ORIGINAL ARTICLE

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Type 2 Diabetes Is Causally Associated With Reduced Serum Osteocalcin: A Genomewide Association and Mendelian Randomization Study

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ABSTRACT

Recent advances indicate that bone and energy metabolism are closely related. However, little direct evidence on causality has been provided in humans. We aimed to assess the association of three bone-related biomarkers—25 hydroxyvitamin D (25OHD), parathyroid hormone (PTH), and osteocalcin (OCN)—with several metabolic phenotypes and investigate any causal relevance to the associations using a Mendelian randomization (MR) study. Serum 25OHD, PTH, and total OCN were measured at baseline in 5169 eligible Chinese participants in Changfeng study. Partial correlation and bivariate GREML analysis were used to estimate phenotypic and genetic correlations, respectively. Multiple linear regression and logistic regression were used to assess linear associations. Genomewide association analysis (GWAS) was performed. Bidirectional two-sample MR analyses were conducted to examine causal relationships between OCN and body mass index (BMI), diastolic blood pressure (DBP), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), glycated hemoglobin A1c (HbA1c), and type 2 diabetes (T2DM), using our GWAS result of OCN and GWAS statistics from Biobank Japan project (BBJ) and the largest meta-analysis of T2DM GWAS in East Asian population. Circulating OCN was significantly associated with higher DBP and HDL-C and decreased TG, blood glucose level, insulin resistance, liver fat content, bone mineral density, BMI, and a favorable body fat distribution pattern. GWAS identified one novel serum PTH locus and two novel serum OCN loci, explaining 0.81% and 1.98% of variances of PTH and OCN levels, respectively. MR analysis suggested a causal effect of T2DM on lower circulating OCN concentration (causal effect: −0.03; −0.05 to −0.01; $p = 0.006$ for T2DM_BBJ and −0.03; −0.05 to −0.01; $p = 0.001$ for T2DM_EAS). These findings indicate that T2DM might impact bone remodeling and provide a resource for understanding complex relationships between osteocalcin and metabolic (and related) traits in humans. © 2021 American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: CAUSAL RELATIONSHIP; GENOMEWIDE ASSOCIATION; MENDELIAN RANDOMIZATION; OSTEOCALCIN; TYPE 2 DIABETES

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Introduction

Osteocalcin (OCN), the most abundant non-collagenous protein in bone, is produced and secreted almost exclusively by osteoblasts during bone formation. OCN secreted by osteoblasts contains three γ-carboxyglutamic acid residues that confer high affinity to the bone hydroxyapatite matrix. During bone resorption by osteoclasts, the acidic environment promotes the carboxyl groups on OCN to be removed, decreasing its affinity for hydroxyapatite and therefore promoting its release into the circulation.[1] While classically linked to bone metabolism, animal studies in the past two decades suggest that OCN may be a key molecule for bone endocrine functions with multiple effects on many aspects of physiology, such as insulin production and glucose metabolism, testosterone production and fertility, fat accumulation, brain development and cognitive function, muscle mass and exercise capacity, and arterial calcification.[2–4] However, recently two studies of independent OCN mouse knockout models showed that loss of OCN function had no effect on bone mineral density (BMD), glucose metabolism, testosterone production, or muscle mass,[5,6] therefore applying results from preclinical animal models to human biology and diseases should be treated with great caution, and evidence in humans is urgently needed.

In humans, apart from its associations with bone remodeling and fractures, circulating OCN has been documented to be inversely associated with adiposity, plasma glucose, insulin resistance, and metabolic syndrome severity.[7–9] However, these are mainly cross-sectional studies, some with small sample sizes. No randomized-controlled trials (RCTs) or Mendelian randomization (MR) studies designed to evaluate the impact of OCN on health outcomes or factors that influence OCN have been conducted so far. Five longitudinal studies evaluated the relationship between total OCN and incidence of diabetes; three reported a significant or near-significant inverse association,[10–12] and the other two showed that OCN was not a risk factor for diabetes.[13,14] Consequently, the meaning of the reported cross-sectional association between a low OCN status and metabolic disorders remains to be further established, and its links with other traits such as blood pressure, lipids, or muscle mass in humans remain to be further studied.

Vitamin D and parathyroid hormone (PTH) are two other key factors in bone mineral metabolism. PTH stimulates osteoclast activity to release calcium, enhances the reabsorption of calcium in distal nephrons, and activates 25-hydroxyvitamin D (25OHD) 1α-hydroxylase in the kidney, which converts 25OHD to its most active metabolite, $1,25(OH)_2D$.[15] The $1,25(OH)_2D$ stimulates intestinal calcium absorption and osteoclast proliferation, increases serum levels of calcium, and exerts negative feedback on PTH secretion. Serum $1,25(OH)_2D$ also regulates OCN, and OCN is increased in enhanced bone formation, including osteoporosis and hyperparathyroidism.[15] MR studies with large sample size have not found causal roles of 25(OH)D concentration on type 2 diabetes, cardiovascular disease, and a range of other health outcomes, although associations were reported in some observational studies.[16–18] Increased circulating PTH level is associated with higher risk of fractures,[19] hypertension,[20] cardiovascular complications,[21] even mortality in some studies.[22] Elucidating their associations with intermediate metabolic phenotypes may be helpful to achieve a comprehensive understanding of their physiological functions.

In the present study, we aim to (i) explore the associations between 25OHD, PTH, and OCN and a wide range of anthropometric and metabolic phenotypes, (ii) identify loci that might influence serum 25OHD, PTH, and OCN levels in the Changfeng population of East Asian ancestry, and (iii) investigate the causal relationships between OCN and several metabolic traits that were significantly associated with OCN, using a bidirectional two-sample MR framework.

Materials and Methods

The Changfeng population

The participants were from Shanghai Changfeng Study, which is a community-based prospective cohort study focusing on chronic metabolic diseases in a middle-aged and elderly Chinese population (aged ≥45 years).[23,24] Among the 6595 participants enrolled at baseline from year 2009 to 2012, 5538 had serum 25OHD, PTH, and OCN measurement data available. In the present study, 369 participants were excluded for any one of the following reasons: (i) missing data of age, sex, height, weight, or body mass index (BMI); (ii) self-reported thyroid diseases or parathyroid diseases that might influence bone metabolism or had been on hormone-replacement therapy or had taken anti-osteoporotic drugs in 1 month before blood draw. The remaining 5169 participants were included in the final analysis. Ethics approval was granted by the ethics committee of Zhongshan Hospital affiliated to Fudan University and all participants provided written informed consent before participation.

Vein blood samples were collected in the morning after overnight fasting for at least 10 hours and were used for biochemical examinations, and further blood sampling was performed 2 hours after 75 g oral glucose tolerance test to measure 2-hour plasma glucose (PPG) levels. Serum 25OHD, PTH, and fasting insulin levels were measured using a Roche Cobas-6001 electro chemiluminescence system with complete reagents (Roche Diagnostics, Basel, Switzerland; coefficient of variation 4.3%, 3.1%, and <5.0%, respectively). Serum total OCN concentration was measured using electrochemiluminescence immunoassay by Mobular E170 automatic electrochemiluminescence analyzer (Roche Diagnostics Ltd., Mannheim, Germany; coefficient of variation 3.1%). Other anthropometric and biochemical measurements and disease definitions are described in Supplemental Materials and Methods.

Phenotypic statistical analyses

Phenotypic correlations between 25OHD, PTH, OCN, and anthropometric traits, biochemical measurements, diseases, BMD, and body fat distribution were estimated by Spearman partial correlation using raw values. Basic covariates included were age, sex, menopause (female), and BMI (except for analyses where BMI was tested as the exposure or the outcome). We modeled sex and menopause in females simultaneously by dividing participants into male, premenopausal female, and postmenopausal female. In analyses with biochemical measurements, medications (yes or no) for hypertension, dyslipidemia, diabetes, or hyperuricemia were also included as covariates when appropriate. As expected,[16,25] 25OHD levels were higher in summer and autumn compared with those in winter and spring (Supplemental Fig. S1*A*). Therefore, we also included season of sample collection as a covariate in analyses with 25OHD.

To investigate the extent to which 25OHD, PTH, and OCN were associated with other traits, multivariable linear regressions and logistic regressions were used with each quantitative or binary

trait as dependent variable and 25OHD, PTH, or OCN as independent variable, respectively. The following dependent variables were log-transformed before analysis because of skewed distribution: triglyceride (TG), fasting plasma glucose (FPG), postprandial glucose (PPG), glycated hemoglobin A1c (HbA1c), homeostatic model assessment of insulin resistance (HOMA-IR), uric acid, LFC, Legs_Total, and Appen_Total. Covariates included were the same as those in Spearman partial correlation analyses mentioned above. Estimates represented per SD change of dependent variables associated with per SD increase of 25OHD (18.91 nmol/L), PTH (16.62 pg/mL), or OCN (7.32 ng/mL). In addition, we carried out sensitivity analysis by excluding individuals on specific medications. We noted that the sample sizes among the traits were similar but different; because not all participants had complete data on all traits, sample sizes of individual analyses were also indicated in results.

All tests performed were two-sided. Bonferroni correction for multiple testing was used with a type 1 error rate of under 0.05. All analyses were performed with R version 3.6.2.

Genotyping, quality control, and imputation

All participants were of Chinese ancestry and genomic DNA was extracted from peripheral blood leukocytes using QIAGEN (Hilden, Germany) QIAamp DNA Mini Blood Kit according to manufacturer's instructions. A total of 5689 Changfeng study population at baseline were genotyped with Illumina (San Diego, CA, USA) Infinium BeadChip genotyping array (707,180 markers). Before analysis, samples were excluded if they had call rates <95%, deviated from the expected inbreeding coefficient (fhet <−0.2 or >0.2), or had a sex discrepancy between reported and genotypic sex (based on inbreeding coefficients calculated from single-nucleotide polymorphisms [SNPs] on the X chromosome). Markers were excluded if they had genotype missingness >2%, Hardy–Weinberg equilibrium (HWE) $p < 1 \times 10^{-5}$, or minor allele frequency (MAF) <1%. After filtering, 5629 participants with 426,070 SNPs remained.

Pre-phasing and phasing were performed using SHAPEIT v2r790.[26] Imputation was performed with IMPUTE2 v2.3.1[27] using the 1000 Genomes (1000G) phase 3 data as the reference. Variants were filtered to include only those with a call rate >98%, HWE $p > 1 \times 10^{-5}$, MAF of >1%, and an INFO score of >0.8, resulting in 8,604,360 variants. All genomic positions are in reference to hg19/build 37.

Genomewide association studies of discovery sample

Principal components (PCs) were calculated with LD pruned (-indep-pairwise 50 5 0.2) genotype calls, a total of 139,522 variants in 5009 unrelated participants (pihat <0.1875) were used to calculate the PCs with PLINK v1.90b4,[28] and the first 8 PCs were chosen for adjustments using EIGENSTRAT.[29] PCs were calculated for unrelated participants and projected onto the complete set 5629 participants.

A maximum of 4292 individuals (1908 males and 2384 females) with genotype and valid 25OHD, PTH, and OCN data at baseline were analyzed. The distribution of 25OHD, PTH, and OCN were right skewed and showed difference between sexes (Supplemental Fig. S1*B–D*), outliers (≥4 SD from mean) were removed separately in males and females, and the raw phenotypes were log-transformed before analysis. We tested autosomal genetic variants for association with 25OHD, PTH, and OCN, separately, assuming an additive allelic effect, using mixed linear models in fastGWA[30] with log(25OHD), log(PTH), and log(OCN) as the response variables and the SNPs as random effects. The following covariates were included as fixed effects: age, sex, menopause (in female), season (only in the analyses with 25OHD), principal components 1 to 8, and sparse genetic relationship matrix (GRM). We also performed additional analyses further adjusting for BMI. We set the significance threshold for our genomewide association study (GWAS) at the conventional genomewide significance threshold $p < 5 \times 10^{-8}$. Conditionally independent variants in each GWAS identified by GCTA-COJO with default parameters (–cojo-p 5×10^{-8} –cojo-wind 10,000 Kb) were annotated to the physically closest gene with the Hg19 Gene range list available in dbSNP (https://www.ncbi.nlm.nih.gov/SNP/).

SNP-based heritability was calculated using GCTA-GREML.[31]

Replication and overlap with previous GWAS results

Because not all participants at baseline underwent DNA extraction, we further genotyped 433 samples collected during follow-up (from year 2014 to 2017) that did not overlap with the discovery samples, using the same genotyping array. Quality control and imputation were consistent with those of discovery cohort, and a total of 396 individuals with 8,705,296 variants were used for replication analyses. Because of the small sample size, GEMMA[32] was used to test associations between autosomal genetic variants and log-transformed 25OHD, PTH, and OCN levels, assuming an additive allelic effect, and age, sex, menopause (in female), season (only in the analyses with 25OHD), and relatedness matrix were included as covariates.

Lead SNPs from all independent signals were cross-referenced with previous GWAS results in NHGRI-EBI GWAS catalog (https://www.ebi.ac.uk/gwas/), GenATLAS (http://geneatlas.roslin.ed.ac.uk/), IEU GWAS database (https://gwas.mrcieu.ac.uk/), and PheWeb for BBJ (http://jenger.riken.jp/en/) to determine other significant associations ($p < 5 \times 10^{-8}$) with the identified signals by the present study. We also looked up previously reported significant associations with 25OHD, PTH, and OCN in our results.

Genetic correlations

Genetic correlation between 25OHD, PTH, OCN, and all other traits were estimated in a bivariate GREML analysis (gcta -reml-bivar) using GCTA v1.93.0 beta.[33] Covariates included were the same as phenotypic correlation analyses plus ancestry informative PCs 1 to 8. Any p values $<4.63 \times 10^{-4}$ (Bonferroni correction: 0.05/108 tests) were considered statistically significant.

Mendelian randomization

Data sources

We performed two-sample MR analyses using our GWAS result of OCN and summary results data from the BioBank Japan Project (BBJ)[34] and the largest type 2 diabetes GWAS meta-analysis of East Asian individuals (referred to as T2DM_EAS, $n = 433{,}540$, ncase = 77,418).[35] The BBJ, one of the largest East Asian biobanks, is a multi-institutional hospital-based registry that has enrolled approximately 200,000 individuals and consists of 47 diseases. GWAS summary data for BMI ($n = 158{,}284$),[36] diastolic blood pressure (DBP; $n = 136{,}615$),[37] TG ($n = 105{,}597$),[37] high-density lipoprotein cholesterol (HDL-C; $n = 70{,}657$),[37] HbA1c ($n = 42{,}790$),[37] and type 2 diabetes (referred to as T2DM_BBJ, $n = 210{,}865$, n case = 40,250)[38] were obtained. Details of the data

sources are presented in Supplemental Table S1. According to the original papers, BBJ phenotypes were retrieved from medical records, and subjects with renal insufficiency or diabetes were excluded for HbA1c.[37] BMI and HbA1c were normalized using rank-based inverse normal transformation, DBP and HDL-C were normalized using *Z*-score, and TG was normalized using log transformation plus *Z*-score (Supplemental Table S1). Basic characteristics of the study participants of the GWAS data used are presented in Supplemental Table S2.

Genetic instrumental variables

Conditionally independent SNPs associated with OCN ($p < 5 \times 10^{-8}$) were used as instrumental variables in MR analyses where OCN was tested as the exposure. Next, bidirectional MR was performed using other traits, respectively, as the exposure and OCN as the outcome. Significant and independent SNPs ($p < 5 \times 10^{-8}$, $r^2 < 0.001$, kb >10,000, MAF >0.01) for each trait were extracted. For SNPs that were not included in our GWAS result data for OCN or were not bi-allelic (2 for BMI, 1 for HDL-C, 3 for T2DM_BBJ, and 6 for T2DM_EAS), 1 proxy SNP and 3 proxy SNPs with $r^2 > 0.8$ were found for BMI and T2DM_EAS, respectively. When harmonizing exposure and outcome data, palindromic SNPs (allele pairs coded as A/T or C/G) with an ambiguous strand (4 for BMI, 1 for DBP, 1 for TG, 2 for HbA1c, 1 for T2DM_BBJ, and 14 for T2DM_EAS) were further excluded to ensure that the effects of a SNP on the exposure and the outcome correspond to the same allele. Finally, 63 SNPs, 17 SNPs, 32 SNPs, 49 SNPs, 23 SNPs, 98 SNPs, and 157 SNPs significantly associated with BMI, DBP, TG, HDL, HbA1c, T2DM_BBJ, and T2DM_EAS, respectively, were included as genetic instrumental variables (Supplemental Table S3). R^2 values (variance of exposure explained by the instrumental variables) were calculated with MR Steiger directionality test and *F* statistics were used to assess weak instrumental variable bias.[39]

MR methods

The conventional inverse variance weighted (IVW) MR[40] was used as the primary analysis. Heterogeneity was assessed by Cochran's *Q* test. "Leave-one-out" analysis was conducted to assess the stability of the instrumental variables. Four complementary sensitivity analyses[41] were performed, including MR-Egger regression,[42] weighted median MR,[43] weighted mode MR,[44] and the outlier-robust method Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO).[45] Horizontal pleiotropy was assessed with the intercept term in MR-Egger regression. MR analyses were performed using the TwoSampleMR R package (version 0.5.5) implemented in the MR-Base platform[46] except for MR-PRESSO, which was performed using the MRPRESSO R package (version 1.0). The number of distributions was set to 10,000 and the outlier-test threshold was set to 0.05. We present results from MR-PRESSO raw tests unless any outliers were detected by MR-PRESSO outlier-test and result of outlier-corrected test was given.

We considered the relationships significant if the directions of the estimates by the five methods (IVW, MR-Egger, weighted median, weighted mode, MR-PRESSO) were consistent, *p* value of the IVW method was <0.007 (0.05/7), and no significant pleiotropy tested by MR-Egger intercept. Power calculations for MR with a continuous exposure were performed using the web-based https://shiny.cnsgenomics.com/mRnd/.[47] For MR with a binary exposure (eg, T2DM_BBJ and T2DM_EAS), R^2 values were calculated as the sum of beta^2*2*MAF*(1-MAF) of each instrumental SNP, and powers were calculated using https://sb452.shinyapps.io/power/.[48]

Results

Phenotypic and genetic correlations between 25OHD, PTH, OCN, and other traits

The baseline characteristics and phenotypes of the 5169 participants are presented in Supplemental Table S4. The mean age of the participants was 63.2 years (SD 9.8) at baseline, and 2884 (55.8%) were female. Compared with male participants, females had lower serum 25OHD levels but higher PTH and OCN levels (Supplemental Table S4; Supplemental Fig. S1*B–D*).

Phenotypic and genetic correlations are illustrated in Fig. 1 and Supplemental Table S5. Overall, genetic correlations were higher than phenotypic correlations with the same directions. PTH was negatively correlated with 25OHD and positively correlated with OCN. Higher PTH was correlated with higher SBP and DBP. All three biomarkers were negatively correlated with TG and positively correlated with HDL-C. OCN was inversely correlated with blood glucose and HOMA-IR, and showed notable negative genetic correlations with PPG ($rG = -0.65$, $p = 1.23 \times 10^{-14}$). Opposite to PTH and OCN, serum 25OHD level was negtively correlated with osteoporosis and positively correlated with BMD at lumbar spine, total hip, and femoral neck. Serum 25OHD and OCN levels were negatively correlated with BMI, and PTH was positively correlated with BMI. Besides, higher OCN exhibited significant correlations with favorable body fat distribution (Fig. 1).

Associations between 25OHD, PTH, OCN, and other traits

As shown in Fig. 2 and Supplemental Table S6, per SD increase of serum 25OHD was associated with 0.068 SD decrease of log(TG), 0.072 SD increase of HDL-C, 0.051 SD decrease of log(FPG), 0.056 SD decrease of log(HbA1c), about 0.07 SD increase of BMD at total hip and femoral neck, and 0.084 SD decrease of BMI (all $p < 0.0001$). Higher PTH showed relatively strong associations with higher DBP, lower TG, higher HDL-C, lower BMD, and higher BMI, with beta coefficients ranging between −0.074 and 0.109 (all $p < 0.0001$). The associations between OCN and other traits were mostly stronger compared with 25OHD and PTH. Per SD increase of OCN level was associated with 0.053 SD increase of DBP, 0.061 SD decrease of log(TG), and 0.074 SD increase of -HDL-C. Strongest associations between OCN and traits regarding glucose status was observed in log(HbA1c) (beta coefficient −0.127 SD, 95% confidence interval [CI] −0.155 to −0.099, $p = 2.46 \times 10^{-18}$). Higher concentration of OCN was most strongly reflective of lower BMD with beta coefficients ranging between −0.186 and −0.147 SD. Per SD increase of serum OCN was also associated with 0.151 SD decrease of BMI. In addition, associations between OCN and favorable fat distribution were rather strong with beta coefficients near ±0.1 (Fig. 2; Supplemental Table S6). Sensitivity analyses that exclude individuals on specific medication before conducting linear regressions instead of adjusting for medication use did not materially change the results (Supplemental Table S7).

After adjusting for age, sex, menopause for female, season, and BMI, higher 25OHD was significantly associated with lower risks of dyslipidemia, hyperglycemia, and osteoporosis, with multivariable-adjusted odds ratios (ORs) of 0.88 (95% CI 0.83–0.94), 0.89 (0.84–0.95), and 0.84 (0.75–0.93) per SD increase of

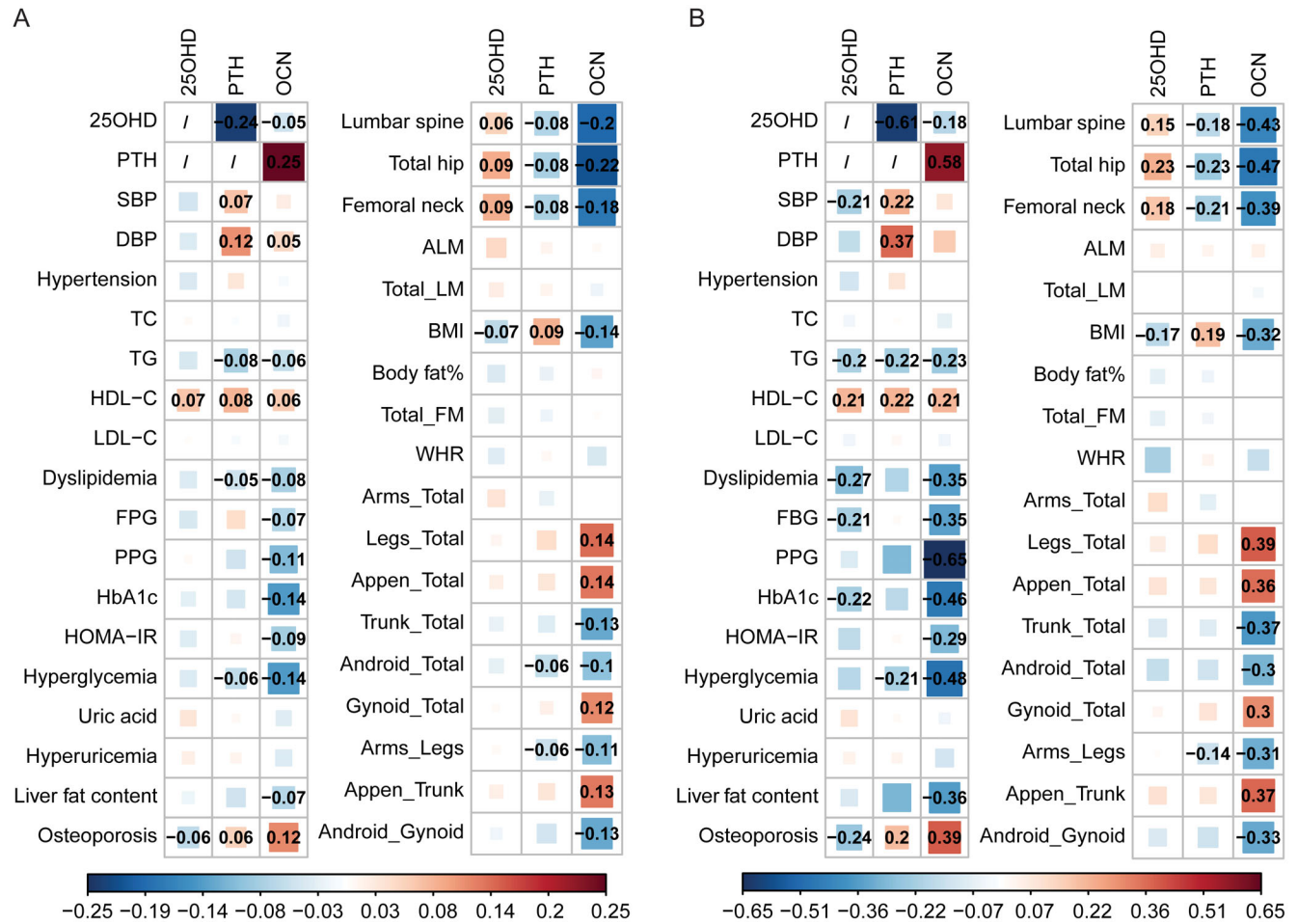

Fig 1. Phenotypic (*A*) and genetic (*B*) correlations of 25OHD, PTH, OCN, and other traits. Significant (p values < 4.63×10^{-4}) results are marked with partial Spearman r values (*A*) or rG values (*B*). Statistical values, covariates adjusted, and sample sizes of each pairwise analysis are presented in Supplemental Table S5.

25OHD, respectively (Table 1). In model 1 adjusting for age, sex, menopause in female, and BMI, higher PTH level was associated with lower risks of dyslipidemia, hyperglycemia, and higher risk of osteoporosis, with adjusted ORs of 0.9 (95% CI 0.85–0.95), 0.89 (0.84–0.95), and 1.24 (1.13–1.36) per SD increase of PTH, respectively. Adjusted ORs for dyslipidemia, hyperglycemia, osteoporosis, and non-alcoholic fatty liver disease (NAFLD) per SD increase of OCN was 0.85 (0.79–0.90), 0.74 (0.69–0.79), 1.46 (1.33–1.61), and 0.84 (0.76–0.91) (all $p < 0.001$). Further adjusting for smoking and drinking in model 2 showed similar results (Table 1).

GWAS results of 25OHD, PTH, and OCN in discovery and replication

A total of 4269, 4250, and 4260 individuals with both phenotype data (25OHD, PTH, and OCN levels) and genotype data were included in the discovery GWA analyses, respectively. We analyzed 8,604,360 SNPs; the Manhattan and quantile–quantile plots are shown in Supplemental Fig. S2. The SNP-based heritability of 25OHD, PTH, and OCN were 0.090 ($p = 0.152$), 0.383 ($p = 1.47 \times 10^{-5}$), and 0.245 ($p = 5.84 \times 10^{-3}$), respectively.

The only one significant association for 25OHD, the missense variant rs4588 ($p = 5.67 \times 10^{-19}$) at 4q13.3 in *GC* (group-specific component) gene (Supplemental Fig. S3), explained 1.83% of the variance in circulating 25OHD concentration and was successfully replicated in our study ($p = 4.03 \times 10^{-5}$). It has been reported to be associated with serum 25OHD levels, vitamin D levels, and hemocytes (Table 2).

One novel genomewide significant locus (rs35610898 at 5q35.3, discovery $p = 9.62 \times 10^{-10}$, replication $p = 2.02 \times 10^{-2}$) explaining 0.81% of the variance of PTH concentration was identified. It is intronic within *SLC34A1* (solute carrier family 34 [type 2 sodium/phosphate cotransporter], member 1) gene (Supplemental Fig. S4) and was previously reported to be associated with hemocytes, estimated glomerular filtration rate, urolithiasis, and so on (Table 2).

A total of 182 variants, all common, at 2 novel loci were significantly associated with OCN (Supplemental Table S8). The top SNP rs2842871 (discovery $p = 4.35 \times 10^{-17}$, replication

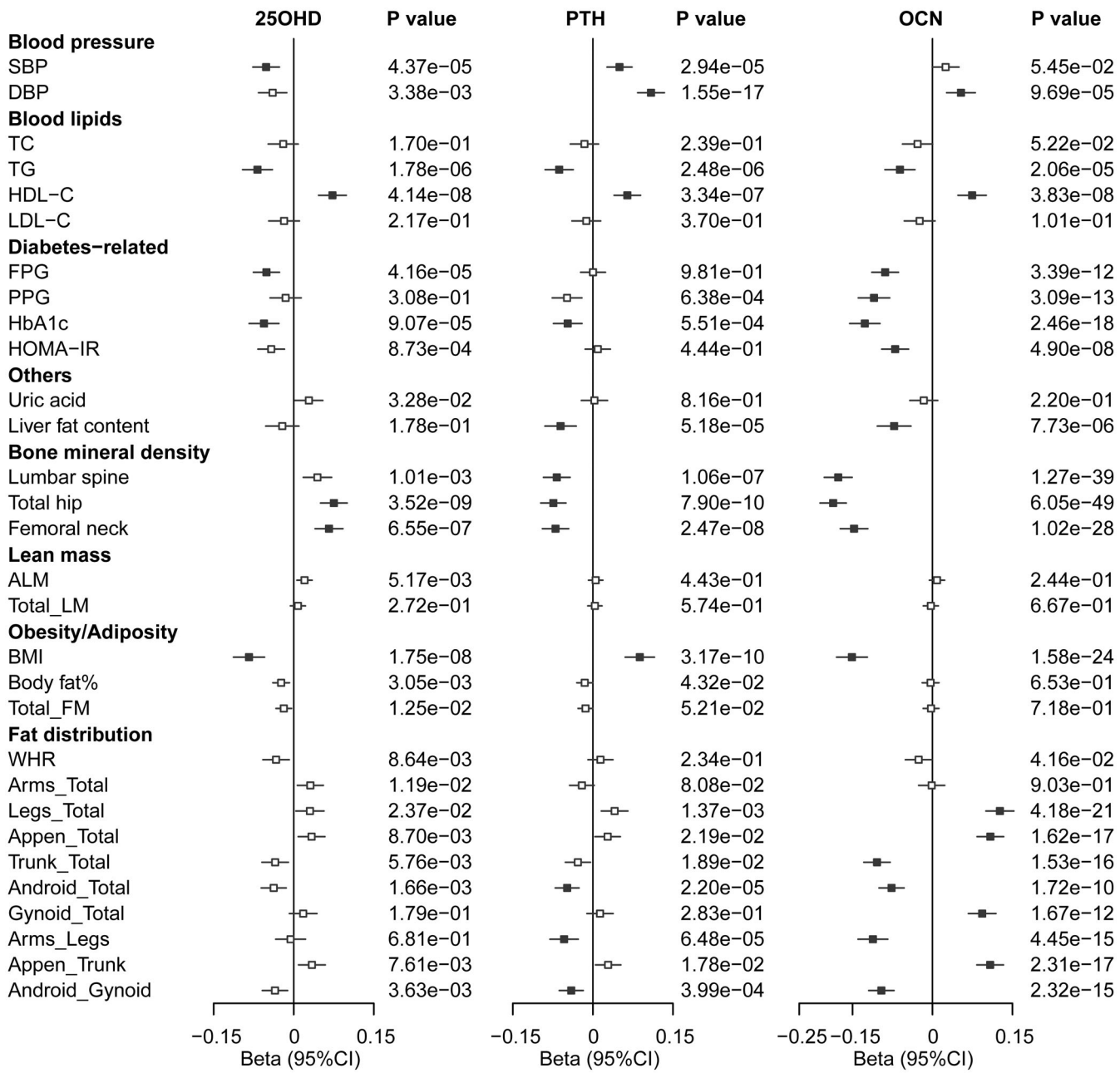

Fig 2. Linear associations between 25OHD, PTH, OCN, and other traits. Data are beta (95% confidence interval). Estimates indicate SD change of dependent variables associated with per SD increase of 25OHD (18.91 nmol/L), PTH (16.62 pg/mL), or OCN (7.32 ng/mL). Covariates adjusted for pairwise analyses are the same as those in partial Spearman correlations indicated in Supplemental Table S5. Statistical values and sample sizes of each pairwise analysis are presented in Supplemental Table S6. Solid squares represent p values $<5.56 \times 10^{-4}$ (0.05/90).

$p = 8.07 \times 10^{-2}$) is located in *PMF1* (polyamine modulated factor 1) or its paralog *PMF1-BGLAP* (PMF1-BGLAP readthrough) on chromosome 1q22 (Fig. 3*A*), which was associated with mosaic loss of chromosome Y (Table 2). The second was rs2062377 (discovery $p = 8.94 \times 10^{-9}$, replication $p = 3.55 \times 10^{-3}$) mapped to 8q24.12 and was previously reported as a genetic regulator of bone mineral density, sitting height, osteoporosis, alkaline phosphatase, and hemocytes (Table 2). It is in strong linkage disequilibrium (LD) with SNPs in the gene *TNFRSF11B* (TNF receptor superfamily member 11b) encoding osteoprotegerin (Fig. 3*B*), which is an osteoblast-secreted decoy receptor that specifically binds to its ligand, osteoprotegerin ligand, and functions as a negative regulator of bone resorption through inhibiting RANK/RANKL signaling and osteoclast development.[49] The two SNPs explained 1.98% of the variance in serum OCN levels.

Further adjusting for BMI in the GWAS did not change the findings (Supplemental Table S9), and results without adjusting for BMI were used in following analyses.

Lookup results of previously reported associations with 25OHD, PTH, and OCN are presented in Supplemental Notes.

Table 1. Multivariable Adjusted Odds Ratios of Metabolic Diseases for Per SD Increase of 25OHD, PTH, and OCN, Respectively

	25OHD			PTH			OCN		
	Cases/*N*	OR (95% CI)	*p* Value	Cases/*N*	OR (95% CI)	*p* Value	Cases/*N*	OR (95% CI)	*p* Value
Model 1									
Hypertension	2868/5137	0.91 (0.85–0.97)	5.50×10^{-3}	2852/5117	1.07 (1–1.14)	4.10×10^{-2}	2856/5127	1.02 (0.95–1.08)	6.58×10^{-1}
Dyslipidemia	2070/5131	0.88 (0.83–0.94)	$\mathbf{5.31 \times 10^{-5}}$	2062/5111	0.9 (0.85–0.95)	$\mathbf{3.30 \times 10^{-4}}$	2066/5121	0.85 (0.79–0.9)	$\mathbf{3.17 \times 10^{-7}}$
Hyperglycemia	2250/5098	0.89 (0.84–0.95)	$\mathbf{3.68 \times 10^{-4}}$	2241/5078	0.89 (0.84–0.95)	$\mathbf{2.12 \times 10^{-4}}$	2246/5088	0.74 (0.69–0.79)	$\mathbf{6.17 \times 10^{-19}}$
Hyperuricemia	975/5133	1.01 (0.94–1.09)	7.44×10^{-1}	963/5113	1.05 (0.98–1.13)	1.59×10^{-1}	966/5123	0.92 (0.85–0.99)	3.77×10^{-2}
Osteoporosis	633/4815	0.84 (0.75–0.93)	$\mathbf{1.10 \times 10^{-3}}$	619/4797	1.24 (1.13–1.36)	$\mathbf{5.03 \times 10^{-6}}$	624/4808	1.46 (1.33–1.61)	$\mathbf{2.33 \times 10^{-15}}$
NAFLD	953/3566	0.92 (0.84–1.01)	7.77×10^{-2}	955/3551	0.93 (0.86–1)	6.60×10^{-2}	956/3559	0.84 (0.76–0.91)	$\mathbf{9.48 \times 10^{-5}}$
Model 2									
Hypertension	2868/5137	0.9 (0.84–0.96)	$\mathbf{1.22 \times 10^{-3}}$	2852/5117	1.07 (1–1.14)	4.15×10^{-2}	2856/5127	1.02 (0.96–1.09)	5.13×10^{-1}
Dyslipidemia	2070/5131	0.88 (0.83–0.94)	$\mathbf{1.40 \times 10^{-4}}$	2062/5111	0.9 (0.85–0.95)	$\mathbf{2.48 \times 10^{-4}}$	2066/5121	0.84 (0.79–0.9)	$\mathbf{1.36 \times 10^{-7}}$
Hyperglycemia	2250/5098	0.89 (0.83–0.95)	$\mathbf{2.72 \times 10^{-4}}$	2241/5078	0.89 (0.84–0.95)	$\mathbf{2.22 \times 10^{-4}}$	2246/5088	0.74 (0.69–0.79)	$\mathbf{5.79 \times 10^{-19}}$
Hyperuricemia	975/5133	1 (0.93–1.08)	9.14×10^{-1}	963/5113	1.05 (0.98–1.13)	1.49×10^{-1}	966/5123	0.92 (0.85–1)	5.28×10^{-2}
Osteoporosis	633/4815	0.84 (0.76–0.94)	$\mathbf{1.71 \times 10^{-3}}$	619/4797	1.24 (1.13–1.36)	$\mathbf{5.70 \times 10^{-6}}$	624/4808	1.46 (1.33–1.61)	$\mathbf{3.40 \times 10^{-15}}$
NAFLD	953/3566	0.91 (0.83–1)	4.00×10^{-2}	955/3551	0.93 (0.86–1.01)	7.50×10^{-2}	956/3559	0.84 (0.77–0.92)	$\mathbf{1.37 \times 10^{-4}}$

25OHD = 25 hydroxyvitamin D; PTH = parathyroid hormone; OCN = osteocalcin; OR = odds ratio; CI = confidence interval; NAFLD = non-alcoholic fatty liver disease.
Model 1: Adjusted for age, sex, menopause (female), season (25OHD), and body mass index.
Model 2: Model 1 plus smoking and drinking.
Any *p* values < 0.0028 (0.05/18) are in bold.

Putative causal relationships between OCN and other traits

MR analysis was performed based on phenotypic analysis results, which showed significant and relatively strong associations between OCN and BMI, DBP, TG, HDL-C, HbA1c, BMD, and body fat distribution. The *F* statistics of the instrumental variables (43 for OCN, 55 for BMI, 51 for DBP, 169 for TG, 135 for HDL, 61 for HbA1c, 93 for T2DM_BBJ, and 139 for T2DM_EAS) were much greater than 10, indicating small possibility of weak instrumental variable bias.

We observed no significant causal effect of OCN on BMI, DBP, TG, or HDL, but the MR analyses had low statistical powers (Fig. 4). There was little evidence to provide any causal effect of OCN on HbA1c, T2DM_BBJ, or T2DM_EAS based on IVW MR with powers of 79%, 85%, and 100%, respectively. The IVW causal estimates were −0.27 (95% CI −0.74 to 0.20) with $p = 0.260$ for HbA1c, −0.12 (−0.55 to 0.30) with $p = 0.570$ for T2DM_BBJ, and −0.20 (−0.46 to 0.07) with $p = 0.143$ for T2DM_EAS (Fig. 4). We were unable to apply other MR methods because of the limited number of genetic instruments for OCN. However, single SNP MR by Wald ratio test showed that higher OCN concentration predicted by rs2842871 was significantly associated with lower HbA1c level (beta = −0.47 SD per unit increase of log(OCN), $p = 3.79 \times 10^{-4}$).

Results of bidirectional MR using OCN as the outcome and the powers are presented in Fig. 5. Each effect represents the estimated causal change of log(OCN) per SD change of BMI, DBP, TG, HDL-C, HbA1c, or per log-odds of T2DM. Statistical powers of the MR analyses were lower than 80% except for HbA1c, T2DM_BBJ, and T2DM_EAS. IVW MR estimates of assessing the causal effect of HbA1c on OCN was −0.09 (−0.16 to −0.01), $p = 0.028$. Cochran's Q test indicated heterogeneity ($p = 0.005$) and MR-Egger intercept indicated pleiotropy ($p = 0.030$). Outlier-corrected result from MR-PRESSO suggested no significant causal effect of HbA1c on OCN (Fig. 5). The five MR methods showed that T2DM_BBJ and T2DM_EAS had significant causal effects on reduced serum OCN concentration (IVW causal effect −0.03, 95% CI −0.05 to −0.01, $p = 0.006$ for T2DM_BBJ, and −0.03, 95% CI −0.05 to −0.01, $p = 0.001$ for T2DM_EAS). The scatter plots displaying the effects of the SNPs on both the exposure (eg, T2DM_BBJ or T2DM_EAS) and the outcome (eg, OCN) are presented in Fig. 6. Leave-one-out plots showed that there was no single SNP driving the causal links between T2DM and OCN (Supplemental Fig. S5). The intercept terms in MR-Egger regression models indicated no horizontal pleiotropy ($p = 0.960$ for T2DM_BBJ and $p = 0.125$ for T2DM_EAS). Cochran's *Q* test detected heterogeneous ratio estimates of T2DM_BBJ SNPs ($p = 0.036$), but MR-PRESSO detected no significant outliers at $p < 0.05$. Removal of the SNP with the least *p* value in MR-PRESSO outlier test (rs12507026, $p = 0.167$) reduced the heterogeneity (Cochran's *Q*, $p = 0.102$) and the IVW estimate remained consistent (−0.03, 95% CI −0.05 to −0.01, $p = 0.0080$).

Discussion

In this middle-aged and elderly Chinese population, we found that higher circulating OCN level was phenotypically and genetically associated with higher DBP and HDL-C and decreased TG, blood glucose level, HOMA-IR, liver fat content, BMD, BMI, and a favorable body fat distribution pattern; the associations were generally stronger than those of 25OHD and PTH. We then

Table 2. GWAS Results for Loci That Reached Genomewide Significance ($p < 5 \times 10^{-8}$) for 25OHD, PTH, and OCN

						Discovery				Replication						
Phenotype	Locus	SNP	Position	A1	A2	A1 freq	Beta	SE	*p* Value	A1 freq	Beta	SE	*p* Value	Nearest gene	Functional annotation	Reported traits[a]
25OHD	4q13.3	rs4588	72618323	T	G	0.325	−0.080	0.009	5.67×10^{-19}	0.304	−0.130	0.031	4.03×10^{-5}	*GC*	Missense	Serum 25-hydroxyvitamin D levels; vitamin D levels; white blood cell (leukocyte) count; neutrophil count; platelet crit; eosinophil cell count; sum basophil neutrophil counts; granulocyte count; lymphocyte cell count
PTH	5q35.3	rs35610898	176815766	C	G	0.267	−0.057	0.009	9.62×10^{-10}	0.253	−0.078	0.034	2.02×10^{-2}	*SLC34A1*	Intronic	Hematocrit; estimated glomerular filtration rate; hemoglobin concentration; red blood cell (erythrocyte) count; neutrophil count; lymphocyte percentage; eosinophil count; activated partial thromboplastin time; creatinine; cystatin C; phosphate; urea; urolithiasis; sitting height
OCN	1q22	rs2842871	156201168	G	A	0.157	0.082	0.01	4.35×10^{-17}	0.144	0.062	0.035	8.07×10^{-2}	*PMF1*; *PMF1-BGLAP*	Intronic	Mosaic loss of chromosome Y
OCN	8q24.12	rs2062377	120007420	T	A	0.265	−0.047	0.008	8.94×10^{-9}	0.266	−0.081	0.028	3.55×10^{-3}	*COLEC10*	Intronic	Bone mineral density; sitting height; eosinophil percentage; osteoporosis; alkaline phosphatase; monocyte cell count; neutrophil cell count; eosinophil percentage

GWAS = genomewide association study; 25OHD = 25 hydroxyvitamin D; PTH = parathyroid hormone; OCN = osteocalcin; SNP = single-nucleotide polymorphism.

[a]Significant associations ($p < 5 \times 10^{-8}$) with the SNP in NHGRI-EBI GWAS catalog (https://www.ebi.ac.uk/gwas/), GenATLAS (http://geneatlas.roslin.ed.ac.uk/), IEU GWAS database (https://gwas.mrcieu.ac.uk/), or PheWeb for BBJ (http://jenger.riken.jp/en/).

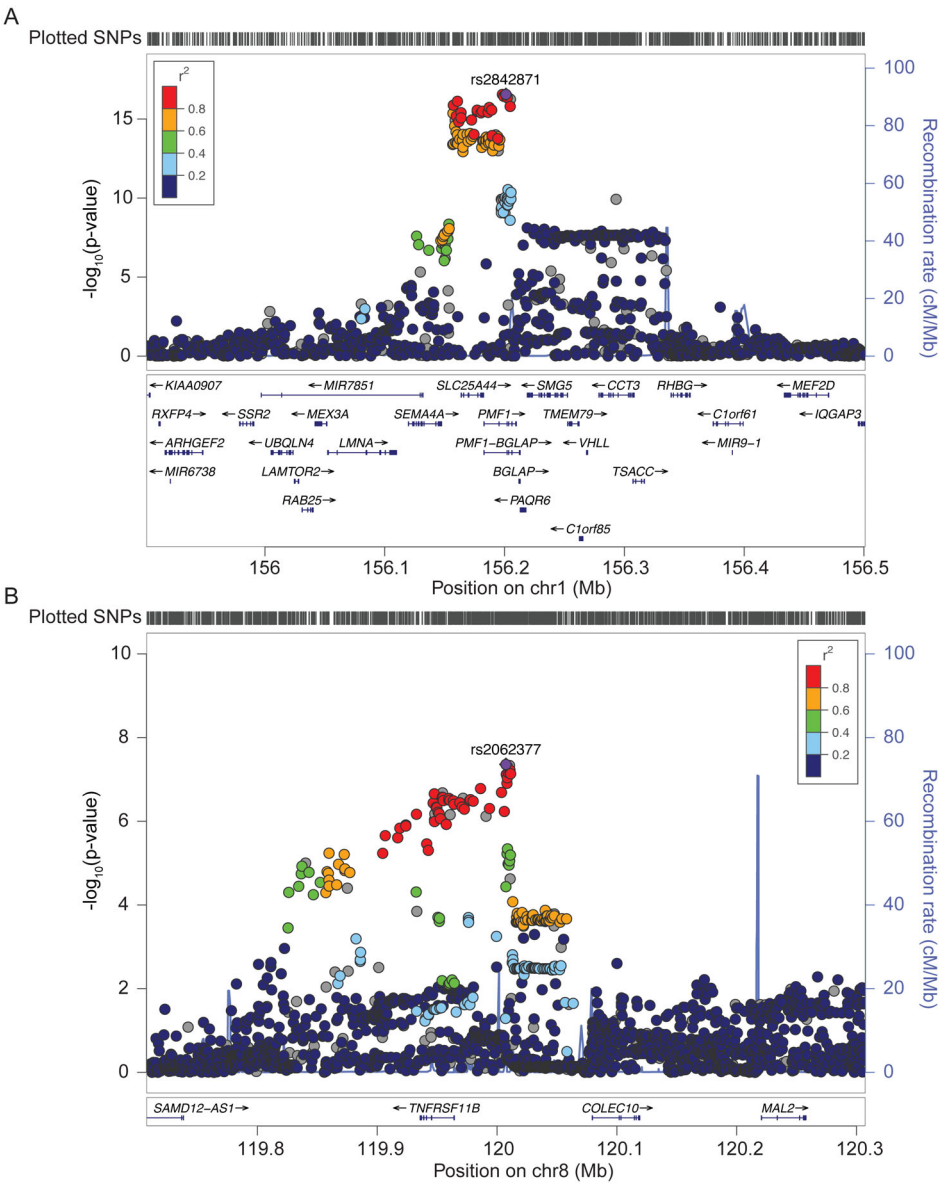

Fig 3. Regional association plots and ENCODE annotation of the two loci that reached genomewide significance ($p < 5 \times 10^{-8}$) in the GWAS of OCN levels. The *x* axis indicates the physical position of each SNP on the chromosome specified, and the *y* axis denotes the evidence of association shown as −log(*p* value). (*A*) The *PMF1-BGLAP* region. (*B*) The *TNFRSF11B* region.

performed GWAS of OCN and identified two genomewide significant loci (*PMF1-BGLAP* and near *TNFRSF11B*), explaining 1.98% of the variance of serum OCN. Bidirectional two-sample MR analysis revealed genetic evidence of a causal effect of T2DM on lower serum OCN level. Taken together, these findings provide a resource for understanding complex relationships between OCN and metabolic traits in humans.

As the first GWAS of 25OHD, PTH, and OCN in East Asian population, the present study validated the previously reported *GC* gene as a major genetic regulator of vitamin D status in middle-aged and elder population. *GC* encodes the vitamin D binding protein that binds 85% to 90% of total circulating 25OHD and its plasma metabolites and transport them to target tissues.[50] *SLC34A1* is a candidate gene for PTH concentration, as it encodes the kidney-specific sodium-phosphate type 2a transporter (Npt2a) responsible for phosphate reabsorption in the proximal tubule.[51] Locus *PMF1-BGLAP* represents naturally occurring read-through transcription between the neighboring *PMF1* and *BGLAP* genes on chromosome 1. While *PMF1* (and *PMF1-BGLAP*) was reported associated with mosaic loss of chromosome Y, ischemic stroke, intracerebral hemorrhage, white matter hyperintensity burden, appendicular lean mass, longevity, C-reactive protein levels, estimated glomerular filtration rate, and acute myeloid leukemia (NHGRI-EBI GWAS catalog—up to August 5, 2020), no significant associations with *BGLAP*, the OCN-encoding gene, had been reported.

Our MR analysis did not support a causal role of OCN on BMI, DBP, TG, HDL-C, HbA1c, or T2DM, but the statistical powers were

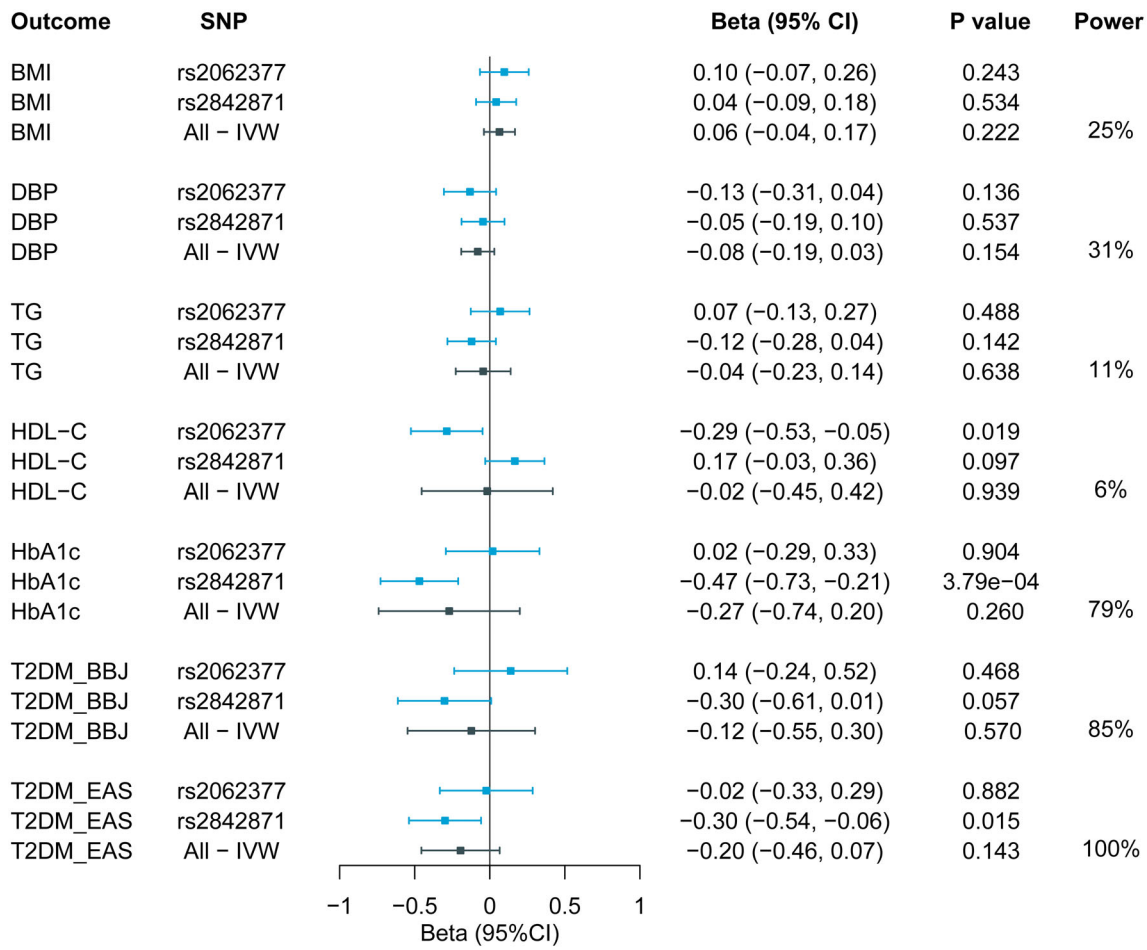

Fig 4. Forest plot displaying causal effect estimates of OCN on other traits. Estimates indicate SD causal change of BMI, DBP, TG, HDL-C, or HbA1c level or log-odds of T2DM associated with per unit increase of log(OCN). IVW = inverse-variance weighted.

below 75% except for HbA1c, T2DM_BBJ, and T2DM_EAS. To date, there is little data on whether decreased levels of OCN is a cause or effect of metabolic disorders in humans, with a few longitudinal studies focusing on glucose metabolism. A retrospective cohort study on 1229 nondiabetic middle-aged men observed baseline associations between higher serum OCN level and favorable metabolic phenotypes including BMI, body fat percentage, TG, HOMA-IR, and HDL-C levels, but no difference in fasting glucose and HbA1c levels. In addition, no statistical differences in the development of type 2 diabetes during an 8.4-year follow-up across the osteocalcin tertiles were evident after adjustment of other risk factors for incident diabetes.[13] Similarly, a nested case-control study on 833 incident diabetes cases and 802 nondiabetic control participants with a 10-year follow-up showed that serum OCN concentration was not associated with the risk of type 2 diabetes.[14] However, in another retrospective study including 1870 middle-aged Chinese with a follow-up period of 3 years, researchers found that low serum osteocalcin concentrations at baseline were independently related to an increased risk of incident type 2 diabetes.[12] Interestingly, both OCN and C-terminal cross-linked telopeptide of type I collagen (CTX), two highly correlated bone turnover biomarkers, were inversely correlated logitudinally with risk of diabetes in 1455 late-postmenopausal women, and CTX but not OCN was the dominant biomarker for this relationship (hazard ratio [HR] = 0.85 [95% CI 0.71–1.02], $p = 0.075$ per SD increment of OCN and HR = 0.82 [95% CI 0.69–0.98], $p = 0.031$ per SD increment of CTX).[11] These findings suggest that lower bone turnover and its underlying factors may be more relevant determinants of dysregulated glucose metabolism than the possible direct effects attributable to OCN. Nevertheless, no secondary analyses of randomized trials showed medications such as antiresoptive agents, warfarin, or vitamin K that lead to alterations in OCN levels had meaningful effects on any metabolic measurements. The conflicting results from different studies may be due to some unknown confounding factors.

Results from bidirectional MR analysis suggested that genetically predicted T2DM may reduce circulating OCN levels. Epidemiological studies have demonstrated that patients with T2DM have increased risk of fracture despite having normal or even high BMD, and MR studies have also found causal effects of both T2DM and increased fasting glucose on higher BMD.[52] Bone is a dynamic tissue, and BMD is a reflection of the balance between bone formation and bone resorption. Hyperglycemia with accumulation of advanced glycation end products in bone matrix has been shown experimentally to inhibit differentiation and

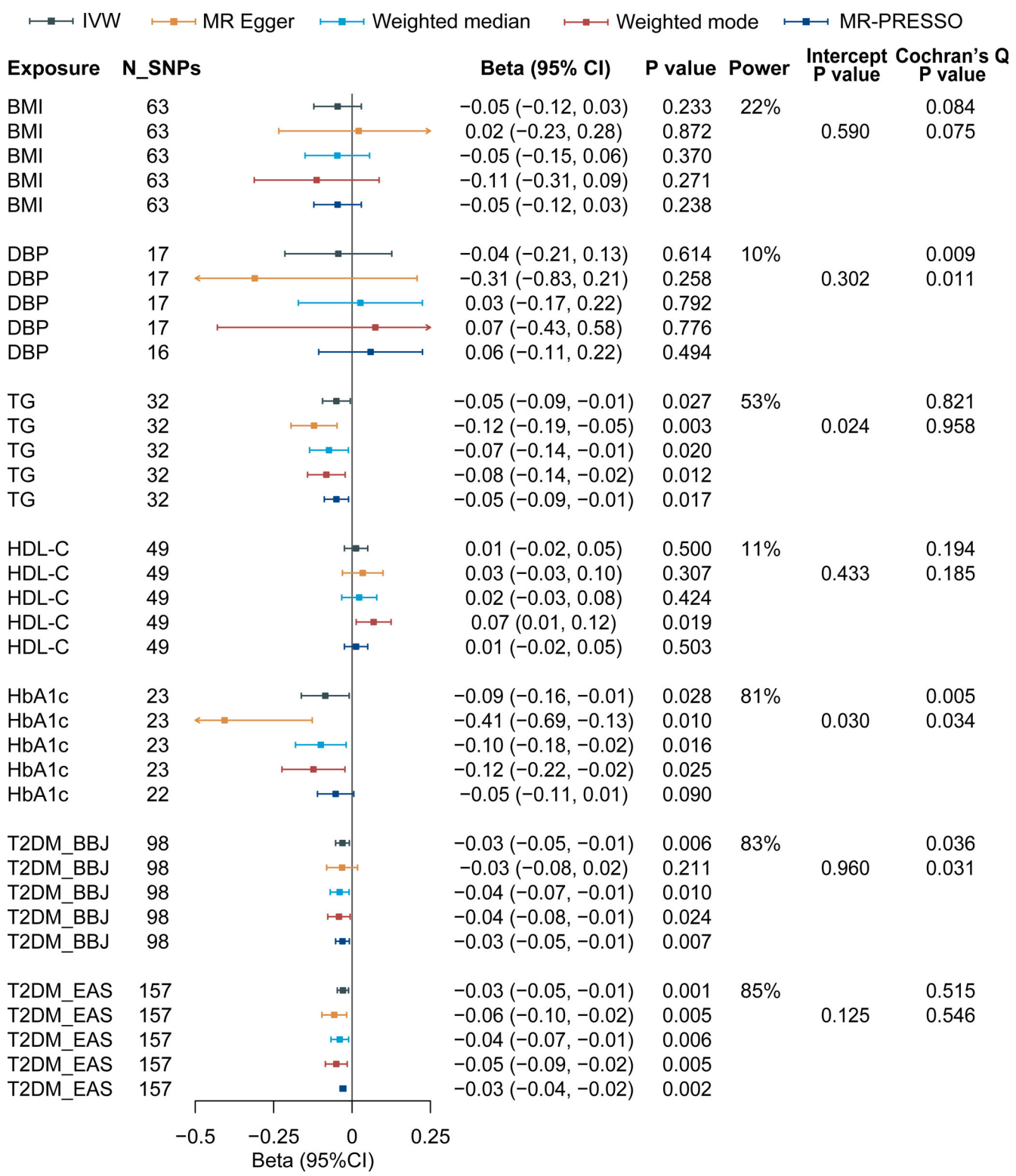

Fig 5. Forest plot displaying causal effect estimates of other traits on OCN. Each effect represents the estimated causal change of log(OCN) per SD change of BMI, DBP, TG, HDL-C, HbA1c, or per log-odds of T2DM. IVW = inverse-variance weighted. N_SNPs = number of SNPs used as genetic instruments in MR analyses.

stimulate apoptosis of osteoblasts,[53] which are the main resource of OCN production. In addition, patients with T2DM are known to have impaired bone formation and reduced bone remodeling reflected by decreased serum procollagen type 1 amino terminal propeptide (P1NP) and CTX and increased sclerostin and osteoprotegerin;[54] hence, the weakend acidic environment may contribute to reduced decarboxylation of OCN and its release into circulation. Besides, we also found that

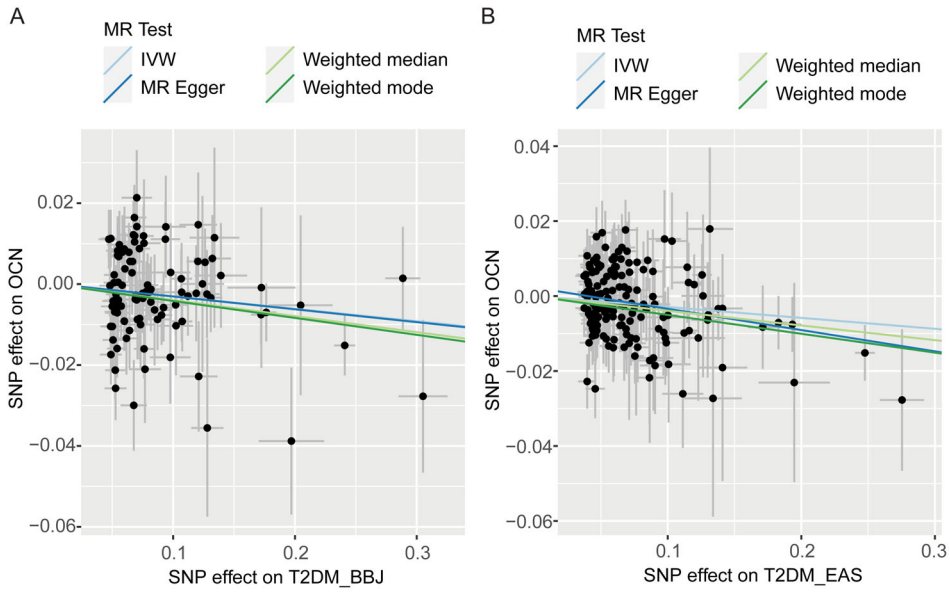

Fig 6. Scatter plots for MR analyses of the causal effect of T2DM_BBJ (*A*) and T2DM_EAS (*B*) on OCN. IVW = inverse-variance weighted.

genetically predicted HbA1c in individuals without diabetes had insignificant causal effects on OCN levels. Taken together, decreased bone turnover and remodeling may be pivotal in diabetic bones, which impair the renewal of aging osteocytes, leading to higher BMD but defective bone strength and quality and therefore bone fragility and fracture.

The cross-sectional associations between the three bone markers and lower TG and higher HDL-C but not TC or LDL-C, after accounting for potential confounding variables including BMI, indicated a specific lipid-bone connection. MR studies on causality of lipid profile and BMD or fractures in recent years reported very contradictory findings.[55–57] Our MR analyses on TG, HDL-C, and OCN unfortunately suffered from low statistical power, and further investigations by larger prospective or MR studies or clinical trials are needed.

Strengths of this study include revealing phenotypic and genetic associations between the three bone-related biomarkers and a wide range of metabolic and intermediate traits, giving first insights into genetic regulations of serum 25OHD, PTH, and OCN levels in East Asian population and providing the first human genetic evidence on the causal effect of T2DM on circulating OCN levels. The major limitation is that the sample size of our GWAS was relatively small, and only two top SNPs were used as genetic instruments for OCN, which led to insufficient power in bidirectional MR analyses with BMI, DBP, TG, and HDL-C. Therefore, we focused on results about HbA1c and T2DM with reasonable power. Secondary, similar to some other studies,[10,11] the present study analyzed total OCN, as concentrations of carboxylated and decarboxylated OCN were not available in Changfeng population. However, in our recently published study, serum total OCN was highly correlated with uncarboxylated OCN ($r = 0.528$, $p < 0.001$).[58] Lastly, the present study was conducted in East Asian populations, and the findings may not be applicable to other ethnicities. In addition, we were not able to perform MR analyses on other traits including 25OHD, PTH, BMD, and fat distribution because of a paucity of large GWAS results in East Asian population.

In summary, serum OCN was cross-sectionally associated with a wide range of metabolic traits. Rs2842871 in *PMF1-BGLAP* and rs2062377 near *TNFRSF11B* were significant genetic regulators of serum OCN levels, explaining 1.98% of the variance of serum OCN. Evidence from MR analysis suggested causal effect of T2DM on reduced circulating OCN concentration, indicating decreased bone turnover and remodeling in patients with T2DM.

Disclosures

All authors state that they have no conflicts of interest.

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Authors' roles: XG, SW, and HL designed the study, interpreted the data, and revised the manuscript. HL, HM, LC, MX, and BP collected the data. HZ analyzed, interpreted the data, and drafted the manuscript. JG and WX analyzed the data and revised the manuscript. All authors approved the final version.

Author contributions

Hailuan Zeng: Conceptualization; formal analysis; methodology; resources; software; visualization; writing-original draft; writing-review & editing. **Jieyu Ge:** Formal analysis; methodology; software; visualization; writing-review & editing. **Wenjie Xu:** Formal analysis; methodology; software; visualization; writing-review & editing.

Hui Ma: Data curation; resources. **Lingyan Chen:** Data curation; resources. **Mingfeng Xia:** Data curation; resources. **Baishen Pan:** Data curation; resources. **Huandong Lin:** Conceptualization; data curation; investigation; project administration; resources; writing-review & editing. **Sijia Wang:** Conceptualization; funding acquisition; methodology; project administration; resources; software; supervision; writing-review & editing. **Xin Gao:** Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; resources; supervision; writing-review & editing.

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