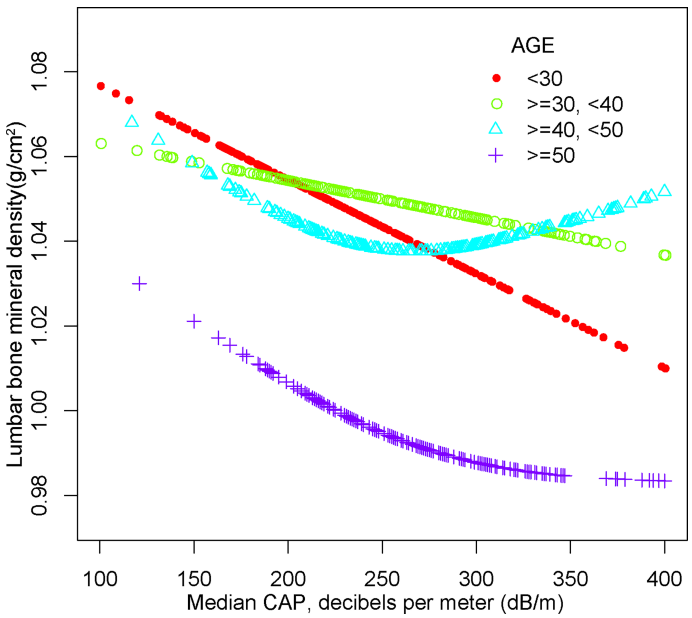

FIGURE 5 | The association between NAFLD and lumbar bone mineral density stratified by age. Age, gender, race, body mass index, poverty to income ratio, education, diabetes status, waist circumference, Glycated hemoglobin, Total cholesterol, Triglyceride, LDL- cholesterol, HDL- cholesterol, ALT, ALP, GGT, AST, Serum creatinine, Serum iron, Lumbar bone mineral density, CAP and LSM were adjusted.

postmenopausal women (26, 27). However, there are few studies on racial differences in NAFLD and BMD and further epidemiological studies based on racial stratification analysis are needed to clarify the causes.

Clinical investigations on the link between steatosis severity and BMD are scarce and controversial. Kim et al. discovered that substantial liver fibrosis as measured by hepatic transient elastography is independently linked with low BMD in a cross-sectional study of 231 asymptomatic Korean participants (15). A new study in NAFLDs looked at the relationship between liver fibrosis and BMD (28). They discovered that NAFLD-related hepatic fibrosis was linked to lower BMD in postmenopausal women with T2DM or IGR. According to a remarkable study (17), severe steatosis defined as CAP ≥ 302, advanced fibrosis defined as LSM ≥ 9.7 kPa, and cirrhosis defined as LSM ≥ 13.6 kPa were noticed. We investigated the association between steatosis severity and BMD by this definition (29). In contrast to previous findings, we discovered that liver advanced fibrosis

TABLE 6 | Association between degree of hepatic steatosis and lumbar bone mineral density (g/cm2).

Exposure	Model 1:β(95% CI), *p*	Model 2:β(95% CI), *p*	Model 3:β(95% CI), *p*
Severe steatosis			
CAP<302	Reference	Reference	Reference
CAP≥302 (n = 489)	-0.015 (-0.030, 0.001) 0.05991	-0.008 (-0.023, 0.008) 0.33794	-0.020 (-0.051, 0.020) 0.35510
Significant fibrosis			
LSM<8.0	Reference	Reference	Reference
LSM≥8.0 (n = 59)	0.011 (-0.015, 0.037) 0.40881	0.013 (-0.013, 0.039) 0.31822	-0.013 (-0.065, 0.046) 0.78231
Advanced fibrosis			
LSM<9.7	Reference	Reference	Reference
LSM≥9.7 (n = 39)	0.049 (0.014, 0.084) 0.00631	0.051 (0.017, 0.086) 0.00367	0.059 (-0.054, 0.101) 0.13638
Cirrhosis			
LSM<13.6	Reference	Reference	Reference
LSM≥13.6 (n = 33)	0.067 (0.021, 0.112) 0.00387	0.068 (0.024, 0.112) 0.00256	0.150 (0.031, 0.264) 0.02010

Model 1: No covariates were adjusted. Model 2: Age, gender, race were adjusted.
Model 3: Age, gender, race, body mass index, poverty to income ratio, education, smoking behavior, Moderate activities, Diabetes status, Waist circumference, HbA1c (%), Total cholesterol, Triglyceride, LDL- cholesterol, HDL- cholesterol, ALT, ALP, GGT, AST, Serum creatinine, Serum iron, Lumbar bone mineral density, CAP and LSM were adjusted.
**In the subgroup analysis stratified by gender or race, the model is not adjusted for the stratification variable itself.*

and liver cirrhosis were independently connected with higher BMD, while no significant differences were detected in severe liver steatosis and BMD.

The mechanisms behind the relationship between NAFLD and BMD are unclear. There are various probable causes for this phenomenon, according to relevant studies. NAFLD can worsen insulin resistance and trigger the production of a slew of pro-inflammatory cytokines and bone-influencing molecules, all of which can contribute to bone demineralization and osteoporosis (30, 31). In addition to this, there is growing evidence that NAFLD causes alterations in the production of several molecular coordinators that may be detrimental to bone health, such as overproduction of TNF-a (32) and deficiencies in vitamin D (33), osteopontin (34) and osteoprotegerin (35). Moreover, circulating molecules may have different effects on bone metabolism by affecting early childhood obesity (36) or the progression of NAFLD (37). However, it is reasonable to believe that increased body weight is a prevalent trait of people with NAFLD (38), may help to prevent bone loss by increasing mechanical loads and improving cortical bone growth. Observations in people with obesity or type 2 diabetes are similar (39). Long-term fracture risk in patients with NAFLD may be underestimated by BMD values alone. It is conceivable to assume, based on the findings of this study, that NAFLD may have a sex-related differential influence on fracture risk. However, given that an increased risk of self-reported osteoporotic fractures among patients with NAFLD has only been seen in two cross-sectional studies conducted in China, it is still unclear if these findings can be generalized to other ethnic communities (22, 23). Furthermore, sex hormone levels and body fat deposition might be plausible causes for the discrepancies between men and women. In postmenopausal women, estrogen insufficiency is believed to be the leading cause of low bone mineral density (40, 41). Estrogen works to retain bone mass by reducing bone resorption by regulating osteoclast activity through the estrogen receptor (42, 43). NAFLD and the effect of estrogen insufficiency in women may contribute to the development of low BMD in an additive or synergistic manner. More research is needed to properly understand the function of NAFLD in the development of bone loss, taking into account the fact that various effects exist depending on gender. However, we feel that further prospective studies and mechanistic research are needed to better understand this crucial subject, particularly in non-Asian populations.

Most cohort and cross-sectional research have focused on postmenopausal women and Asians to yet. Little is known regarding the relationship between NAFLD and BMD in non-Asian, younger populations. Our findings are extremely relevant to the entire population since we used a nationally representative sample. We were also able to undertake subgroup analyses of NAFLD and lumbar spine BMD across gender and ethnicity, and evaluate the relationship between the degree of hepatic steatosis and bone mineral density, thanks to our large sample size. However, it is crucial to acknowledge the study's limitations. First, our study's cross-sectional design makes it hard to conclude a causal association between NAFLD and lumbar BMD in adults. To understand the specific mechanism of the relationship between NAFLD and BMD, further fundamental mechanistic research and large sample prospective studies are required. Second, NAFLD was diagnosed based on vibration controlled and transient elastography, which may have understated the prevalence of the disease. Third, the part of missing data from the NHANES database 2017-2018 on the usage of medication, history of fracture that can alter BMD could have skewed the results. Fourth, due to the limitations of the NHANES database, we were unable to obtain data on T score or Z score, which could also affect our assessment of the participants' osteoporosis.

CONCLUSION

Our study found an independently unfavorable relationship between NAFLD and lumbar BMD in persons aged 20 to 59. This connection followed a U-shaped pattern among blacks and persons aged 40-49 years. We also discovered a positive link between BMD and advanced fibrosis and cirrhosis. Our findings may provide insight into prospective osteoporosis preventative and treatment approaches. More high-quality prospective studies are needed to corroborate or refute our findings on this research issue, as well as a more in-depth analysis of gender and ethnic disparities.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: www.cdc.gov/nchs/nhanes/.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RX and ML designed the research. RX collected, analyzed the data, and drafted the manuscript. RX and ML revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.857110/full#supplementary-material

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Discovery of Potential Biomarkers for Postmenopausal Osteoporosis Based on Untargeted GC/LC-MS

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Purpose: As an important public health problem, osteoporosis (OP) in China is also in an upward trend year by year. As a standard method for diagnosing OP, dual-energy X-ray absorptiometry (DXA) cannot analyze the pathological process but only see the results. It is difficult to evaluate the early diagnosis of OP. Our study was carried out through a serum metabolomic study of OP in Chinese postmenopausal women on untargeted gas chromatography (GC)/liquid chromatography (LC)–mass spectrometry (MS) to find possible diagnostic markers.

Materials and Methods: 50 Chinese postmenopausal women with osteoporosis and 50 age-matched women were selected as normal controls. We first used untargeted GC/LC-MS to analyze the serum of these participants and then combined it with a large number of multivariate statistical analyses to analyze the data. Finally, based on a multidimensional analysis of the metabolites, the most critical metabolites were considered to be biomarkers of OP in postmenopausal women. Further, biomarkers identified relevant metabolic pathways, followed by a map of metabolic pathways found in the database.

Results: We found that there may be metabolic pathway disorders like glucose metabolism, lipid metabolism, and amino acid metabolism in postmenopausal women with OP. 18 differential metabolites are considered to be potential biomarkers of OP in postmenopausal women which are a major factor in metabolism and bone physiological function.

Conclusion: These findings can be applied to clinical work through further validation studies. It also shows that metabonomic analysis has great potential in the application of early diagnosis and recurrence monitoring in postmenopausal OP women.

Keywords: biomarkers, postmenopausal osteoporosis, metabolomics, mass spectrometry, gas chromatography, liquid chromatography

INTRODUCTION

With over 200 million people worldwide with osteoporosis (OP) (1), the main features of the disease are low bone mineral density (BMD), bone loss, microstructure deterioration, and bone quality decline (2), which puts up the fracture vulnerability and the risk of individual hip, spine, and other bone fractures (3). From the clinical data, the prevalence and fracture rate of OP in postmenopausal women are much higher than those in elderly men, so OP is usually considered as a "woman's disease" (4). OP has become a major health problem in developed countries. The existing data show that compared with other Caucasian populations, the prevalence of OP in the Chinese population is higher (5); this will inevitably lead to huge medical costs caused by osteoporosis in China.

As a standard method for diagnosing OP (6), dual-energy X-ray absorptiometry (DXA) cannot detect the pathological process of OP; these changes can only be displayed on DXA for many years. Some bone turnovers are now also used in the diagnosis and drug efficacy evaluation of patients with OP. Some of these markers have been clinically used to determine bone resorption, such as type I collagen cross-linked C-telopeptide (CTX), deoxypyridinoline, serum tartrate-resistant acid phosphatase 5b (TRACP5b), and type I collagen cross-linked N-telopeptide (NTX); there are also some indicators of bone formation, such as osteocalcin and procollagen type I N-terminal propeptide (P1NP) and bone alkaline phosphatase (BAP). In the case of the gold standard for the diagnosis and drug efficacy, no specific marker can be used to determine them. Therefore, it is difficult to evaluate the early diagnosis of OP.

More than 100 years ago, Sir Hans Krebs, an early biochemist, discovered the urea cycle and the citric acid cycle and made a pioneering study on metabolites for the first time. The metabonomics technology is widely used in clinical and biomedical research and has gradually become a new overall diagnostic tool using both advanced analytical technology and bioinformatics. Because it can reflect the current phenotype of specific biological systems, metabonomic measurement can really improve the understanding of pathophysiological process of disease progression and the discovery of new biomarkers for disease diagnostics or prognosis in various organisms (7). At present, there are many mass spectrometry (MS)-based high-throughput platforms, which can analyze 1,000–10,000 samples per day; it has been applied to a variety of metabolomics studies. Microfluidics and miniaturization of separation techniques, as a commonly used emerging technology, can analyze quickly and accurately (8).

Liquid chromatography (LC)–mass spectrometry and gas chromatography (GC)–mass spectrometry are the most common analytical platforms for mass spectrometry in metabolomics research. GC-MS is one of the most effective, repeatable, and commonly used analysis platforms in metabonomics research with the characteristics of robustness, excellent separation ability, selectivity, sensitivity, and reproducibility (9). Due to the ion suppression and matrix effect by co-eluting compounds, GC-MS obtains a higher chromatographic resolution than LC-MS to some certain extent (10). Because it can be used only to distinguish volatile compounds and low molecular weight (about 50–600 DA), chemical derivatization is required before GC-MS is used to detect polar, heat-resistant, and non-volatile metabolites, which makes GC-MS have an inherent limitation (11). LC-MS combines the separation ability of LC and the mass analysis ability of MS; it can not only separate pure or near-pure parts from a chemical mixture but also identify compounds with polymer specificity and detection sensitivity (12). Therefore, LC-MS often analyzes thermally labile, non-volatile, and polar compounds (13). According to the available literature, there are 6 metabonomics studies on patients with osteoporosis, of which two have studied the plasma of postmenopausal women with osteoporosis with nuclear magnetic resonance (NMR) (14) and LC-MS (15), respectively, and three have studied the serum of postmenopausal women with osteoporosis with NMR (16), GC-MS (17), and LC-MS (18) respectively; another study used LC-MS (19) to study the serum of patients with osteoporosis. They all used a single method; the recognition area of metabolites was relatively narrow, and the number of patients involved in most studies was small. Therefore, the results of these studies had some limitations. By combining the two technologies, we can make full use of the technical advantages of GC-MS and LC-MS to study metabolomics more comprehensively and accurately.

This study measured the metabolites in the serum of postmenopausal women with OP and postmenopausal women with normal BMD based on untargeted GC/LC-MS. Untargeted metabonomics can collect as much material information as possible and has a wide material coverage. Serum is easily available and contains molecules that represent the current state of the body and short-term changes. Compared with other compartments, it can better understand the metabolic processes of animal models and humans over a period of time (20). The serum of postmenopausal women with OP was collected in our hospital, and the serum of postmenopausal women with normal BMD without other basic diseases was selected for control. Then, by analyzing the differences of the two groups, biomarkers that can detect postmenopausal osteoporosis early were selected. This is the first metabolic study with our knowledge of OP in postmenopausal women in China based on untargeted GC/LC-MS.

MATERIAL AND METHODS

Participants

In this study, the case group comprised postmenopausal women diagnosed as OP during a physical examination in the General Hospital of Western Theater Command from June 2020 to June 2021. The inclusion criteria are as follows: (1) postmenopausal women with independent signing rights, (2) participants with OP who were definitely diagnosed with clinical manifestations combined with DXA, and whose T value of BMD of spine was less than -2.5. The exclusion criteria are as follows: (1) participants who suffer from a health complication that may impact bone metabolism, (2) participants who have received

drugs or treatments that may affect BMD, and (3) participants with addiction aggression. In addition, the healthy control group was composed of age-matched postmenopausal volunteers who had normal BMD of spine and femur neck; the exclusion criteria for the control group were the same as in the case group. This study requires that all volunteers review and sign a form of informed consent carefully. At the same time, the Ethics Committee of the hospital (General Hospital of Western Theater Command) approved the clinical study.

Sample Collection and Processing

Each serum sample was collected by volunteers on an empty stomach in the morning. First, the collected fresh whole blood was loaded into an untreated sterile non-anticoagulant tube at room temperature. After collection, it was allowed to stand for 30 min and centrifuged at 16,000 × g for 15 min at room temperature (20). Finally, the upper serum was collected in an Eppendorf tube, 300 µl of each serum was collected, in liquid nitrogen frozen for 30 s, and the stored temperature was -80°C for the next analysis.

We thawed the samples at room temperature. First, a 150-µl sample was added to a 1.5-ml Eppendorf tube, and 10 µl 3, 4-dichlorophenylalanine (0.3 mg/ml) with methanol dissolved in the tube was used as the internal standard, then the tube was vortexed for 10 s. Next, 450-µL mixtures of methanol and acetonitrile (2/1, vol/vol) were added and vortexed for 30 s, and the whole sample was extracted by ultrasonication in an ice water bath for 10 min and stored at -20°C for 30 min. The extract was centrifuged for 10 min (4°C 13,000 RPM). In a freeze concentration centrifugal dryer, the 150-µl supernatant was dried in a glass bottle. The glass-derived vial was filled with 80 µl of 15 mg/ml methoxyamine hydrochloride in pyridine. We first vigorously rotated the mixture for 2 min and then cultured it at 37°C for 90 min. 50 µl of BSTFA (with 1% TMCS) and 20 µl n-hexane were added, and the mixture was vigorously rotated for 2 min and derivatized for 60 min at 70°C. Finally, after the samples were left for 30 min at ambient temperature, they were analyzed by GC-MS.

A 150-µl sample was added into another 1.5-ml Eppendorf tube with an internal standard of 10 µl of L-2-chlorophenylalanine (0.3 mg/ml) dissolved in methanol (0.3 mg/ml); the test tube was rotated for 10 s. Next, a 450-µl mixture of acetonitrile and methanol (1/2, vol/vol) was added in ice-cold state and rotated for 1 min, and the whole sample was extracted for 10 min in an ice water bath using ultrasound and then left at -20°C for 30 min. The extract was centrifuged for 10 min (4°C 13,000 RPM). 0.22-µm microfilters were used to filter the 150-µl supernatant in the tube collected using a crystal syringe and then transferred to LC vials. The vials were left at -80°C and then were analyzed by LC-MS.

Metabolite Measurement

The derivative was separated using an AHP-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 µm, Agilent J&W Scientific, Folsom, CA, USA); the derived samples were analyzed by GC-MS on an Agilent 7890B gas chromatography system and Agilent 5977B MSD system (Agilent Technologies Inc., CA, USA). A Vion IMS QTof Mass Spectrometer (Waters Corporation, Milford, USA) and ACQUITY UPLC I-Class system (Waters Corporation, Milford, USA) are used for LC-MS analysis of samples at the same time, and the metabolic spectra in ESI-positive ion and ESI-negative ion modes were obtained. For evaluating the data repeatability, QCs were injected every 10 samples throughout the analysis. Quality control samples (QC) are prepared by mixing the extracts of all samples in equal volume. The original data were processed by the Progenesis QI v2.3 (Nonlinear Dynamics, Newcastle, UK), and peak detection, peak identification, MS2Dec deconvolution, characterization, peak alignment, wave filtering, and missing value interpolation were performed. In each sample, all peak signal intensities were segmented and normalized according to the internal standards with a relative standard deviation (RSD) greater than 0.3 after screening. After the data were normalized, redundancy removal and peak merging were conducted to obtain the data matrix. For the extracted data, the ion peak with the missing value (0 value) >50% was deleted in the group, and the 0 value was replaced with half of the minimum value.

Multivariate Data Analysis

To understand the metabolic variety of OP in postmenopausal Chinese women and postmenopausal Chinese women with normal BMD, principal component analysis (PCA), partial least square discriminant analysis (PLS-DA), and orthogonal projection to latent structure with discriminant analysis (OPLS-DA) are used as statistical analysis tools.

We performed these multivariate data analyses using the R Programming Language. PCA, an analytical pattern recognition tool without supervision, captures most of the variation of the whole data set with transforming high-dimensional data into a group of smaller orthogonal variables or components. PCA is usually applied to multiple data sets, and the generated two- or three-dimensional plots are visually compared to evaluate the differences (21). The spatial coordinates of each sample are composed of the projection score values on the plane composed of the first principal component and the second principal component, which can intuitively reflect the similarity or difference between samples. A unit variance scaling method was used for PLS-DA and OPLS-DA. PLS-DA is a method with supervision; by modeling the relationship of prediction space and response space, the potential corresponding variables to the principal components of principal component analysis are determined, and the covariance (PLS-DA score) between the two matrices is explained as much as possible, which can be used to predict the response of the population (22). Using MS data to perform OPLS-DA can more effectively facilitate the loading interpretation. By inversing the calculation of the coefficient, we obtained the model coefficient containing variable weights and drew it using color-coding coefficients to increase the interpretability of the model (23). To assess the PLS-DA and OPLS-DA, two parameters, R2Y and Q2, are used. R2Y shows the possibility of a difference between the square sums of all Xs and Ys. Q2 can show the percentage of cumulative cross validation in total predictable changes in current potential

variables. The higher R2Y coefficient values and Q2 coefficient values (>0.5) show better ability of discrimination and prediction (24). At the same time, the PLS-DA model is cross validated by a 200-times permutation test; the permutation test is evaluated by cross validation, and the correlation coefficients R2 and Q2 of cross validation are used to verify whether there is overfitting. If the Q2 regression line intercept on the Y-axis is less than 0, the model can be reliable and effective, which is not overfitting (25).

Find Key Biomarkers and Analysis Metabolic Pathway

After multidimensional statistical analysis, we screened out the metabolites with an absolute value of $p < 0.05$ and variable importance for the projection (VIP) >1.0, which is considered to have great potential as a potential biomarker of OP in postmenopausal women (26). Then, the Kyoto Encyclopedia of Genes and Genomes (KEGG) was searched to find metabolic pathways related to these key metabolites, the relevant literature was reviewed to verify their pathological relationship with OP, and the screened metabolic pathway was finally drawn. At the same time, we will also analyze the correlation between the metabolites we screened and the other two bone turnover markers (TRACP5b and BAP).

Statistical Methods

The means ± SDs were expressed. The Kolmogorov–Smirnov test was used to inspect the normality and homogeneity of variance of all the data. A comparative study of the results from 2 groups was conducted by Student's 2-sided t-test, and a 1-way analysis of variance was performed to explain differences in more than 2 groups. The correlation between two continuous variables was assessed using Pearson correlation analysis. The significance standard is $p < 0.05$. The Statistical Package for the Social Sciences, version 25.0 (SPSS, Chicago, IL), is used to statistical analysis.

RESULTS

Participants

During this study, 50 postmenopausal osteoporosis women and 50 healthy postmenopausal women whose BMD was normal were eventually incorporated in our study. As shown in the **Table 1**, the healthy control group basically matched to those in the case group from the age, menopausal age, body mass index, and T value of the spine BMD. Through statistical data analysis, the values of these conform to the normal distribution.

Untargeted GC/LC-MS Analysis of Samples

We performed a comprehensive metabolomic analysis of the serum of two groups of postmenopausal women. The identification of compounds is based on the accurate mass number, secondary fragments, and isotopic distribution, and the Human Metabolome Database (HMDB), LIPID MAPS (v2.3), and A Metabolite Mass Spectral Database (METLIN) are used for qualitative analysis. 48 compounds by GC-MS and 306 compounds by LC-MS were identified respectively in serum, including fatty acids, amino acids, and some carbohydrates. After multivariate analysis, according to the value of VIP, fold change (FC), and P of metabolites, 18 metabolites are considered as potential biomarkers of postmenopausal women with OP (**Table 2**). **Table 2** shows the specific metabolites designated by GC/LC-MS.

Multivariate Data Analysis Base on MS Data

Through the PCA score plots (**Figures 1A, B**), it can be seen that there is a significant difference in serum samples between the postmenopausal women with the OP group and the healthy control group, which indicated that the OP group and the control group have a significant and complete difference.

Two clusters corresponding between the case group and control group can be highlighted by the two detection methods from the PLS-DA plots. R2Y and Q2 are 0.940 and 0.813 (GC-MS), 0.966, and 0.994 (LC-MS), respectively, in PLS-DA (**Figures 1C, D**). These results show that the model has good recognition and prediction ability. R2Y and Q2 were 0.940 and 0.824 (GC-MS) and 0.996 and 0.995 (LC-MS) in OPLS-DA, respectively (**Figures 1E, F**), which also reveals that the model with good discrimination is predictive to be accurate and accurately defined.

The volcanic map shows the p value and fold change value, thus proving the effectiveness of differential metabolites. Hierarchical clustering is carried out through the expression of all metabolites with significant differences, which can reflect the relationship among samples and the metabolite expression differences among different samples more directly. **Figure 2** indicates that the differences of the metabolite we chose are significant.

TABLE 1 | Participant characteristics at the time of sampling.

Characteristics	Case group	HCG	p[a]
Number of participants	50	50	—
Age (y, mean ± SD)	69.3 ± 9.3	66.3 ± 10.0	0.130
Menopausal age (y, mean ± SD)	49.5 ± 5.4	48.9 ± 5.6	0.562
BMI (kg/m^2, mean ± SD)	23.8 ± 3.2	23.5 ± 4.4	0.672
BMD of spine (T, mean ± SD)	-3.2 ± 0.3	0.05 ± 0.6	—

[a]*Calculated by Student's t-tests for continuous variables and chi^2 tests for categorical variables between case group and healthy control group.*
BMI, body mass index; SD, standard deviation; HCG, healthy control group; BMD, bone mineral density.

TABLE 2 | Summary of potential biomarkers of the case group by serum GC/LC-MS analysis.

Metabolite	Status[a]	VIP value[b]	FC[c]	p[c]	Data origin	Pearson correlations	
						TRACP5b	BAP
Isothreonic acid	↑	1.91	2.1	<0.001	GC-MS	0.18	0.10
Ornithine	↑	1.07	1.5	<0.001	GC-MS		
Lactobionic acid	↑	2.36	2.8	<0.001	GC-MS		
Tartaric acid	↓	1.29	0.6	<0.001	GC-MS		
Glyceric acid	↓	1.29	0.7	<0.001	GC-MS		
Stearic acid	↓	1.10	0.8	<0.001	GC-MS		
PC	↑	1.60	3.6	<0.001	LC-MS	0.12	-0.006
Linoleic acid	↓	6.50	0.005	<0.001	LC-MS		
LysoPC	↑	4.05	26.2	<0.001	LC-MS		
PE	↑	3.74	19.7	<0.001	LC-MS		
DG	↓	3.62	0.004	<0.001	LC-MS		
PS	↓	2.02	0.07	<0.001	LC-MS		
SM	↓	2.00	0.06	<0.001	LC-MS		
Docosahexaenoic acid	↓	1.96	0.04	<0.001	LC-MS		
D-Glucose	↓	1.32	0.2	<0.001	LC-MS		
Lipoxin C4	↑	1.29	11.1	<0.001	LC-MS		
Heneicosanedioic acid	↓	1.16	0.005	<0.001	LC-MS		
PA	↓	1.15	0.03	<0.001	LC-MS		

[a]*Relative concentrations compared to healthy controls: ↑ = upregulated, ↓ = downregulated.*

[b]*Correlation coefficient and VIP value were obtained from OPLS-DA analysis.*

[c]*Fold change between PWOP patients and healthy controls.*

[c]*p value determined from Student's t-test.*

PC, phosphatidylcholine; LysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; DG, diacylglycerol; PS, phosphatidylserine; SM, sphingomyelin; PA, phosphatidic acid; FC, fold change; HC, healthy control; VIP, variable importance for projection.

Potential Biomarkers and Pathway Analysis

Significant differences between groups can also be shown by potential biomarker box-and-whisker plots (**Figure 3**). By database searching (KEGG) and consulting relevant literature, we found that these metabolites are mostly related to glucose, amino acids, and choline metabolism and also have some relationship with inflammatory response. These metabolic pathways often have a close relation to the changes in the marrow microenvironment in the bone marrow, which can eventually lead to the changes in osteoclast differentiation and oxidative stress. As shown in **Figure 4**, we can more intuitively reflect the relationship between these metabolites by drawing the metabolic pathway map of these metabolic markers with significant differences.

DISCUSSION

Metabonomics is widely regarded as the most phenotypic omics by identifying and quantifying small molecular metabolites (27). Because of its inherent sensitivity, metabonomics is the most powerful method to study local and specific stimulus responses and pathogenesis. It can detect subtle changes in biological pathways to obtain clear biochemical information about disease mechanisms, to help us understand the process of various physiological conditions and abnormal processes (28). OP is a metabolic disease that eventually causes the continuous decrease in bone mass and the deterioration of the bone microstructure. Metabonomics analysis can further examine the pathological process of OP and identify the reaction to drugs in each stage of OP treatment (29). Untargeted GC-MS combined with LC-MS was used for the first time to describe the metabolism of 50 Chinese postmenopausal women with OP and 50 Chinese postmenopausal women with normal BMD for our study. Through multivariate analysis, we found that postmenopausal women with OP/normal bone mass had a large number of metabolites with significant differences. This shows that the PCA and PLS-DA/OPLS-DA models established by using normal serum metabolites of Chinese postmenopausal women with OP and Chinese postmenopausal women with normal bone mass have high sensitivity and specificity.

In previous metabonomics studies, some potential biomarkers of osteoporosis have been found. These metabolites are mainly concentrated in fats [e.g., phosphatidylinositol, phosphatidic acid, sphingolipid (15), linoleic acid, oleic acid, arachidonic acid, and 11, 14-eicosadienoic acid (17)] and amino acids [e.g., glutamine (14), 4-aminobutyric acid, proline, aminopropionitrile, threonine, methionine (15), leucine, isoleucine, and taurine (17)]. Our study also found some new potential biomarkers, which greatly enriched the database of potential markers of osteoporosis and provided more directions for the diagnosis of osteoporosis. At the same time, we also found that some potential biomarkers we detected this time were also found in previous experiments, which further shows that metabonomics has a certain repeatability in the study of osteoporosis. It also shows that these repeatedly verified potential biomarkers have greater potential to become markers for the diagnosis of osteoporosis.

Adult bone is a multifunctional organ that is constantly reconstructed. In adults who have normal bone mass, the resorption of osteoclast bone and the formation of osteoblast

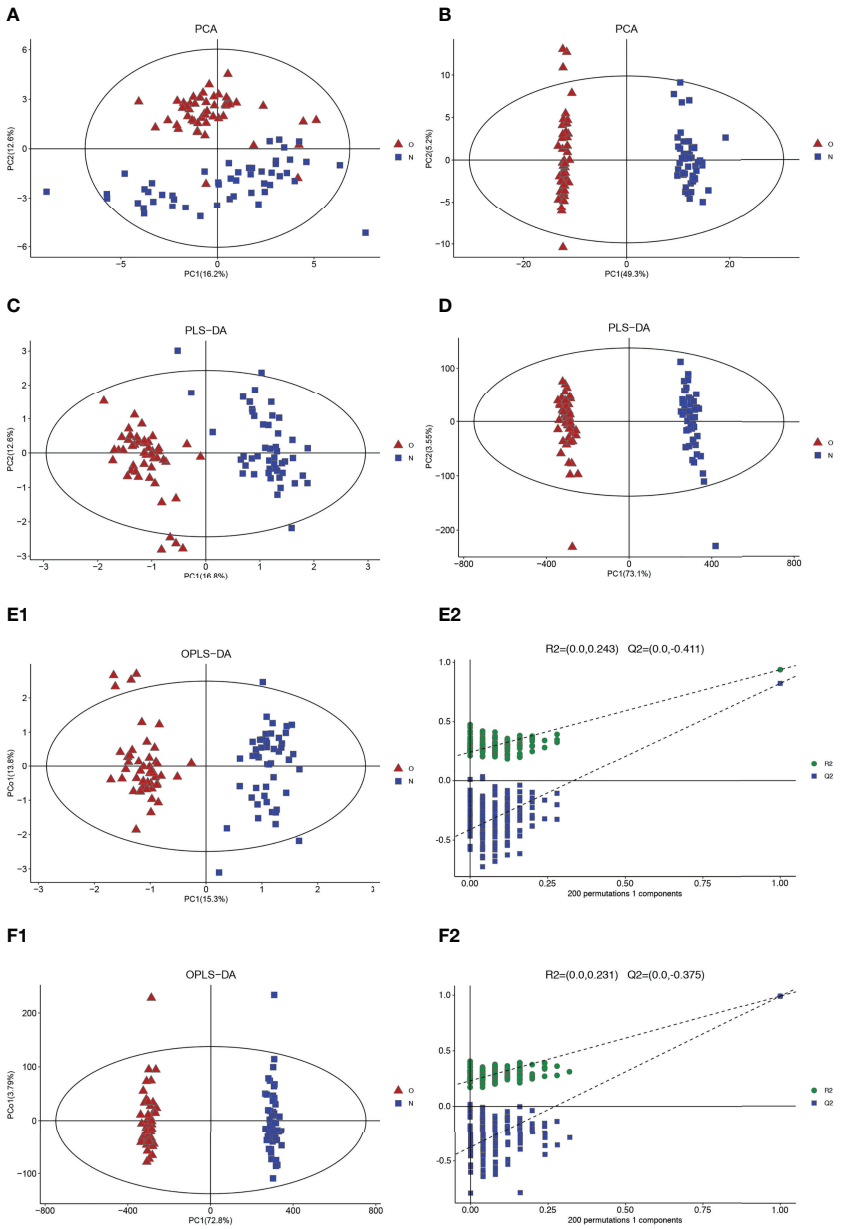

FIGURE 1 | Multivariate date analysis of date from serum between the case group (O red triangle) and healthy control group (N blue squares) base on GC/LC-MS. **(A)** PCA score plots based on the GC-MS. **(B)** PCA score plots based on the LC-MS. **(C)** PLS-DA score plots. **(D)** PLS-DA score plots. **(E1, 2)** OPLS-DA score plots (left panel) and statistical validation of the corresponding OPLS-DA model by permutation analysis (right panel) based on the GC-MS. **(F1, 2)** OPLS-DA score plots (left panel) and statistical validation of the corresponding OPLS-DA model by permutation analysis (right panel) based on the LC-MS. The two coordinate points are relatively far away on the score map, indicating that there is a significant difference between the two samples, and vice versa. The elliptical region represents a 95% confidence interval.

bone bones have a delicate balance. When this balance is broken, it leads to OP and other bone diseases (30). Before menopause, estrogen can reduce oxidative stress in bone and bone marrow, so as to maintain the balance of the bone microenvironment and keep bone strength in the normal range (31). However, this balance is slowly broken after menopause. In addition, the expression of the receptor activator of nuclear factor-b ligand (RANKL) will be overexpressed with estrogen deficiency, which is also a reason for the increase in bone resorption to achieve OP (32). Bone resorption of osteoclasts consumes a large amount of energy, which makes glycolysis and oxidative phosphorylation to speed up. Patients with OP usually have fatty acid disorder and abnormal amino acid metabolism, which have promoted the occurrence and development of OP (33).

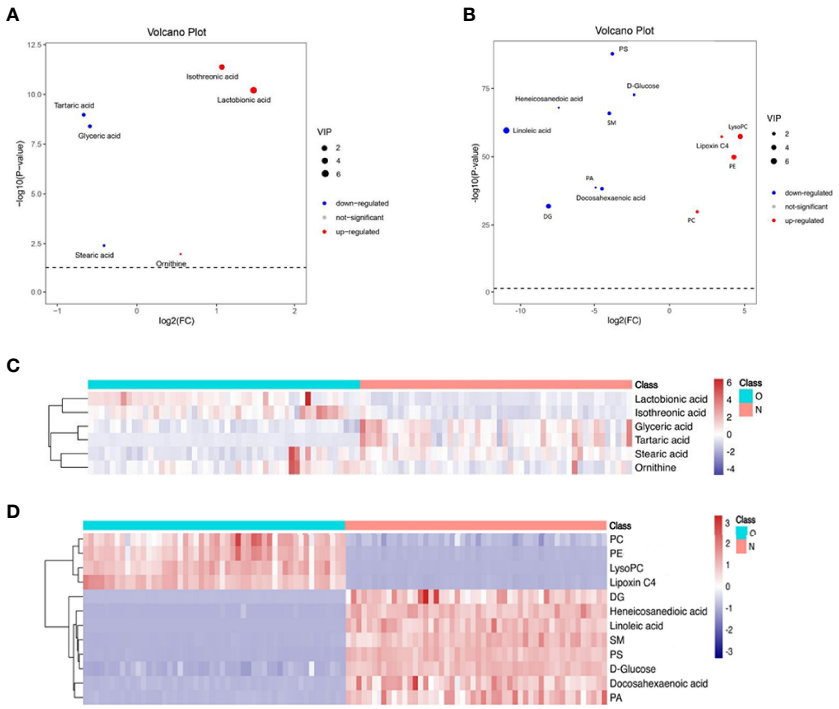

FIGURE 2 | Volcano plot and hierarchical clustering based on the GC/LC-MS of serum metabolites obtained from the case group (O blue) and healthy control group (N red). **(A)** Volcano plot based on GC-MS. **(B)** Volcano plot based on LC-MS. **(C)** Hierarchical clustering based on GC-MS. **(D)** Hierarchical Clustering based on LC-MS. In **(A, B)**, the blue dot represents metabolite with a downward trend, red represents metabolites with an upward trend, and the gray origin represents that the change of metabolites is not obvious. The area size of the point is related to the VIP value. In **(C, D)**, the color from blue to red illustrates that metabolites' expression abundance is low to high in hierarchical clustering. PC, phosphatidylcholine; LysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; DG diacylglycerol; PS, phosphatidylserine; SM, sphingomyelin; PA, phosphatidic acid.

With the enhancement of energy metabolism, the citric acid cycle is also enhanced. As an important intermediate product of the citric acid cycle, the concentration of isotricarboxylic acid in serum is also increased (34), a large amount of glucose is used, and the glucose concentration becomes lower. The above mechanism may explain that the serum glucose concentration is low and the isothreonic acid concentration is high. Lactonic acid has strong antioxidant capacity, can chelate Fe^{3+}, and can reduce the tissue damage caused by hydroxyl free radicals produced by ion catalysis (35). Therefore, the increase in lactonic acid may be related to the reduction in tissue damage caused by hydrogen and oxygen free radicals produced by the enhancement of energy metabolism.

Fat and bone have a very complex relationship with each other, and this correlation is widely reflected in both systematic and local aspects. Local effect is mainly reflected in the change of the bone marrow microenvironment and the expression of fat with other bone cells (36). *In vitro*, under the pro-inflammatory stimulation of TNF-α and IFN-γ, bone marrow mesenchymal stem cells (MSCs) were activated and the metabolism of PE, PS, and lysoPC was affected (37). The content of PE increased during osteoclast differentiation (38), and LysoPC can be transformed into phosphatidylcholine (PC). LysoPC can promote osteoclast differentiation and increase intracellular free calcium concentration (39). Our results also support the positive correlation between PE, lysoPC, and PC and decreased BMD.

Some *in vitro* experiments show that high levels of PS can stimulate osteoblasts and promote the deposition of mineral substances in bone tissue (40). Diacylglycerol (DG), released from membrane lipids, is a cellular mediator that was critical for the regulation of inflammation and disease (41), which can promote protein kinase C (PKC) expression. PKC activates calcium absorption and increases the cAMP concentration in osteoblasts (42). Phosphatidic acid (PA) is the main metabolite in the synthesis of DG, so the decrease of DG, PA, and PS may be related to the inhibition of osteoblast activation in patients with OP, which leads to the further development of OP. In Diana Cabrera's report (15), there are also results consistent with ours.

In other studies, stearic acid (43) and tartaric acid (44) have obvious inhibitory effects on osteoclasts. The final metabolite of tartaric acid is glyceric acid. At the same time, glyceric acid can further isomerize into sugar or further participate in glycolysis to meet the energy metabolism of osteoclasts (45). The decrease in stearic acid, tartaric acid, and glyceric acid may reduce the inhibitory effect of fat on osteoclasts, which will be accompanied by the decrease in human bone mass and eventually develop into OP. The findings suggest that low levels of stearic acid, tartaric acid, and glyceric acid in serum may predict low BMD.

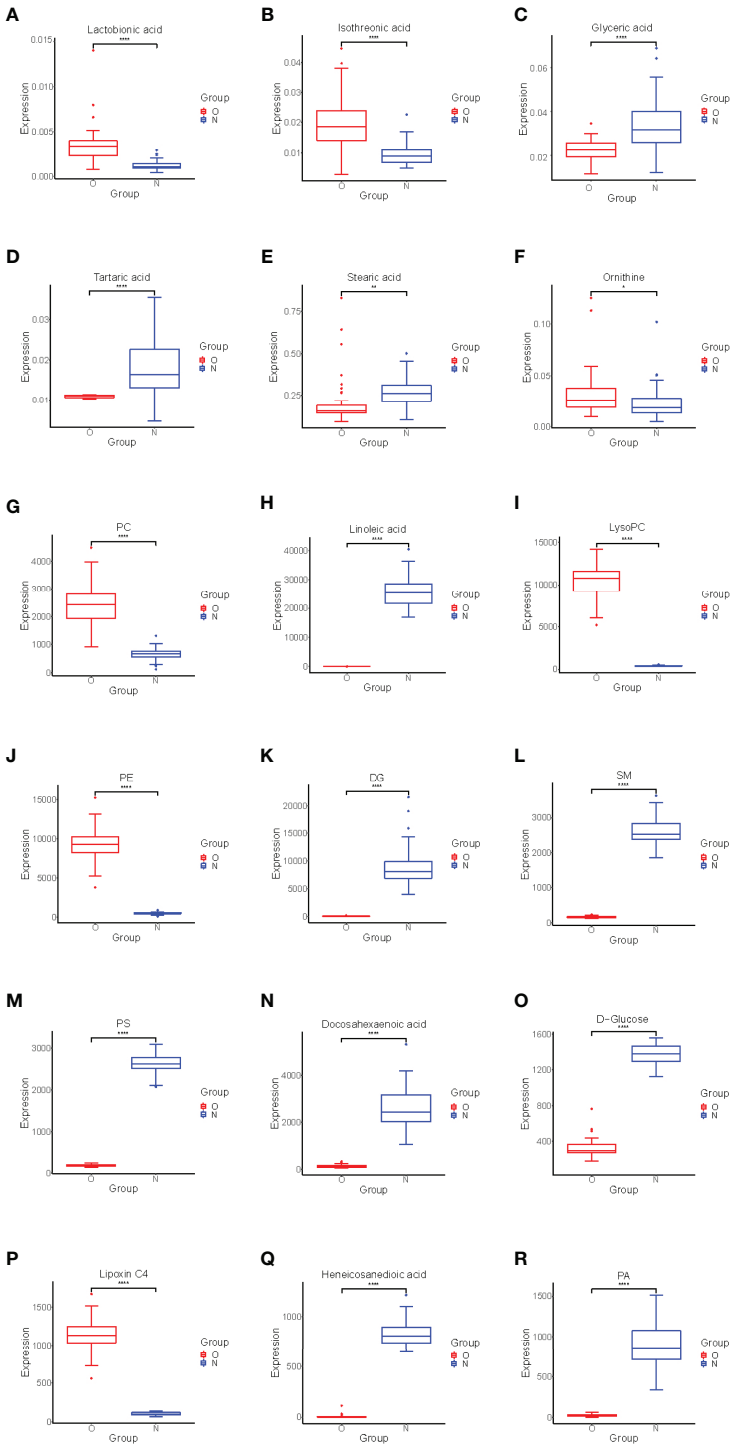

FIGURE 3 | Box-and-whisker plots showing the relative levels of selected potential biomarkers for the postmenopausal women with OP. **(A–F)** were found by GC-MS, **(G–R)** were found by LC-MS. The red box on the left represents the case group, and the blue box on the right represents the healthy control group. Horizontal line in the middle portion of the box, median; bottom and top boundaries of boxes, lower and upper quartiles; whiskers, 5th and 95th percentiles. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. PC, Phosphatidylcholine; LysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; DG, Diacylglycerol; PS, phosphatidylserine; SM, sphingomyelin; PA, phosphatidic acid.

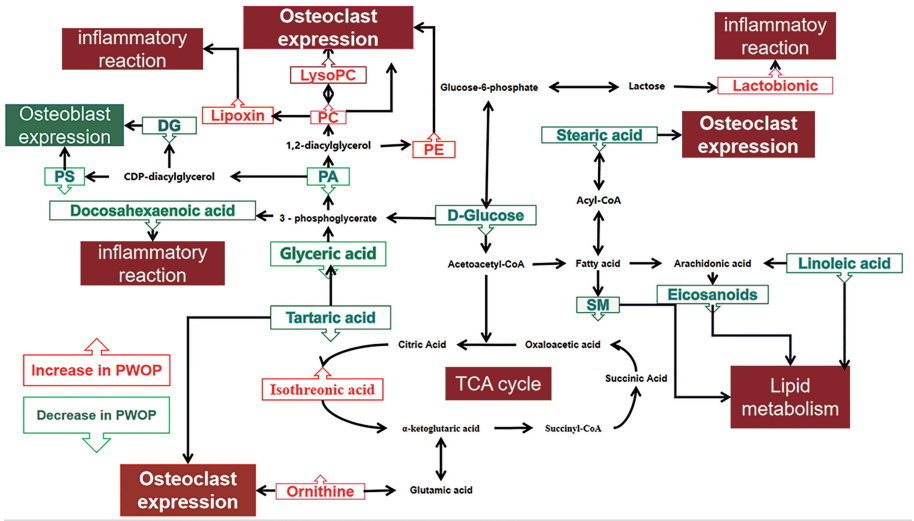

FIGURE 4 | Altered metabolic pathways for the most relevant distinguishing metabolites (potential biomarkers) between the case group and healthy control group. The metabolites with red border were upregulated in the case group, whereas those with green border indicate metabolites that were downregulated. PC, phosphatidylcholine; LysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; DG, diacylglycerol; PS, phosphatidylserine; SM, sphingomyelin; PA, phosphatidic acid.

In the mouse model of OP, lipoxygenase (LOX) gene expression leads to an increase in the concentration of lipoxin, which can produce endogenous anti-inflammatory effects, which is related to the decrease in bone strength in the mouse model of OP. Some eicosanoids are related to allergic reaction and inflammation and play a pro-inflammatory role, which is opposite to lipoxin (46). This helps to explain the results that we detected an increase in lipoxin C4 and a decrease in docosahexaenoic acid in the patient group. In previous studies, it was found that long-term OP would reduce the levels of arachidonic acid, docosahexaenoic acid, and sphingomyelin (SM) (47). Arachidonic acid is formed by linoleic acid metabolism *in vivo*, which is consistent with the change trend. Therefore, in our study, we found that the levels of docosahexaenoic acid, sphingomyelin, and linoleic acid in the experimental group were at a relatively low level. In the study of the macrophage signaling pathway, it was found that the animal model had OP, which showed that the expression of hyperactive osteoclasts increased. These macrophages produced *in vivo* have high arginase levels and produce ornithine (48). This is also similar to our test results, which confirmed that the ornithine concentration of OP patients is higher.

Finally, through the correlation analysis of 18 metabolites screened and 2 bone turnover markers (TRACP5b, BAP) in this study, it is found that only 3 metabolites are significantly correlated with them, while other metabolites have only a non-significant correlation. Firstly, bone turnover markers are not the gold standard for diagnosis of osteoporosis. Bone turnover markers are more effective in determining the response to osteoporosis treatment and as a reference in the diagnosis of secondary osteoporosis, but the prediction effect on primary osteoporosis is not very good. Secondly, there are day-to-day changes in the concentration of bone turnover markers (49), which may be the reason why we get such results.

Overall, the metabolomic profiles we obtained were promising. These potential biomarkers have great biological significance for the diagnosis and recurrence monitoring of postmenopausal OP women. However, we admit that our study is not enough. First, the number of samples is not rich enough, and the samples are mostly from Sichuan Province and surrounding areas; the result should be verified in more postmenopausal OP women in the future. Secondly, this study only uses serum as the sample for exploration, and the results are relatively incomplete. Therefore, more kinds of samples can be selected for OP metabolome research in the future, such as bone marrow and urine, so as to establish a more complete metabolic database. In addition, there is a lack of absolute qualitative and quantitative data of substances that untargeted metabonomics may produce a lot of false positive signals. The potential biomarkers could be studied by targeted metabonomics in the next step. Finally, the results of this study are only for postmenopausal women and can be further explored in OP male patients.

CONCLUSIONS

The metabolism analysis of postmenopausal women with OP is the first time to study by untargeted GC/LC-MS on serum to obtain more comprehensive metabolomic characteristics and screen out a large number of potential biomarkers with significant differences. Through multivariate data analysis and metabolic pathway analysis, most of these metabolic markers are related to the disorder of glucose metabolism, amino acid metabolism, and lipid metabolism and influence the bone

microenvironment and the homeostasis changes of the whole body in OP women. 18 metabolites with significant differences were screened, which is important in these metabolic pathways, which are judged to have great potential as potential biomarkers of OP in postmenopausal women. In a further study, we need to conduct more validation experiments to prove that these biomarkers we found can be widely used in clinical work.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the General Hospital of Western Theater Command. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: JK, CH, LC, ZZ, WW, LT, and DL. Carried out the experiments: JK, WZ, WG, and NX. Analyzed and interpreted data: JK, CH, LC, ZZ, LT, WZ, WG, and NX. Writing—original draft: JK, CH, LC, and ZZ. Review and editing: WW and DL. Funding acquisition: WW. All authors contributed to the article and approved the submitted version.

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The Global Burden of Osteoporosis, Low Bone Mass, and Its Related Fracture in 204 Countries and Territories, 1990-2019

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Background: Low bone mineral density (LBMD), including osteoporosis and low bone mass, has becoming a serious public health concern. We aimed to estimate the disease burden of LBMD and its related fractures in 204 countries and territories over the past 30 years.

Methods: We collected detailed information and performed a secondary analysis for LBMD and its related fractures from the Global Burden of Disease Study 2019. Numbers and age-standardized rates related to LBMD of disability-adjusted life-years (DALYs) and deaths in 204 countries and territories were compared by age, gender, socio-demographic index (SDI), and location.

Results: Global deaths and DALYs number attributable to LBMD increased from 207 367 and 8 588 936 in 1990 to 437 884 and 16 647 466 in 2019, with a raise of 111.16% and 93.82%, respectively. DALYs and deaths number of LBMD-related fractures increased 121.07% and 148.65% from 4 436 789 and 121248 in 1990 to 9 808 464 and 301 482 in 2019. In 2019, the five countries with the highest disease burden of DALYs number in LBMD-related fractures were India (2 510 288), China (1 839 375), United States of America (819 445), Japan (323 094), and Germany (297 944), accounting for 25.59%, 18.75%, 8.35%, 3.29%, and 3.04%. There was a quadratic correlation between socio-demographic index (SDI) and burden of LBMD-related fractures: DALYs rate was $179.985-420.435SDI+417.936SDI^2$($R^2$ = 0.188, p<0.001); Deaths rate was $7.879-13.416SDI+8.839\ SDI^2$($R^2$ = 0.101, p<0.001).

Conclusions: The global burden of DALYs and deaths associated with LBMD and its related fractures has increased significantly since 1990. There were differences in disease burden between regions and countries. These estimations could be useful in priority setting, policy-making, and resource allocation in osteoporosis prevention and treatment.

Keywords: low bone mineral density, osteoporosis, fracture, death, disability-adjusted life years (DALYs), global burden

INTRODUCTION

Low bone mineral density (LBMD), including osteoporosis and low bone mass, is a chronic bone metabolic disease characterized by impaired bone mass and microstructure, leading to increased risk of fractures in various parts of the body. This public health problem has brought a heavy burden to the global economic, social and health development (1, 2). Osteoporosis currently affects more than 10 million people in the United States and is expected to affect approximately 14 million adults over the age of 50 by 2020. Worldwide, about 200 million women suffer from osteoporosis (1, 3).

It is important to note that the most serious complication of osteoporosis is fracture. It was projected that by 2050, the worldwide incidence of hip fracture in men would increase by 310% and 240% in women (4). Results from large prospective studies show that almost all types of fractures increase in patients with LBMD, and that adults who already have one type of fracture are 50% to 100% more likely to have a different type of fracture, regardless of the type (5, 6). The concealment and particularity of osteoporosis are that the osteoporotic population usually lacks clinical symptoms prior to the fracture event, thus fragility fracture becomes the dominant clinical presentation. About one in three women and one in five men, typically aged 50 and older, experience a fragility fracture in the rest of their lives (7, 8). In Europe, fragility fractures are the fourth leading cause of chronic diseases, behind ischemic heart disease, dementia and lung cancer (9). Moreover, older people with osteoporosis are at increased risk for persistent fragility fractures, and factors such as falls can accelerate it (10). The aging of the world's population and changing lifestyles will lead to rising rates of chronic diseases, such as osteoporosis (11, 12). There was a continuous relationship between decreased bone mineral density and increased fracture risk, with a significant increase in fracture risk for each 1SD decrease in bone mineral density (13). Osteoporotic fractures will not only cause pain to individuals, such as deformity and pain, resulting in physical damage and serious psychological disorders such as depression, anxiety and fear, but also cause huge economic pressure to the society (14–17).

To our knowledge, there is few of global data on the disease burden associated with LBMD and fragility fractures. In the Global Burden of Disease Study 2019 (GBD 2019), LBMD is a risk factor to assess its impact on human health and longevity. In GBD 2019, death and health loss from osteoporotic fractures cannot be directly identified because as cause of death data from vital registration and verbal autopsy attribute injury deaths to causes of death (e.g., falls or road injury) and not nature of injury (such as fractures) (18, 19). However, GBD 2019 restricted assessment of the health burden of LBMD to a list of causes that were deemed to cause fractures: falls, pedestrian road injuries, motor vehicle road injuries, motorcyclist road injuries, other exposure to mechanical forces, other transport injuries, cyclist road injuries, physical violence by other means, non-venomous animal contact and other road injuries (20, 21). Most above events can directly result in fractures because of injuries and violence, not LBMD. As this has been proven in previous articles, most osteoporotic fractures limited to falls are expected to be coded (20). Therefore, in this study only disease burden of LBMD-related falls was considered as osteoporotic fractures. We used GBD 2019 to capture data of LBMD and LBMD-related falls on deaths and DALYs as absolute numbers and age-standardized rates for all age groups and 204 countries and territories annually from 1990 to 2019 to investigate the trend of the burden of LBMD and osteoporotic fractures, and to provide evidence for the adjustment of health resources and policies (22).

METHODS

Overview

The Global Health Data Exchange is the world's most comprehensive survey to date, covering census, household surveys, civil registration and vital statistics, disease registration, health service use, air pollution monitoring, satellite imaging, disease notifications and other health-related data. GBD 2019 quantifies health loss, including 369 diseases and injuries, and for 87 risk factors in 204 countries and territories around the world, which the data were assessed using spatiotemporal Gaussian process regression, DisMOd-MR 2.1, a Bayesian meta-regression method, or alternative methods for age-sex-location-year exposure (18, 19). These methods have been introduced before and the data and results are available from GBD Results Tool GHDx (healthdata.org) (December 16, 2021).

Case Definition and Data Sources

GBD 2019 has a risk hierarchy, using CRA to assess the disease burden of risk factors, in which LBMD is defined as a level 3 risk factor, whose exposure is defined as standardized mean bone mineral density values measured by dual X-ray absorptiometry at the femoral neck in g/cm^2. The theoretical minimum risk exposure level is determined based on 99th percentile of NHANES 1988-2014 by age and sex (23).

In healthy adults' population, bone mineral density (BMD) appears to be approximately gaussian normal distribution, therefore an individual's BMD can be valued in standard deviation (SD) units in relation to the reference population. For women, according to WHO and the International Osteoporosis Foundation, low bone mass (osteopenia) is defined as the value for BMD more than 1.0 but less than 2.5 SD below the young adult female reference mean (T-score less than -1 and greater than -2.5 SD) and osteoporosis is the value for BMD 2.5 or more SD below the young adult female reference mean (T-score less than or equal to -2.5 SD). Osteoporosis is also diagnosed based on presence of fragility fractures in the absence of other metabolic bone disorders and even with a normal bone mineral density (T-score) (24–26). International Classification of Diseases (ICD) code list that maps to the global burden of disease cause of death, falls is defined as ICD10 is W00-W19.9 and ICD9 is E880-E886, and E888, as the third level coding strategy in GBD, is one of the unintentional injuries.

The data we are interested in this study consists of: a) global data of LBMD and LBMD-related falls on deaths and DALYs as absolute numbers and age-standardized rates (per 100 000 population) for all age groups; males, females, and both sexes combined; and 204 countries and territories annually from 1990 to 2019. b) Global prevalence, deaths and DALYs of falls as absolute numbers and age-standardized rates (per 100 000 population) by gender and age groups from 1990 to 2019.

Ethical approval and informed consent were not required for this study, as GBD 2019 used DE-idented vetted and approved by the Institutional Review Board of the University of Washington, aggregated data, exempted informed consent, and did not risk disclose personal identity. Used to estimate LBMD and falls, more detailed information of the original data source, see GBD2019 data input source tools website (http://ghdx.healthdata.org/gbd2019/data-input-sources).

Disease Burden of LBMD Risk and Fracture

We used DALYs and mortality to estimate the global burden of LBMD. DALYs is a pooled indicator of population health, which measures the health status of a population. The goal is to give individuals a standard life expectancy in full health, which has two aspects: years of life lost (YLLs) and years lived with disability (YLDs). YLLs was used to show the burden of premature death from LBMD and YLDs was used to reflect disability-weighted years of life with long-term or short-term health loss. YLDs was calculated by the prevalence of different disease sequelae and injury sequelae multiplied by disability.

Attributing the health burden to osteoporotic fractures was searched by setting LBMD as a risk factor and falls as a cause of injury in GBD 2019. This database used available hospital data to estimate the proportion of injury deaths during admission that could be ascribed to fractures (18). Previous studies that looked at data from hospitals in Brazil, Canada, Mexico and the United States found that falls accounted for a large proportion of deaths, especially among the elderly, and hip fractures were the main cause of death. GBD 2019 restricted assessment of the health burden of LBMD to a list of causes that were deemed to cause fractures: falls, pedestrian road injuries, and other violence injuries. Most high-energy injuries can directly result in fractures because of injuries and violence, not LBMD. Therefore, based on the above research and the research purpose of this paper, most osteoporotic fractures limited to falls are expected to be coded (20). LBMD-related falls can be considered as LBMD-related osteoporotic fractures.

Socio-Demographic Index

The socio-demographic index (SDI) is a composite indicator of the social background and economic conditions that affect health in each country and region. It is a geometric mean of 0 to 1 and includes per capita income, the average educational level of the population aged 15 or above, and the fertility rate of women under 25. The GBD 2019 and World Bank standards are divided into five parts, high SDI (> 0.81), high-middle SDI (0.70-0.81), middle SDI (0.61-0.69), low-middle SDI (0.46-0.60), and low SDI (< 0.46).

Statistical Analysis

Data is represented by values with a 95% uncertainty interval (UI). Age-standardized mortality rates and years of life are expressed as figures per 100 000 population. Most statistical analyses were performed using SPSS 24.0(Statistical Product and Service Solutions) software unless otherwise specified, and Prism Version 9 (GraphPad, San Diego, California) was used for all images. Pearson correlation analysis and curve fitting method were used to analyze the relationship between SDI and disease burden. A P value less than 0.05 was considered statistically significant.

RESULTS

Global Trends of DALYs and Mortality Attributable to LBMD by Years

In generally, the global burden of disease attributable to LBMD from 1990 to 2019 increased dramatically, with elevating in the total number of deaths and DALYs (**Figure 1A**). The global DALYs number contributable to LBMD doubled from 8.6 million (95% UI: 10.14-7.04) in 1990 to 16.6 million (95% UI: 20.04-13.50) in 2019. The risk of LBMD was higher in females than in males when it comes to gender. In 2019, compared with LBMD contributed to 4297319 (95% UI: 5 182 046-3 478 535) DALYs in female whereas 4 291 617 (95% UI: 4 989 401-3 521 616) for male in 1990, LBMD doubled in female 8 656 587 (95% UI: 10 586 101-6 935 384) and 7 990 880 (95% UI: 9 429 640-6 480 003) in male (**Figure 1A**). As in 2019, the number of deaths due to LBMD risk increased to 209 586 (95% UI: 236 460-173 630) among male and 228 298 (95% UI: 266 439-177 697) among female. Contrast with 1990, there was an increase of 111.16%, in global death toll (**Figure 1B**).

After the data were standardized for age, the DALYs rate showed a slight downtrend from 226.57 (95% UI: 268.08-185.26) in 1990 to 206.85 (95% UI: 248.69-167.92) per 100 000 population in 2019, as well as, the mortality rate showed a similarly trend from 6.26 (95% UI: 6.88-5.36) in1990 to 5.74 (95% UI: 6.51-4.72) per 100,000 population in 2019 (**Figures 1A, B**).

Disease Burden of Fractures Due to LBMD by Years

LBMD was a risk factor for many injuries. According to DALYs, fractures accounted for the highest proportion (58.9%) of all injuries related to LBMD worldwide in 2019, followed by pedestrian road injuries (9.99%), motor vehicle road injuries (9.81%), motorcyclist road injuries (6.74%), other exposure to mechanical forces (5.59%), other transport injuries (3.28%), cyclist road injuries (3.26%), physical violence by other means (1.35%), non-venomous animal contact (0.53%) and other road injuries (0.52%) (**Figure 1C**).

The absolute values of deaths and DALYs were growing year by year as a result of fractures with LMBD. Among males, DALYs increased from 1 678 544 (95% UI:2 001 436-1 392 292) in 1990 to 3 704 444 (95% UI:4 440 007-3 031 796) in 2019, while females climbed by 2.21 times from 2 758 245 (95% UI:3 361 499-2 217 636) to 6104020 (95% UI:7 540 557-4 860 689). In addition, the

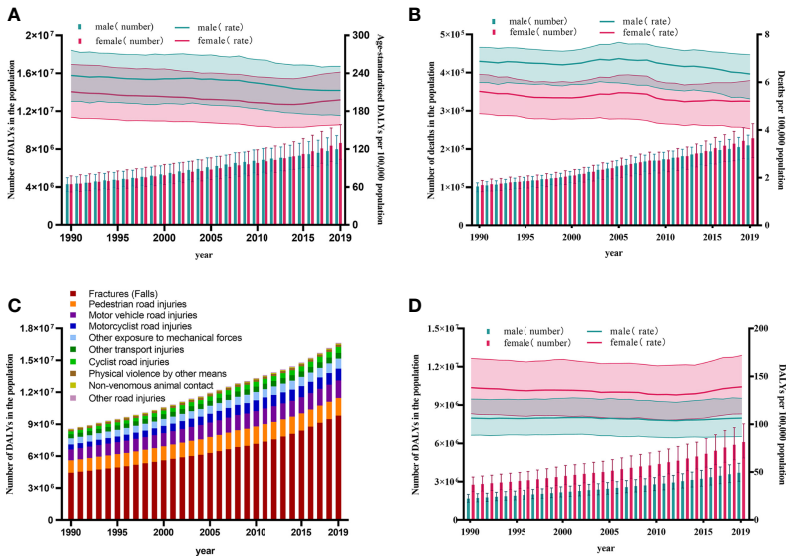

FIGURE 1 | Trend of LBMD and its related fractures at the global level from 1990 to 2019. **(A)** Trends in numbers and age-standardised rates of DALYs of LBMD at the global level,1990-2019; **(B)** Trends in numbers and age-standardised rates of deaths of LBMD at the global level, 1990-2019; **(C)** Composition of different causes at the risk of LBMD by DALYs at the global level, 1990-2019; **(D)** Trends in numbers and age-standardized rates of DALYs of fractures at LBMD risk at the global level, 1990-2019.DALYs, Disability-adjusted life years; LBMD, Low bone mineral Density; Error bars indicate the 95% uncertainty interval (UI) for numbers and rates.

death toll has risen. The number of deaths of fractures caused by LBMD reached 301,482 (95% UI:345 110 -240 323) worldwide in 2019, 2.49 times that in 1990: Males accounted for 39.8% of all deaths, despite female deaths decreasing marginally from 62.5% to 60.2%, to 181,635 (95% UI:213 852-136 974), still more than 1.5 times that of males (**Figure 1D**).

Differences of Disease Burden in LBMD and Fractures by Gender

LBMD led to a rapidly increase in DALYs and deaths in female from 4 297 319 (95% UI: 5 182 046-3 478 535) and 105 267 (95% UI: 117 964-88 278) in 1990 to 8 656 587 (95% UI: 10 586 101-6 935 384) and 228 297.905 (95% UI: 266 439-177 697) in 2019, with an increase of 101.44% and 116.87%. As for male, the DALYs and deaths improved from 4 291 617 (95% UI: 4 989 401-3 521 616) and 102 100 (95% UI: 111 585-87 870) in 1990 to 7 990 880 (95% UI: 9 429 640-6 480 003) and 209 586 (95% UI: 236 460-173 630) in 2019, with an increase of 86.20% and 105.28%. Female, on average, has a higher risk of LBMD than male, and this gender disparity was projected to widen in the future. Deaths of LBMD-related fractures has increased dramatically in both male and female over the last few decades, reaching 119 846 (95% UI:136 010-98 206) for male and 181 635 (95% UI:213 8512-136 6974) for female in 2019. Impressively, from 1990 to 2019, female deaths rates were higher than male, and the discrepancy appeared to have widened over time. Furthermore, LBMD-related fractures in DALYs and deaths was primarily found in adults ≥40 years old. Males in the 85-89 age had the greatest deaths burden in 2019, accounting for 20.58% of all male deaths (95% UI:26 948-18 268), and the 80-89 age group accounted for nearly half of all male deaths. As for female, deaths number of LBMD-related fractures was 37 790 (95% UI:45 229-26 976), 1.60 times as many as males at the age of 85-89. The gender divide among those over 40 years old widened as they became older: deaths of female was 0.31-fold those of males at the age of 40-44, a shocking 1.70-fold between 80-84 years old, and 1.874-fold between 90-94 years old. Female had a higher health burden than male, which was reflected not only in the number of fatalities, but also in DALYs. Both male and female had a unimodal trend in DALYs. Male aged 70-74 years had the highest DALYs: 50 1500 (95% UI: 612 276-404 681), with 70-89 years group accounting for 50.36% of total burden of male. Between the age of 80-84, the peak number of females was 971 411 (95% UI:1 193 500-774 109). Female growth rates were higher than male beyond the age of 40. Female aged 40-44 have 0.73 times the disease burden of males, while female aged 90-94 have 2.43 times that of male (**Figure 2**).

The Burden of Fractures Due to LBMD by Countries and Regions

The study found that the burden of LBMD-related fractures was greater in developed countries and in developing countries with larger populations. In the GBD 2019 study, data from 204 countries and territories were analyzed. The five countries with the highest DALYs number in fractures due to LBMD in 2019 were India 2 510 288 (95% UI:2 971 348 -2 072 778); China 1 839 375 (95% UI:2 316 329-1 346 044); United States of America 819 445 (95% UI:1 041 431-644 729); Japan 323 094 (95% UI:419 012-248 280); Germany 297 944 (95% UI:380 978-228 142). They, in turn, accounted for 25.59%, 18.75%, 8.35%, 3.29%, and 3.04% of the global total LBMD-related fractures burden. The countries with the highest number of deaths due to LBMD-

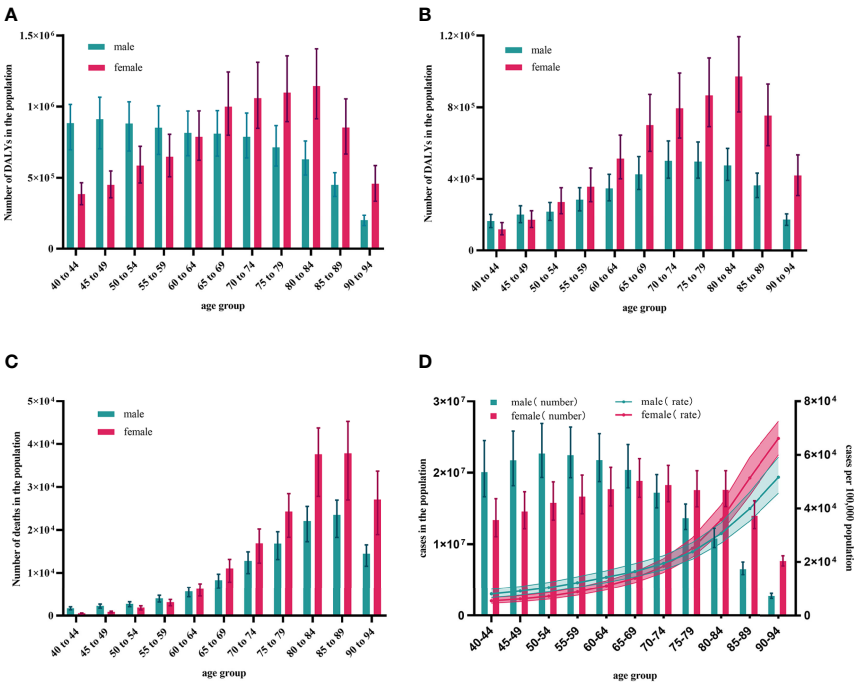

FIGURE 2 | Global burden of LBMD and its related fractures by age groups in 2019. **(A)** DALYs number of LBMD at the global level, 2019; **(B)** DALYs number of fractures at LBMD risk at the global level, 2019; **(C)** Deaths number of fractures at LBMD risk at the global level, 2019; **(D)** Age-specific numbers and rates of fractures prevalent cases by gender, 2019. DALYs, Disability-adjusted life years; LBMD, Low bone mineral density; Error bars indicate the 95% uncertainty interval (UI) for numbers.

related fractures in 2019, in order, India 93 675 (95% UI:110 965-74 053), China 56 639 (95% UI:71 875-30 514), United States of America 22 174 (95% UI:24 927-18 183), France 9321 (95% UI:11 214-6840), Germany 8542 (95% UI:9892-6815), with the proportion of 31.07%,18.79%,7.36%, 3.09%,2.83% (**Figure 3**).

The Association Between SDI and Fractures Due to LBMD

According to the SDI classification, the 204 countries and territories was divided into 33 low, 40 low-middle, 44 middle, 44 high-middle and 43 high SDI countries or territories. We found that the three countries with the highest DALYs and deaths were India, China and the United States. The number of deaths and DALYs in all three countries is increasing year by year. The combined DALYs and deaths rate of male and female were positively correlated with SDI (linear regression) in all countries and regions: DALYs rate was 179.985-420.435SDI+417.936SDI^2(R^2 = 0.188, p<0.001); Deaths rate was also positively correlated with SDI, deaths rate 7.879-13.416SDI+8.839 SDI^2(R^2 = 0.101, p<0.001). And DALYs number was -66 944.230 + 308 436.893SDI-187 612.227 SDI^2 (R^2 = 0.005, p=0.594); Deaths number was -2647.442+12 850.800SDI-9325.708SDI^2 (R^2 = 0.003, p=0.714) (**Figures 4C–F**).

For countries and regions with high SDI, the number of DALYs increased from 1 348 472 (95% UI:1 699 379-1052555) in 1990 to 2 681 727 (95% UI:3 409 105-2 086 644) in 2019, an increase of 98.87%. The number of countries and regions in the high-middle SDI increased from 1 153 070 (95% UI:1 431 916-917 557) to 2 168 703 (95% UI:2 739 295-1 706 491), an increase of 88.08%. The Middle SDI increased 167.96% from 901 213 (95% UI:105 619-747 672) to 2 414 849 (95% UI:2 929 129-1 905 732). The number of low-middle SDI countries and territories reached 1 985 931 (95% UI:2 340 410-1 649 307) in 2019, 2.64 times that of 1990. The DALYs with low SDI countries and territories also reached 552 813 (95% UI:642 702-467 246) in 2019, 2.40 times that of 1990. In female, the increase of fractures associated with LBMD was significantly higher in low-middle and middle SDI countries than in male. And in middle SDI countries and territories, female DALYs number increased by 1.74 times from 529 735 (95% UI:627 607-427 067) in 1990 to 1 449 610 (95% UI:1 768 625-1 132 004) in 2019. In low-middle SDI, an increase of 1.62 times (**Figures 4A, B**). The upward trend of males in different SDI countries was the same as that of females, and the fastest upward trend was mainly in low-middle and middle SDI countries and territories.

DISCUSSIONS

Based on an in-depth analysis of the data from GBD study 2019, this study estimates the global burden of LBMD and LBMD-related fractures. From 1990 to 2019, the total number of deaths and DALYs from LBMD and LBMD-related fractures increased significantly, consistent with the rising trend in the number of patients and deaths from osteoporosis (27). After age was

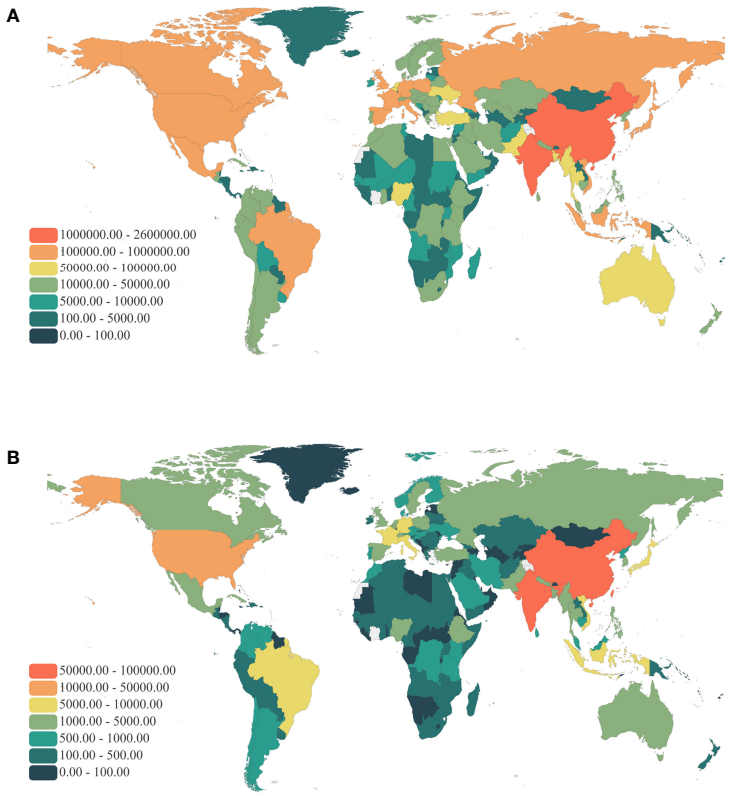

FIGURE 3 | Global map of health burden of LBMD related fractures in 2019. **(A)** DALYs number; **(B)** Death number. DALYs, Disability-adjusted life years; LBMD, Low bone mineral density.

standardized to adjust for population and age structure, LBMD-related fractures death rate and DALYs rate were slowly declining. The changes in rates showed that the death of absolute number rose is largely due to population growth, moreover also increasing the number of cases related with the incidence of falls. This study showed that LBMD is responsible for half of the fall in recent years. Fragility fractures due to osteoporosis caused a significant and growing economic burden on healthcare systems and societies worldwide, so IOF (International Osteoporosis Foundation) calls for priority to be given to prevention to support the effective management of fragility fractures, thereby avoiding the escalation of pain and suffering and associated costs.

The results of the analysis by age showed that there was a unimodal distribution of deaths of LBMD-related fractures both in male and female. In all age groups between 40 to 59 year, LBMD-related fractures caused more deaths in male than in female, while in the age groups 60 year and above, the result was reversed. The higher mortality of male in early life may be related to their social work. In the current social division of labor, male have more physical labor, and the probability of violent injury is higher than female (28). DALYs surpassed male as early as age 50. Our findings are consistent with most previous researches (9, 11). The result was Partly because of differences in bone mineral density between males and females at maturity, and also because of the rate of cortical bone loss in males and females: the bone mass of the human body reached the peak in early adulthood, and then from roughly the fifth decade began to decline with the growth of the age, females have begun to have a large number of cortical bone loss in middle age, and males began at 75-year-old. Besides, females in a variety of factors influence, had an acceleration period of bone loss in the menopausal transition, with an annual rate of bone loss of 1-2% (29). Before menopause, estrogen played a protective role against women, and in older women. Reduction in bone mass was associated with low level of biological activity sex steroids and higher levels of follicle stimulating hormone and bone turnover markers. Similarly, as the growth of the age, male and female fracture frequency was increasing, a combination of reduced bone density and an increased tendency to falls (30). In addition, females generally lived longer than males, so they may be exposed to low bone mineral levels for longer periods of time than males, and 61% of osteoporosis fractures occurred in females, for a ratio of 1.6 to 1. And studies have shown that females are twice as likely as males to have sustained fractures (7).

Based on the disease burden of LBMD related fractures, we found that countries with high DALYs rates were mostly located in Western Europe, Northern Europe and The Indian Peninsula after standardizing the data according to population and age. Geographic and ethnic differences may be associated with the distribution of osteoporotic fractures. The incidence of

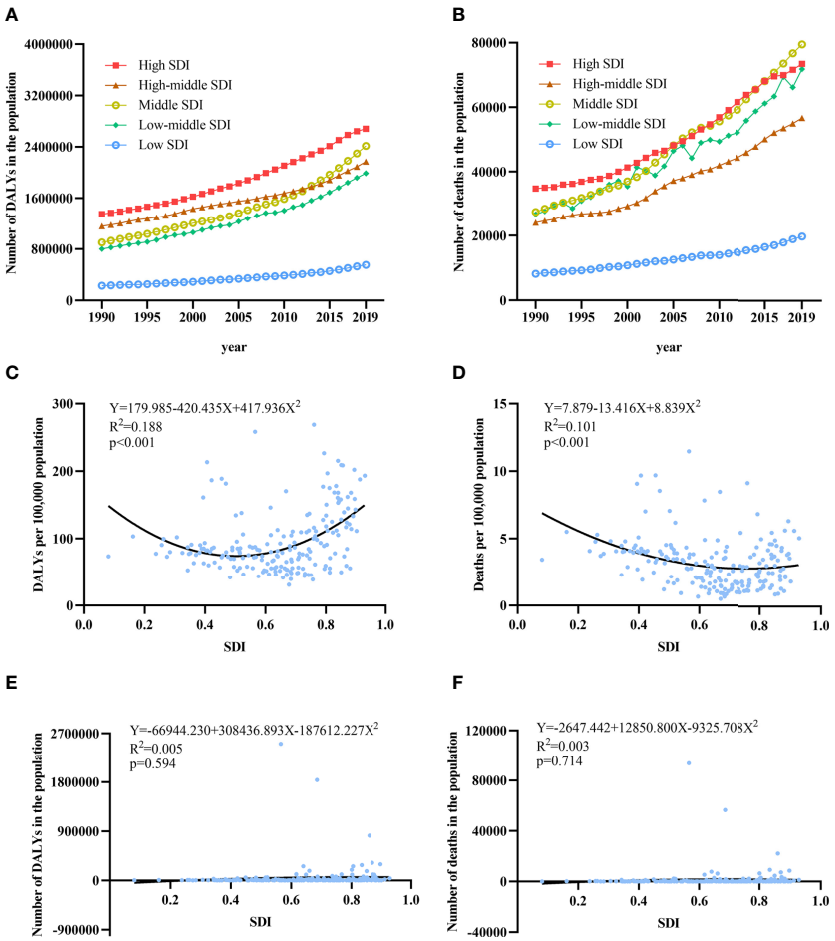

FIGURE 4 | The association between SDI and burden of LBMD related fractures. **(A)** Trends in DALYS number of fractures at LBMD by SDI quintiles in both sexes,1990-2019; **(B)** Trends in DALYS rate of fractures at LBMD by SDI quintiles in both sexes,1990-2019; **(C)** Association between DALYs rate and SDI in both sex in 2019; **(D)** Association between deaths rate and SDI in both sex in 2019; **(E)** Association between DALYs number and SDI in both sex in 2019; **(F)** Association between deaths number and SDI in both sex in 2019; SDI, Socio-demographic Index.

osteoporotic fractures due to LBMD may be ethnically related, with racial differences in fragility fractures being greater than in any other fracture. Black people have the lowest fracture rates for both male and female, so white female are 4.7 times more likely to have a fragility fracture than black female, and white male are 2.7 times more likely than black male. South Asian male also had higher fracture rates than black and mixed-race male (31). In general, people who live further away from the equator might have higher rates of fractures (32). These might be partly responsible for the high osteoporotic fracture burden in places such as Northern Europe. Other studies have found that incidence of fractures in whites are higher than in blacks and Asians over the age of 50, but the rates in blacks, Asians and Hispanics gradually surpass those in whites as people age (33). This might explain the higher fracture burden associated with LBMD in South Asia, particularly India, in recent years.

We found that countries or territories with a higher burden of fractures associated with LBMD were found in both economically advanced and less developed regions. This suggested that osteoporotic fractures were not only related to gene, but also economic situation. We found that from 1990 to 2019, countries and regions with high SDI had the highest DALYS number, especially in the United States and The European Union. After standardizing the population structure of the data, the increase rate of osteoporotic fractures has decreased in countries and regions with high SDI in recent years. On the contrary, countries and regions with middle and low-middle SDI levels showed a trend of rapid rise, and even in recent five years, DALYs rate exceeded that of countries and regions with high SDI, among which India was the most outstanding. The higher burden of disease in regions such as developed countries might be related to their current large populations, and the recent decline in the rate of osteoporotic fractures coincide with the approval of bisphosphonates for the treatment of osteoporosis. The use of anti-osteoporosis drugs has been observed to reduce the incidence of hip fractures in

Belgium, the United States, Denmark and elsewhere (34, 35). Combined estrogen-progestin replacement therapy was associated with significant reduction in hip fracture was also proved in the Women's Health Initiative, whereas the announcement of the Women's Health Initiative (WHI) in 2002 showed that hormone replacement therapy (HRT) had more detrimental than beneficial effects (36, 37). Since then, despite HRT remains the most effective treatment for vasomotor symptoms (VMS) and the genitourinary syndrome of menopause, the popularity and use of hormone therapy declined (38, 39). However, BMS (British Medical Society), IMS (International Menopause Society) and NAMS (The North American Menopause Society) have confirmed the benefits of HRT in the treatment of osteoporosis, arguing that the conclusion of the 2002 WHI study was biased. They claimed that for women aged younger than 60 years or who are within 10 years of menopause onset and have no contraindications, estrogen-based treatments still have a major role in the treatment and risks can be minimized and benefits maximized with selection of individualized circumstances (40, 41). With the development of modern medicine, a number of drugs such as selective estrogen receptor modulators, bisphosphonates, denosumab, have been developed for the treatment of osteoporosis fractures. The use of these drugs may further reduce the incidence of osteoporotic fractures (2).

In this study, there was a U-shaped relationship between SDI and DALYs rate, so did SDI and mortality. SDI is a great indicator in the assessment of social development and the relation between SDI and LMBD-related fractures was influenced by multiple factors. The occurrence of osteoporotic fractures is affected by the interaction of various factors such as exercise and nutrition. Lower socioeconomic status means heavier lifting and more bending motion which can lead to vertebral fractures and contingencies. On the contrary, unhealthy lifestyle habits increase as the result of economic status development, and exercise loses its protective effect on bone density (42). Despite exercise, nutrition condition can also affect osteoporotic fractures. There is a correlation between average body weight and the incidence of fractures, with both underweight and overweight increasing the incidence of fractures, especially in people with a body mass index <20 kg/m^2 (43). Furthermore, medical conditions and life expectancy are two other reasons explaining the relationship. Due to the poor medical care and lack of health education, timely treatment cannot be obtained lead to higher risk of the disability and mortality in people with fractures in countries with low level of social development. Although the death rate from osteoporotic fractures has declined due to improvements in medical care and life expectancy, the duration of disability and loss of quality of life is more pronounced, which explains why osteoporosis is a heavier problem in developed countries (44, 45). Therefore, the prevention and treatment of osteoporotic fracture must be emphasized in the transition of economic and social development.

Osteoporosis, as a chronic disease, was closely related to the aging of the population. Previous researches showed that the number of men and women aged 65 and over in Europe wound increase by 50.6 percent over the next 25 years. For developing countries, where the burden of disease wound be more pronounced in the future and the total population and life expectancy of the elderly will more than triple in the next 25 years. The higher DALYs and death rates reflected potential modifiable factors that wound be more significant. As the burden of disease increases, so will the economic burden of osteoporotic fractures, both directly and indirectly. With the cost of osteoporotic fractures rising faster than the general rate of inflation in almost every country worldwide (3), osteoporotic fractures will have greater significance for healthcare planning. Paying more attention to the prevention and care of osteoporotic fractures will bring more benefits and reduce social and medical pressure.

Genetic factors were important in the development of osteoporotic fractures, but differences between ethnic groups can be modified by reversing lifestyle. Some lifestyles such as low milk intake, smoking, lack of sunlight exposure, lower BMI, and physical activity (46), can cause the incidence of hip fracture to rise further. The high prevalence of unhealthy lifestyle habits such as smoking and alcohol abuse in lower socioeconomic levels, coupled with more pronounced poor diet structure and quality, has been shown to have adverse effects on bones (1, 47). Therefore, the most logical and cost-effective prevention strategy is to encourage the population to quit smoking and avoid excessive alcohol use. And provide advice on consuming adequate calcium and vitamin D, as well as medical advice (48). Of course, inadequate understanding of osteoporosis in developing countries may also play a role, with knowledge of osteoporosis and its risk factors currently low in the Indian cohort of men and women. There is a need to create awareness programmers for both female and male, especially for those with lower education, lower socio-economic status and a history of osteoporosis.

This study also has some limitations. The current disease burden of osteoporotic fractures may be underestimated for several reasons. Firstly, osteoporosis is likely to be missed as a potential cause of death because osteoporotic fractures and death has long time interval (49). Secondly, DXA screening for diagnosis and monitoring of osteoporosis is generally reserved for high-risk patients currently. Compared to other chronic diseases such as hypertension, obesity, and diabetes mellitus, bone mineral density test is relatively expensive and need higher technical requirement, therefore relevant data source is limited. The parameters of DXA devices in different countries are divergent, leading to some changes in the values. Besides, there are many diagnostic criteria for osteoporosis, such as the international standard recommended by WHO, NOF (National Osteoporosis Foundation) and ISCD (International Society for Clinical Densitometry) (50–52). There are information gaps in different health information systems in different countries and regions. In addition, the deficiency of this study also include that the disease burden associated with LBMD in GBD 2019 is restricted to hip fractures. However, for patients with osteoporotic fractures, there are also lumbar vertebrae, thoracic compression fractures, and upper limb fractures, etc. Besides, population with the same LBMD has different fracture rates at

different risks. Due to limited data, it was not possible to quantify bone mineral density, and other information was lacking on subjects' history of fracture, bone metabolic diseases, or treatments that might affect bone metabolism (53). We recommend that future studies include multiple osteoporotic fractures, such as vertebrae and radius fractures, and quantify individual risk factors to provide as comprehensive patient information as possible.

In conclusion, this study suggested that LBMD and fracture is a growing global health burden. Female had a higher burden of disease than male, and the gap widened with age. Increasing education and dissemination of osteoporosis, improving resource allocation, and paying more attention on screening and treatment of osteoporosis could help reduce the global burden of disease attributable by LBMD and fracture, especially in low-middle and middle SDI countries and territories.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

YS and XH conceived the study, collected data, performed the statistical analysis and participated in writing and preparation of the report. JW, XL, XZ and ZZ were involved in the data collection, interpretation of the data and preparation of the report. XP, JX, and JQ analyzed the data and revised the report. TZ, and LY revised the report. P-FS, YR, and HJ designed and coordinated the study, acquired funding, performed the statistical analysis and participated in writing and editing the final report. P-FS assumes full responsibility for the overall content of this report. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.882241/full#supplementary-material

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Genetically Predicted Milk Intake Increased Femoral Neck Bone Mineral Density in Women But Not in Men

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Background: Cow milk contains more calcium, magnesium, potassium, zinc, and phosphorus minerals. For a long time, people have believed that increasing milk intake is beneficial to increasing bone density. Many confounding factors can affect milk consumption, and thus the association described to date may not be causal. We explored the causal relationship between genetically predicted milk consumption and Bone Mineral Density (BMD) of the femoral neck and lumbar spine based on 53,236 individuals from 27 studies of European ancestry using the Mendelian randomization (MR) study. 32,961 individuals of European and East Asian ancestry were used for sensitivity analysis.

Methods: A genetic instrument used for evaluating milk consumption is rs4988235, a locus located at 13,910 base pairs upstream of the *LCT* gene. A Mendelian randomization (MR) analysis was conducted to study the effect of selected single nucleotide polymorphisms (SNPs) and BMD. The summary-level data for BMD of the femoral neck and lumbar spine were obtained from two GWAS meta-analyses ['Data Release 2012' and 'Data Release 2015' in the GEnetic Factors for OSteoporosis Consortium (GEFOS)].

Results: we found that genetically predicted milk consumption was not associated with FN-BMD(OR 1.007; 95% CI 0.991–1.023; P = 0.385), LS-BMD(OR 1.003; 95% CI 0.983–1.024; P = 0.743) by performing a meta-analysis of several different cohort studies. High levels of genetically predicted milk intake were positively associated with increased FN-BMD in Women. The OR for each additional milk intake increasing allele was 1.032 (95% CI 1.005–1.059; P = 0.014). However, no causal relationship was found between milk consumption and FN-BMD in men (OR 0.996; 95% CI 0.964–1.029; P = 0.839). Genetically predicted milk consumption was not significantly associated with LS-BMD in women (OR 1.017; 95% CI 0.991–1.043; P = 0.198) and men (OR 1.011; 95% CI 0.978–1.045; P = 0.523).

Conclusion: Our study found that women who consume more milk have a higher FN-BMD. When studying the effect of milk consumption on bone density in further studies, we need to pay more attention to women.

Keywords: milk intake, bone mineral density, mendelian randomization study, cause effect, female

INTRODUCTION

In bones, osteoporosis is a common metabolic skeletal disorder that has a causal relationship with aging and is characterized by poor bone strength. The microarchitecture of bone tissues deteriorate, and the risk for fractures increases (1). There is a growing prevalence of osteoporosis globally due to the rapidly aging population worldwide. The International Osteoporosis Foundation recently released statistics that report that, on average, 1 in 3 women over 50 years of age and 1 in 5 men will sustain osteoporotic fractures in their lifetimes. This disease significantly impacts on patients' emotional, physical, and financial health. It can result in permanent disability, poor quality of life for elderly patients, and heavy financial burdens that patients must bear in attending to the high cost of treatment they must endure (2). Osteoporosis is primarily diagnosed by measurements of bone mineral density (BMD), which is the most common method of determining this condition, either by Dual Energy X-Ray Absorption (DXA) or bone densitometry (3). Research on twins and families has shown that cross-sectional BMD is highly heritable (50-85%) (4, 5). Many methods have been explored in preventing osteoporosis, and the dairy diet has received the most attention.

Compared with any other typical food in the adult diet, cow milk contains more calcium, magnesium, potassium, zinc, and phosphorus minerals (6). It is widely acknowledged that an enriched diet consisting of milk products would reduce the chances of osteoporosis. Research has suggested that taking dairy products equivalent to at least three or four glasses of milk a day will reduce at least 20% of the costs associated with osteoporosis (6). Even though several studies have suggested that dairy products and milk consumption can prevent fractures and osteoporosis (7–9). Many studies believe that milk intake has nothing to do with increasing bone density (10, 11). Some studies even think that drinking milk more than two times a day will increase fracture risk by 50% (12). There is no evidence to suggest that dairy products have any causal effect on preventing osteoporosis, and research is still ongoing regarding the issue (13, 14). Finnish study reports that high calcium intake in older and younger women is positively associated with non-weight-bearing radius but not with weight-bearing tibia (15). Another study also showed that the intake of calcium-rich foods such as milk was positively related to radial bone density, and it seemed that non-weight-bearing bone density benefited from high calcium intake, while weight-bearing bones like the femur and spine benefited from physical activity (16). As most studies on milk consumption and BMD have been observational or experimental, it is difficult to determine whether confounding factors or reverse causality, eliminated by MR, is responsible for the result. As no studies have studied the causal association between milk consumption and BMD, we decided to make an MR study to investigate the causation. MR is a genetic epidemiological method that uses genetic variants as instrumental variables. Reverse causation and potential confounding factors can be eliminated by MR (13). Single nucleotide polymorphism sites (SNPs) are assigned randomly at conception, avoiding reverse causation bias and residual confounding (14).

Lactase is encoded by the lactase gene (*LCT*), secreted by small intestinal cells, used to break down milk sugar. There is a single nucleotide polymorphism (SNP) upstream of the *LCT* gene that is related to lactase persistence (the presence of lactase in adulthood) and the increased consumption of milk by the European population during the 20th century (17, 18). We used *LCT* gene variation as an instrumental variable to represent milk consumption and assessed the potential causal relationship between milk consumption and bone density.

METHODS

Data Resources

The femoral neck and lumbar spine are the two common osteoporotic fractures sites of postmenopausal women and men 50 years or older. The summary-level data for BMD were obtained from a GWAS meta-analysis, which included 53,236 individuals from 27 studies of European ancestry. We extracted data on BMD in the femoral neck (n=49988) and lumbar spine (n=44731) from this GWAS meta-analysis (**Supplementary Table 1**). Measurement of BMD was recommended utilizing dual-energy X-ray absorptiometry. GWAS summary statistics for BMD were downloaded from 'Data Release 2015'(http://www.gefos.org/?q=content/data-release-2015) in the GEnetic Factors for OSteoporosis Consortium (GEFOS). It is the latest summary statistics of BMD in the femoral neck and lumbar spine. Besides, GEFOS is an extensive international collaboration comprising numerous research groups. This organization regularly publishes large samples of bone mineral density-related GWAS data.

In addition, we included another GWAS meta-analysis, 'Data Release 2012' (http://www.gefos.org/?q=content/data-release-2012), in the GEFOS for sensitivity analysis. This meta-analysis provides BMDs in the femoral neck (n=34,910) and lumbar spine (n=34,632) in different genders, which including 17 genome-wide association studies (**Supplementary Table 2**). For further analysis, we study the causal effect of milk intake on BMD in different genders.

Genetic Instrument

Lactase breaks down the lactose in milk. People with lactase deficiency will experience diarrhea, bloating, and abdominal pain after eating cow's milk (19). The milk intake of this group of people will be significantly lower than that of the regular group (20). A genetic instrument used for evaluating milk consumption is rs4988235, a locus located at 13,910 base pairs upstream of the *LCT* gene. Rs4988235(NC_000002.12:g.135851076G>A), located in the *MCM6* gene but with influence on the lactase *LCT* gene. Rs4988235 is one SNP associated with hypolactasia, more commonly known as lactose intolerance in European populations (21, 22). Several studies have demonstrated that rs4988235 is strongly associated with milk consumption among individuals in Europe, thus supporting the first MR assumption (17, 18) (**Figure 1**). Participants with the lactase persistent genotype TT/TC can digest more milk than do participants with the lactase nonpermanent genotype CC. During the EPIC-InterAct study, extra lactase persistence alleles (T) of rs4988235 increased daily milk consumption from 162 grams per day to 179 grams per day (11). In a Danish cohort of 73,715 individuals, weekly milk intake increased by 0.58 cups for each additional T allele of rs4988235 (18). According to genetic studies, the genetic variant rs4988235 is estimated to account for 2% of the variance in milk intake (18). This genetic variant has an F-statistic of 515, indicating an association between milk consumption and the variant (18). So using rs4988235 as an instrumental variable for milk intake is in line with the first assumption of MR studies (instrument variables are strongly correlated with exposure factors). For the second assumption of Mendelian randomization, we did not find any confounding factors associated with rs4988235 and affects BMD using phenoscanner database (http://www.phenoscanner.medschl.cam.ac.uk). The third assumption is that the instrumental variables affect outcomes only through causal pathways of exposure of interest and cannot be checked (23).

Statistical Analysis

To calculate the ratio estimate for rs4988235, we divided the resultant beta coefficient by the beta coefficient for milk consumption. For each additional increase in milk intake, ORs and 95% CIs were computed for the T-allele of rs4988235, which increases milk intake. The meta-analysis used the MR random-effects model to pool individual outcomes. The analyses were performed using in RStudio version 1.4.1717. The following R packages were used during the study: TwoSampleMR package; meta package; forestplot package; ggplot2 package; grid package.

RESULTS

By performing a meta-analysis of several different cohort studies, we found that genetically predicted milk consumption was not associated with FN-BMD(OR 1.007; 95% CI 0.991–1.023; P = 0.385), LS-BMD(OR 1.003; 95% CI 0.983–1.024; P = 0.743) (**Figure 2**).

High levels of genetically predicted milk intake were positively associated with increased FN-BMD in Women (**Figure 3**). The OR for each additional milk intake increasing allele was 1.032 (95%CI 1.005–1.059; P = 0.014). However, no causal relationship was found between milk consumption and FN-BMD in men (OR 0.996; 95% CI 0.964–1.029; P = 0.839). Genetically predicted milk consumption was not significantly associated with LS-BMD in women(OR 1.017; 95% CI 0.991–1.043; P = 0.198) and men (OR 1.011; 95% CI 0.978–1.045; P = 0.523) (**Figure 3**).

DISCUSSION

We used the MR design to study the causal relationship between genetically predicted milk consumption and BMD of the femoral

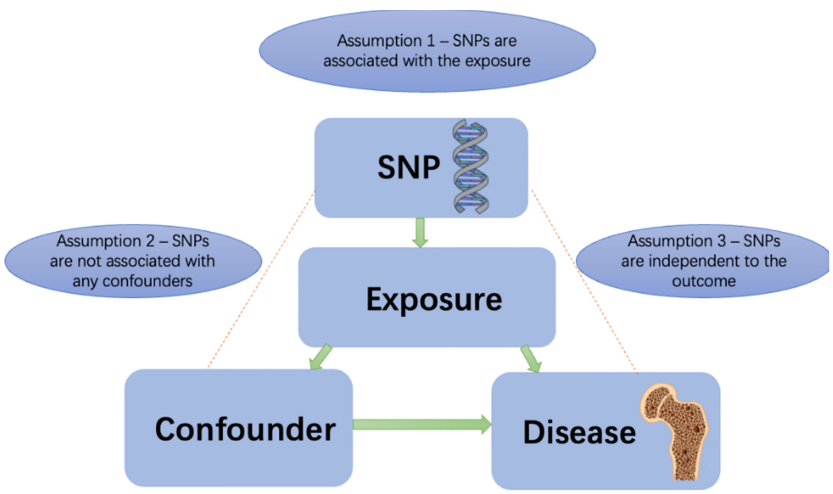

FIGURE 1 | Principles of Mendelian randomization study.

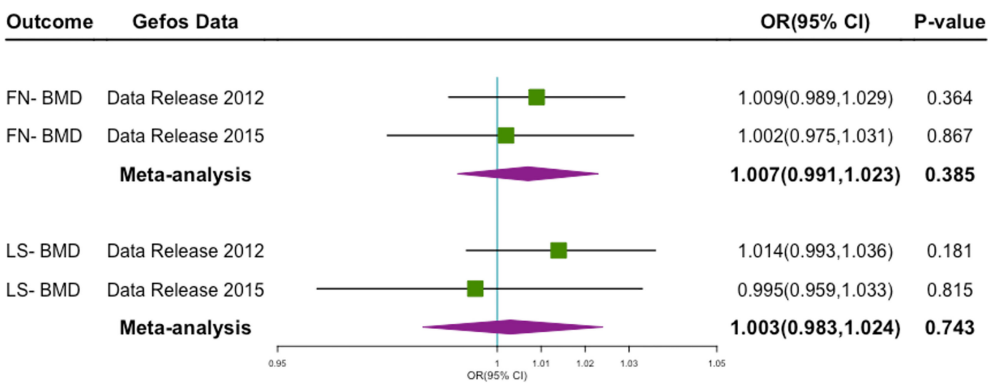

FIGURE 2 | Forest plot of MR study using genetic instruments with FN-BMD, LS-MD, OR, odds ratio; CI, confidence interval; FN-BMD, femoral neck Bone Mineral Density; LS-MD, lumbar spine Bone Mineral Density.

neck and lumbar spine. To analyze the difference in this causal relationship in different genders, we also used the other data in GEFOS to do a gender stratification analysis. As far as we know, this is the first MR study to evaluate the relationship between milk consumption and BMD.

Throughout the world, milk is a widely consumed beverage, and it provides essential macro and micronutrients that are essential for the health and well-being of millions of individuals (24). For a long time, people have believed that increasing milk intake is beneficial to increasing bone density (25, 26). In a prospective cohort study, researchers measured the BMD of the radius and tibia with ultrasound equipment. They found that intake of dairy products may reduce the incidence of radial osteoporosis in Korean postmenopausal women, but there is no significant effect in the tibia (15). The study also confirmed that milk might affect on bone density in other parts of the body (15). Consumption of high-calcium skim milk can effectively reduce bone loss in the hip in postmenopausal Chinese women in Malaysia, which is consistent with our study (27). A review study of older women using regular or fortified milk reports significant changes in bone biomarkers and some changes in bone density but no reduction in fracture risk (28). Using genetic MR analysis, this study found that women who consume more milk have a higher FN-BMD. However, this causality was not seen in male FN-BMD and LS-BMD.

Milk intake has a positively correlated causal effect on FN-BMD in women but not in men. There may be the following reasons: First, osteoporosis may be influenced by childhood or teenage years. Adequate milk intake during adolescence may reduce osteoporosis in adulthood. As a result of consuming a serving of milk per week (low intake) during childhood compared to consuming more than one serving per day (high intake), bone mineral content was 5.6% lower in women aged 20-49 (29). 18-year-old men and 20-year-old women can reach 90% of their peak bone mass (30). Both women and men continue to gain a relatively small amount of bone mass; however, men do so more rapidly than women do. Second, the risk of osteoporosis is related to estrogen levels. Women's estrogen levels decline rapidly in the years after menopause, and the rate of bone loss at this time is much faster than at any other time in their lives. In a follow-up study of up to 15 years, researchers found a linear

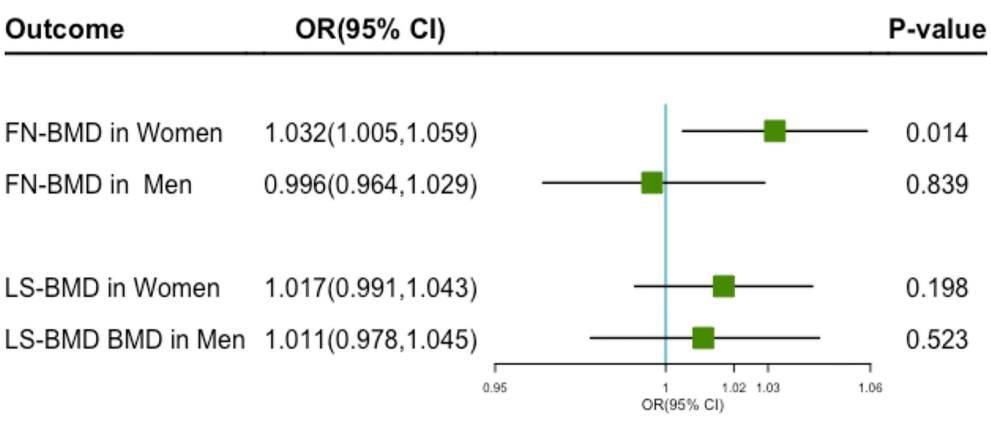

FIGURE 3 | The plot of the MR study used genetic instruments with FN-BMD and LS-MD in different genders. OR, odds ratio; CI, confidence interval; FN-BMD, femoral neck Bone Mineral Density; LS-MD, lumbar spine Bone Mineral Density.

decline of 1.67% per year in femoral neck bone loss in women aged 45-68 (31). These differences in bone loss between men and women may explain the difference in the causal benefit of milk consumption. Some studies support our overhead view. Calcium from dairy products (fortified with calcium) can increase the bone mineral density of Caucasian women by 0.7 to1.8%, but not for men (32).

Our analysis has several strengths. We utilize the largest summary statistics data of BMD, which could overcome limitations of conventional epidemiological study designs, such as confounding and reverse causality. It is more time-efficient and less expensive than RCT. There were some limitations in this study. Firstly, our datasets included the European populations, which limited the applicability of results to non-European populations. Secondly, MR's linear effect assumption could not further investigate nonlinear causality (33). Thirdly, the possibility cannot be ruled out that genetic instruments used to represent milk intake might affect BMD in ways other than milk intake, thus contradicting the second and third MR hypotheses. Finally, most studies on the mechanism of milk's different effects on bone mineral density in men and women focus on the difference in bone loss. More detailed mechanism studies are still relatively rare, which is also an issue that our team will study in the future.

CONCLUSION

According to our study, we found that women who consume more milk have a higher FN-BMD. Women may improve femoral neck bone density and prevent osteoporotic fractures by consuming more milk. When studying the effect of milk consumption on bone density in further studies, we need to pay more attention to women.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SoC, TC participated in Study design. Data was acquired and analyzed by SoC, JC, YP. SYC participated in the study's supervision. The manuscript was drafted by SoC. CZ has the same contribution to the article as SoC. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.900109/full#supplementary-material

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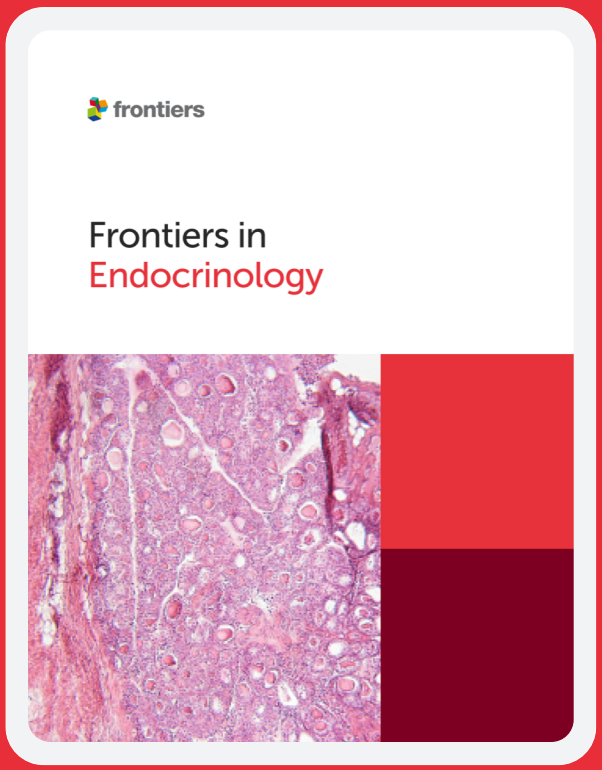

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