Positive effects of low LDL-C and statins on bone mineral density: an integrated epidemiological observation analysis and Mendelian Randomization study

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Key Messages

- Genetically predicted decrease in blood LDL-C levels was associated with increase in total-body BMD (TB-BMD) and estimated BMD (eBMD), which was comparable with the results of epidemiological observation analyses conducted in two independent epidemiological cohorts.
- TB-BMD played a negative causal role on the variation of blood LDL-C level.
- Reduced blood LDL-C level caused by genetic proxies of statin therapy was associated with increase in TB-BMD and eBMD; while null causation was observed between other LDL-C-lowering drugs and BMD.
- There was insufficient evidence to demonstrate BMD was causally associated with coronary artery disease.

Abstract

Background

Low-density lipoprotein cholesterol (LDL-C) is suggested to play a role in osteoporosis but its association with bone metabolism remains unclear. Effects of LDL-C-lowering drugs on bone are also controversial. We aim to determine whether LDL-C is linked causally to BMD and assess the effects of LDL-C-lowering drugs on BMD.

Methods

Association between blood lipid levels and BMD was examined by epidemiological observation analyses in US representative cohort NHANES III (N=3,638) and Hong Kong Osteoporosis Study (HKOS; N=1,128). Two-sample Mendelian Randomization (MR), employing genetic data from GWAS of blood lipids (N=188,577), total body BMD (TB-BMD) (N=66,628) and estimated BMD (eBMD) (N=142,487), was performed to infer causality between LDL-C and BMD. Genetic proxies for LDL-C-lowering drugs were used to examine the drugs' effects on BMD.

Results

In NHANES III cohort, each SD decrease in LDL-C was associated with 0.045 SD increase in femoral neck BMD (95% CI: 0.009 to 0.081; P=0.015). A similar increase in BMD was observed in HKOS at femoral neck and lumbar spine. In MR analysis, decrease in genetically predicted LDL-C was associated with increase in TB-BMD [estimate per SD decrease, 0.038 (95% CI: 0.002 to 0.074); P=0.038] and eBMD [0.076 (0.042 to 0.111); $P=1.20x10^{-5}$]. Reduction of TB-BMD was causally associated with increased LDL-C [0.035 (0.033 to

0.066); P=0.034]. Statins' LDL-C-lowering proxies were associated with increased TB-BMD [0.18 (0.044 to 0.316); P=9.600×10^{-3}] and eBMD [0.143 (0.062 to 0.223); P=5.165×10^{-4}].

Conclusions

Negative causal association exists between LDL-C level and BMD. Statins' LDL-C-lowering effect increases BMD, suggesting its protective effect on bone.

Keywords: LDL-C, statins, bone mineral density, fracture, coronary artery disease, mendelian randomization

Medical Subject Headings (MeSH)

Mendelian Randomization Analysis
Bone Density
Hydroxymethylglutaryl-CoA Reductase Inhibitors
Lipoproteins, LDL
Fractures, Bone
Coronary Artery Disease

Introduction

Osteoporosis and atherosclerosis are two major causes of morbidity and mortality. Emerging evidences have suggested a link between osteoporosis and coronary artery disease (CAD), and lipid metabolism was involved in the progression of both diseases(1). Low-density lipoprotein cholesterol (LDL-C) is a well-known causal factor for atherosclerotic cardiovascular disease(2). Other than this, the causal relationship among bone mineral density (BMD), CAD and LDL-C is still largely unknown.

The relationship between LDL-C and BMD has been investigated by different studies but the results remained inconclusive. Positive(3) (4), null(5) (6) and inverse(7-10) associations between LDL-C and BMD were reported. Similar inconsistent findings are also observed for the association between BMD and CAD. A recent meta-analysis demonstrated that individuals with low BMD had an increased risk of developing cardiovascular disease and CAD(11). Yet, a Mendelian Randomization (MR) study showed that one standard deviation (SD) increase in genetically predicted estimated BMD (eBMD) was associated with 5% higher risk of CAD(12).

LDL-C has been a key target for CAD interventions and statins are one of the most commonly used LDL-C-lowering drug classes for treating CAD. Besides CAD, statins were also suggested to influence bone health though the evidence was inconsistent(13-15). Another LDL-C-lowering drug, ezetimibe, showed an inverse association with BMD, although the

association was not statistically significant(16). While different levels of benefits and adverse effects were observed for different LDL-C-lowering drugs(17), their effects on bone health have yet been investigated.

According to Mendel's laws of inheritance, genetic variants are inherited randomly and exert a life-long effect on phenotypes. They could be used as instrumental variables to infer causality which is free of biases from confounding and reverse causation commonly found in observational studies. This approach is known as MR. Univariable MR has several key assumptions (Figure 1). A well-conducted MR study should provide reliable evidence that is comparable to those provided by randomized clinical trials (RCTs). The reliability could be further improved by cross-validation with evidence from other study designs(18).

Due to the inconsistent findings of previous observational studies, we firstly tested the association between LDL-C and BMD in two independent observational cohorts with distinct genetic compositions: the third National Health and Nutrition Examination Survey (NHANES III) and the Hong Kong Osteoporosis Study (HKOS) in the present study. Next, we determined genetic correlation and inferred causality between different traits using MR approach. We further tested if multiple LDL-C-lowering drugs (represented by genetic proxy) were associated with BMD variation.

Materials and Methods

Epidemiological observation analyses

Two independent cohorts of different genetic dispositions (NHANES III and HKOS) were employed in the epidemiological observational studies. The participants included in this study were described in Supplementary Methods. For the analyses in the NHANES and HKOS, blood lipid levels and BMD were standardized with a mean of 0 and standard deviation (SD) of 1. The relationship between the blood lipid traits (including LDL-C, HDL-C and triglycerides) and BMD was evaluated using multivariable linear regression with adjustment for age, sex, ethnicity/race, height, weight, serum LDL-C levels (for the analysis with serum HDL-C and triglycerides levels), serum HDL-C levels (for the analysis with serum LDL-C and triglycerides levels), and serum triglyceride levels (for the analysis with serum HDL-C and LDL-C levels). Serum lipid levels were adjusted as covariates to avoid their potential pleiotropy with the lipid under investigation. For the NHANES analysis, sample weights that account for the unequal probabilities of selection, oversampling, and non-response were applied for all analyses using complex sampling module in SPSS version 22.0 software (SPSS Inc, Chicago, IL). All values presented were weighted to represent the U.S. civilian population. All statistical analyses were conducted using R or SPSS.

Data sources for estimation of genetic correlation and MR

Summary statistics from large-scale genome-wide association study (GWAS) or meta-analysis of GWAS were used for both estimation of genetic correlation and MR. Two BMD

phenotypes were assessed: total body BMD (TB-BMD) and eBMD at heel calcaneus as differences might exist between the two(9). In addition to the difference in skeletal sites measured, TB-BMD is measured by dual-energy X-ray absorptiometry (DXA), the gold-standard method of BMD measurement while eBMD is estimated by quantitative ultrasound. Summary statistics for TB-BMD was obtained from the currently largest GWAS of DXA-derived BMD: a GWAS meta-analysis of 66,628 individuals from populations across America, Europe and Australia(19) publicly available from GeFOS. Summary statistics for eBMD was obtained from a recent GWAS conducted in 142,487 participants of primarily European ancestry from UK Biobank, which was publicly available through the GEnetic Factors for OSteoporosis Consortium (GeFOS)(9). While summary-level data obtained for lipids(20), fracture(21) and CAD(22) were used in estimation of genetic correlation and evaluation of causal association, summary statistics of diabetes(23) and BMI(24) were utilized as confounding factors in MR analyses. Details of the data sources were provided in Supplementary Methods.

Estimation of genetic correlation

LD score regression, which requires only GWAS summary statistics instead of individual-level data(25, 26), was employed to estimate the genetic correlation among LDL-C, TB-BMD, eBMD, fracture and CAD. Pre-computed LD scores suitable for European-ancestry samples and Python command line tool (http://github.com/bulik/ldsc) were adopted.

Study design for MR and MR analyses

The current two-sample MR study utilized publicly available summary statistics from GWAS or GWAS meta-analysis: the instrument-risk factor and instrument-outcome associations were obtained from two different sets of participants (Figure 2). In brief, summary statistics of SNPs significantly associated with the risk factors (genome-wide significance: $p<5x10^{-8}$) were extracted from the confounding and outcome datasets. If the SNPs were not included in the datasets, proxies for the missing SNPs ($r^2>0.8$) were identified from all the risk factors, confounding and outcome datasets. The SNPs were excluded from MR analyses if proxies were not identified. Selection and summary statistics of genetic instruments for each MR analysis was provided in Supplementary Methods, and Supplementary Tables 1 to 10. Inverse-variance weighted (IVW)(27) and multivariable IVW methods(28) were used for main MR analysis. Weighted median(29) and MR-Egger(30) were used for sensitivity analysis. Genetic variants may affect the outcome independent of the risk factor (known as pleiotropy) and this violates the third assumption of MR analysis (Figure 1). Although MR-Egger is able to detect bias arising from pleiotropy, we conducted an additional sensitivity analysis for the observed causality by repeating the MR analyses with exclusion of genetic instruments which were associated with potential confounders via the web-interfaced PhenoScanner, a curated database of publicly available GWAS(31). All MR analyses were conducted using the 'MendelianRandomization' package in R(32). Detailed description of MR analyses was included in Supplementary Methods. mRnd (http://cnsgenomics.com/shiny/mRnd/)(33), an online web tool was employed to perform

power calculation in our MR study. Power calculation and strength of genetic instruments are presented in Table 1.

Drug target analysis using genetic proxy

Lotta *et al*. made use of LDL-C-lowering variants in or near genes encoding molecular targets of current or prospective LDL-C-lowering therapies as genetic proxies to study the efficacy of drugs on type 2 diabetes(34). These genes included Niemann-Pick C1-Like 1 (*NPC1L1*) targeted by ezetimbe, 3-hydroxy-3-methylglutaryl-coenzyme A (*HMG-CoA*) reductase (*HMGCR*) targeted by statins, proprotein convertase subtilsin-kexin type 9 (*PCSK9*) targeted by Evolocumab and Alirocumab, the ATP-binding cassette subfamily G member 5 (*ABC5/ABC8*) targeted by bile acid sequestrants, and a prospective drug target low-density lipoprotein receptor (*LDLR*). In this study, we employed the same sets of SNPs as genetic instruments to examine if the LDL-C-lowering effects of the drugs would have causal association with TB-BMD and eBMD. Summary statistics were extracted from GLSC's GWAS meta-analysis(35), TB-BMD GWAS meta-analysis(19) and eBMD GWAS(9) (Supplementary Table 5).

Results

Epidemiological observational analyses – Blood lipids and BMD

The association between serum lipids and BMD was evaluated in two epidemiological cohorts: the NHANES III and the HKOS. Demographic characteristics of participants from the two cohorts were shown in Table 2. The association between LDL-C and BMD was reported in Table 3. In the NHANES III cohort, each SD decrease of LDL-C was associated with 0.045 SD increase in BMD at femoral neck (95% CI: 0.009 to 0.081; P=0.015), after adjusting for age, sex, ethnicity/race, height, weight, serum HDL-C and triglyceride levels. A similar increase (0.039 SD) in BMD at femoral neck was also observed in the HKOS cohort but with a wider 95% CI (95% CI: -0.011 to 0.089; P=0.123). At lumbar spine, each SD decrease in LDL-C was associated with 0.083 SD increase in BMD (95% CI: 0.03 to 0.135; P=0.002). For HDL-C and triglycerides, no evidence of association was observed (Supplementary Tables 11 and 12 respectively).

Genetic correlation

Genetic correlation among the studied traits were shown in Table 4. Moderate and positive genetic correlation was observed between TB-BMD and eBMD (r=0.59; 95% CI: 0.555 to 0.628; P= 5.47×10^{-59}). Weak and inverse genetic correlation was observed between LDL-C and both BMD phenotypes (TB-BMD: r=-0.079; 95% CI: -0.107 to -0.0519; P=0.0038; eBMD: r=-0.082; 95% CI: -0.1045 to -0.0597; P=0.0003). In addition, eBMD was weakly correlated with HDL-C (r=-0.0724; 95% CI: -0.0949 to -0.0499; P=0.0013) and CAD (r=0.0669; 95% CI: 0.0416 to 0.0922; P=0.0082). None of the remaining trait pairs had evidence of genetic correlation.

MR analyses – blood lipids, TB-BMD and eBMD

The causal association between blood lipids and BMD were examined by MR. Primary analysis was done for LDL-C (Table 3). In IVW analysis, 1 SD decrease in genetically predicted blood LDL-C level was associated with 0.038 SD (95% CI: 0.002 to 0.074; P=0.038) increase in TB-BMD in IVW analysis (Supplementary Figure 1a). Similar estimate with a wider confidence interval was obtained for multivariable MR analysis adjusting for blood HDL-C and triglycerides levels, which are highly correlated with LDL-C. The result was no longer significant in weighted median (Estimate: 0.018; 95% CI: -0.026 to 0.063; P=0.416) and MR-Egger (Estimate: 0.011; 95% CI: -0.045 to 0.068; P=0.694) analysis. As GWAS meta-analysis of TB-BMD was also performed across five age stratum, MR was conducted in age-stratified manner (Supplementary Table 13).

Meanwhile, each SD decrease in genetically predicted blood LDL-C level was associated with 0.076 SD (95% CI: 0.042 to 0.111; $P=1.20x10^{-5}$) increase in eBMD (Supplementary Figure 1b). Similar estimate was obtained in multivariable IVW model adjusting for beta estimates of HDL-C and triglycerides, as well as in weighted median (0.065 SD increase in eBMD per SD decrease in LDL-C; 95% CI: 0.04 to 0.09; $P=3.59x10^{-7}$) and MR-Egger method (0.052 SD increase in eBMD per SD decrease in LDL-C; 95% CI: -0.002 to 0.106;

P=0.059). There was no evidence of pleiotropy for both phenotypes (MR-Egger intercept: 0.002; P=0.235 for TB-BMD; 0.002; P=0.257 for eBMD).

Based on the web-based PhenoScanner, eight of the genetic instruments (rs10401969, rs1535, rs1800961, rs2000999, rs579459, rs653178, rs6859 and rs7703051) were associated with potential confounder (BMI or / and Type II diabetes) in large-scale GWAS conducted by representative consortiums and they were excluded as an additional sensitivity analysis. For TB-BMD, similar results were obtained for univariable (0.038 SD increase in TB-BMD per SD decrease in LDL-C; 95% CI: -1.7×10^{-4} to 0.076; P=0.051) and multivariable (0.036 SD increase in TB-BMD per SD decrease in LDL-C; 95% CI: -0.004 to 0.075; P=0.077) IVW analyses. For eBMD, causal association was still observed in univariable (0.063 SD increase in eBMD per SD decrease in LDL-C; 95% CI: 0.027 to 0.999; $P=5.78 \times 10^{-4}$) and multivariable (0.067 SD increase in eBMD per SD decrease in LDL-C; 95% CI: 0.029 to 0.105; P=0.001) IVW analysis. Weighted median method also resulted in causal association with wider confidence intervals (0.034 SD increase in eBMD per SD decrease in LDL-C; 95% CI: 0.007 to 0.060; P=0.012).

Reverse causation of BMD on LDL-C was also tested (Table 5). Each SD decrease in TB-BMD was associated with 0.035 SD increase in LDL-C (95% CI: 0.003 to 0.066; P=0.034) in univariable IVW analysis (Supplementary Figure 2a). Similar estimate with a wider confidence interval was obtained from weighted median method (0.043 SD increase in LDL-

C per SD decrease in TB-BMD; 95% CI: -0.001 to 0.087; P=0.055) but insignificant association was suggested by MR-Egger method (Estimate: 0.004; 95% CI: -0.087 to 0.095; P=0.927). There were no signs of pleiotropy (MR-Egger intercept: 0.002; P=0.486). The causal association remained significant in multivariable IVW analysis adjusting for HDL-C and triglycerides (0.037 SD increase in LDL-C per SD decrease in TB-BMD; 95% CI: 0.011 to 0.063; P=0.006). Out of the 68 genetic instruments, none were found to be associated with potential confounding factors (BMI or Type II diabetes) in large GWAS conducted by representative consortiums so further sensitivity analysis was not performed. No reverse causation of eBMD on LDL-C was detected (Supplementary Figure 2b).

For HDL-C, no evidence of association was observed with both BMD phenotypes (Supplementary Table 11 and Supplementary Figure 3). For triglycerides (Supplementary Table 12 and Supplementary Figure 4), univariable IVW analysis showed that 1 SD decrease was causally associated with 0.064 SD increase in TB-BMD (95% CI: 0.011 to 0.118; P=0.019). The causal association remained significant in multivariable IVW analyses adjusting for beta estimates of LDL-C and HDL-C (Estimate: 0.084; 95% CI: 0.023 to 0.144; P=0.007). Thirteen genetic instruments (rs10401969, rs1260326, rs1515110, rs1535, rs3741414, rs4921914, rs687339, rs749671, rs7607980, rs7897379, rs9686661, rs9693857 and rs998584) were associated with BMI or / and Type-II diabetes based on web utility PhenoScanner. They were excluded in the sensitivity analysis. The causal association (0.037 SD increase of TB-BMD per SD decrease of triglycerides; 95% CI: -0.018 to 0.092; P=0.187)

was no longer significant in the sensitivity analysis. There was null causal association between triglycerides and eBMD.

Drug target analysis using genetic proxies of LDL-C-lowering drugs

The effects of LDL-C-lowering drugs on BMD were examined by IVW analysis of their genetic proxies (Table 6). Statistical significance was detected for statins' proxies where 1 SD decrease in genetically predicted decrease in LDL-C was associated with 0.18 SD increase in TB-BMD (95% CI: 0.044 to 0.316; $P=9.600x10^{-3}$). Similar association was observed for eBMD (estimate: 0.143; 95% CI: 0.062 to 0.223; $P=5.165x10^{-4}$). Genetic proxies for other LDL-C-lowering drugs did not show causal association with TB-BMD and eBMD.

MR and drug target analysis – LDL-C and fracture

Causal association between LDL-C and fracture was evaluated. Null association was observed in univariable and multivariable MR analyses adjusted for HDL-C, triglycerides and eBMD (Supplementary Table 14 and Supplementary Figure 5). In view of statins' LDL-C-lowering effects on increased BMD, drug target analysis was also performed on fracture. Null causal association was detected for genetic proxies of statin therapy on fracture (Table 6).

MR analysis –TB-BMD, eBMD and CAD

The effects of TB-BMD and eBMD on CAD were assessed by univariable and multivariable MR adjusted for blood lipid levels, diabetes and BMI (Table 7 and Supplementary Figure 6). Null association was observed for TB-BMD on CAD. For eBMD, 1 SD decrease was associated with 5.1% reduced risk of CAD (odds ratio: 0.949; 95% CI: 0.903 to 0.998; P=0.042) in univariable IVW analysis. However, there was no evidence of association in sensitivity analyses, and after taking risk factors (including blood LDL-C, HDL-C and triglycerides levels, diabetes and BMI) into account in multivariable analysis. Reverse causation of CAD on BMD was also tested but no evidence of association was observed (Supplementary Table 15 and Supplementary Figure 7).

Discussion

This study confirmed the association between LDL-C and BMD in two independent cohorts in epidemiological observation analyses and provides high-level evidence that a decrease in blood LDL-C level was causally associated with increased TB-BMD and eBMD. Whereas, decrease in TB-BMD, but not eBMD, was causally associated with increased LDL-C levels. The estimates derived from MR analyses were in line with the results from epidemiological observation analyses. MR analyses using genetic proxies of statin therapy consistently demonstrated that statins' LDL-C-lowering effect was causally associated with increased TB-BMD and eBMD. We also examined the causal association between LDL-C and fracture,

but null association was observed. There was insufficient evidence to prove the causation of BMD on CAD.

Our LD score regression analysis demonstrated moderate and positive genetic correlation between TB-BMD and eBMD (r=0.59; 95% CI: 0.555 to 0.628; P= 5.47 x 10^{-59}). TB-BMD and eBMD are measured at different skeletal sites. eBMD is a quick and relatively inexpensive estimate of BMD, but it is not a proxy of DXA-measured BMD, which is the gold standard in the clinical management of osteoporosis. Discordant results between eBMD and DXA-measured BMD are often observed(36). Notably, Kemp *et al*. reported that six eBMD-associated loci had opposite directions of effects when compared with GWAS of DXA-measured BMD(9). DXA-measured BMD at sites prone to fracture (femoral neck and lumbar spine) has strong and positive genetic correlation with TB-BMD (r>0.9)(19) but is just moderately correlated with eBMD (r=0.5–0.6)(9). eBMD measurement does not include the most critical and valuable diagnostic skeletal sites at lumbar spine or femoral neck. Only a minority of quantitative ultrasound devices for measurement of eBMD at calcaneus heel have been scientifically validated by clinical studies(37). Another limitation of quantitative ultrasound measurement is the absence of standard definition of osteoporosis(36). In predicting hip fracture, eBMD is a much weaker predictor than femoral neck BMD(38). DXA-measured TB-BMD is therefore more clinically relevant in diagnosis of osteoporosis than eBMD. We investigated TB-BMD and eBMD as two different phenotypes in MR analyses. Importantly, LDL-C was shown to be genetically correlated with both TB-BMD

and eBMD, suggesting that LDL-C level and BMD variation may have shared genetic etiology. It also indicates that causality between LDL-C and bone metabolism may exist.

There has been controversy on the role of LDL-C in bone metabolism. Previous observational studies showed inconsistent association between LDL-C and BMD (3-7, 10), which may arise from unmeasured confounding and presence of reverse causation. As MR better accounts for these biases, our study, which cross-validated results of epidemiological observation analyses and MR, should provide a more reliable casual inference that reduction in LDL-C level is associated with increased BMD. It is worth-noting that power for the MR analysis of LDL-C on age-stratified TB-BMD is below 40% due to the small sample size in sub-groups (Table 1). The null association in sub-group analyses is likely attributed to inadequate power. Causal linkage between LDL-C level and life-course TB-BMD should be re-visited when future GWAS with larger age-stratified subgroups becomes available. In addition, our bi-directional MR analysis suggested that TB-BMD, but not eBMD, played a negative causal role in blood LDL-C level. Taken together, our study suggests a positive feedback loop between bone and lipid metabolism. This reinforces the role of bone in feedback control of energy homeostasis, and the concept of mutual regulation of bone and energy metabolism(39).

Three recent studies suggested that blood LDL-C level had a negative causal association with eBMD variation, supporting part of our findings(40-42). The most important difference that

distinguishes the current study from the three studies is that all of them investigated the eBMD at heel only but not DXA-derived BMD, which is more relevant to the clinical diagnosis of osteoporosis. As aforementioned, intrinsic difference exists between eBMD and the gold-standard DXA-derived BMD, and eBMD is only modestly correlated with DXA-derived BMD (r=0.5-0.6)(9). Thus, cautious interpretation is required to claim the causal association between LDL-C and BMD by using eBMD alone as the outcome in MR analyses. Based on both eBMD and DXA-derived TB-BMD in bi-directional MR analysis, we suggested positive feedback loop exists between bone and lipid metabolism. The feedback loop feature was not observed in studies using eBMD as the phenotype due to the difference between eBMD and TB-BMD. Among the three studies, the first study conducted by O'Connor *et al* developed a latent causal variable (LCV) model to identify causal relationships among genetically correlated pairs of complex traits. They detected a negative genetically causal effect of LDL-C on eBMD ($p=7x10^{-34}$)(40). Unlike our multivariable MR analysis, LCV can only model two traits at a time without conditioning the effects of confounders(40) so they could not provide evidence that the causal association of LDL-C on eBMD might be mediated by other risk factors, such as HDL-C and triglycerides. The second study conducted by Cherny *et al* used univariable MR approach to examine the bi-directional causal association between LDL-C and eBMD at heel(41). Similar to our findings for eBMD, they found that LDL-C was causally associated with eBMD (both total, left and right heel) but null association was observed for the reverse direction. However, their univariable MR approach could not assess the role of the highly correlated HDL-C or triglycerides in LDL-C's causality on eBMD. In addition to the multivariable MR analysis adjusting for beta

estimates of HDL-C and triglycerides, we also performed a sensitivity analysis by excluding pleiotropic genetic instruments which might influence BMD variation through pathways other than LDL-C. The third study conducted by Zheng *et al* merely made use of bi-directional MR approach to investigate the causal association between plasma lipids and eBMD(42). They also repeated the MR analysis with and without genetic instruments located in statins' target gene, *HMGCR*. Their study findings were consistent with ours that LDL-C had a negative causal effect on eBMD and effects of statins on eBMD might be partly mediated by lowering the LDL-C level. Nevertheless, reliability of a MR study could be further improved by cross-validation with evidence from other study designs(18). In contrast to the pure MR approach adopted by Zheng *et al*, we integrated the results of genetic correlation and observational analysis in two independent cohorts in addition to MR analysis, providing robust evidence supporting the negative causal role of LDL-C on eBMD, as well as TB-BMD. Moreover, Zheng *et al* did not identify proxies as alternative genetic instruments even though the original ones could not be matched between the exposure and outcome datasets. The major drawback is that only summary statistics of 28.6% conditionally independent SNPs (404 out of 1,410) significantly associated with eBMD were available in the plasma lipids dataset when they examined the reverse causation of eBMD on LDL-C(42). Such selection of genetic instruments by data availability is prone to bias.

We demonstrated that the LDL-C-lowering effect of statins was causally associated with increased TB-BMD and eBMD. In a recent meta-analysis investigating the effect of statins

on BMD in both RCT and cohort studies, use of statins was significantly associated with increased BMD at lumbar spine (standardized mean difference [SMD]: 0.20; P=0.002), marginally significant at total hip (SMD: 0.18; P=0.05), but statistically insignificant at femoral neck (SMD: 0.08; P>0.05)(13). Notably, only two small RCTs (total N=138) were included in the analysis, thus the significant result was mainly driven by the observational studies. In fact, null association with BMD was observed in the subgroup analysis of RCTs only. Another meta-analysis of RCTs, including the two small RCTs aforementioned and five other RCTs of larger sample size (N=27,754), suggested that statin use increased BMD(14). With consistent evidence from both observational and MR analyses, this study suggested causal inference of statin use on increased BMD.

Although causality could be inferred through MR, whether the finding could be translated clinically is unknown. Our findings suggested that lifelong genetic exposure to each SD decrease in LDL-C will increase TB-BMD by 0.038 SD or eBMD by 0.076 SD, but whether reducing LDL-C by 1 SD using pharmacological agent (e.g. statins) would lead to increase in eBMD / TB-BMD by the same amount is unknown, and such effect could not be tested in our observational cohorts due to the unavailability of pharmacological data. The effect of statins on LDL-C reduction was approximately 1.07mmol/L per year(43) (~1.13 SD based on NHANES III data). In a meta-analysis of RCTs, statin treatment for one year was associated with 0.03g/cm^2 increase in BMD (95% CI: 0.006, 0.053; P<0.001)(14), which was approximately equivalent to 0.2 SD based on NHANES III data. Thus, it seems that

reduction of LDL-C by statins may have an additional beneficial effect on BMD. These findings suggested the potential beneficial effects of statins on bone health, in addition to its protective role in cardiovascular diseases. As genetic predisposition to lower blood LDL-C levels is associated with increase in BMD, reduction of LDL-C level is therefore a common goal for the management of osteoporosis and CAD, the two prevalent diseases associated with increased immobility, morbidity and mortality.

Animal and cell studies provided mechanistic explanation regarding the role of LDL-C on bone metabolism. High cholesterol diet reduced BMD in mouse model(44), probably via increased osteoclastogenesis(44, 45). On the other hand, statins target the mevalonate pathway, which is also the target of nitrogen-containing bisphosphonates (N-BPs), the first-line therapy for osteoporosis. Treatment of statins reduced bone loss via reduced osteoclastogenesis in ovariectomized rat(46) and promoted osteoblast differentiation in ovariectomized rabbits(47) respectively. As statins were reported to decrease bone resorption by inhibiting osteoclast differentiation and osteoblast apoptosis downstream of the mevalonate pathway(48), this additional effect on bone resorption may explain why the reduction of LDL-C by statins may have an additional beneficial effect on BMD. While the mevalonate pathway is mainly responsible for cholesterol biosynthesis, the isoprenoid lipids produced are essential for prenylation and activation of small GTPases, which play a crucial role in the regulation of osteoclast morphology(49). Studies reported that patients treated with intravenous N-BPs had reduced LDL-C level(50-53) although studies examining

patients treated with oral N-BP gave inconsistent results(54). Use of N-BPs was also associated with lower risk of cardiovascular mortality and incident myocardial infarction in hip fracture patients(55). Mevalonate pathway is the possible link between bone and lipid metabolism.

Our MR analyses demonstrated that decrease in LDL-C level was causally associated with increase in BMD but not fracture. Although LDL-C is a causal factor of BMD regulation, other non-bone related factors are also important in predicting fracture, such as muscle strength and risk of falls. These risk factors might explain the missing link between LDL-C level and fracture. Similarly, MR analyses suggested that the LDL-C-lowering effect of statins was causally associated with increased BMD, but it had null association with fracture. Meanwhile, statin use was found associated with reduced osteoporotic fracture in a meta-analysis of RCTs and observational studies(13) though the finding was postulated to be confounded by healthy adherer effect(15). The discrepancy may be explained by the fact that genetic proxies of statins just account for its LDL-lowering effects but could not fully cover its mechanism of action involving osteoblast differentiation and reduced osteoclastogenesis.

On the other hand, our MR analyses showed that decrease in triglycerides was causally associated with increase in TB-BMD in both univariable and multivariable IVW analyses. Yet, null association was observed for sensitivity analyses, which may be attributed to the low statistical power (25%).

Regarding the causal relationship between BMD and CAD, a MR study showed that increase in genetically-instrumented eBMD was associated with higher risk of CAD(12). It was inconsistent with the findings from a recent meta-analysis which showed that lower BMD was associated with a higher risk of CAD(11). In our study, we showed that the effect of eBMD on CAD was indirect and it could be confounded by blood lipids levels(56), diabetes(57, 58) and BMI(56), which all shared common pathophysiological pathways with bone metabolism. In addition, our MR analyses suggested TB-BMD had a null effect on CAD. Insufficient evidence was present to prove the causal relation between BMD and CAD.

The present study has several strengths. Firstly, the epidemiological observation analyses were conducted in two independent cohorts: Mexican Americans, hispanic and non-hispanic in the U.S. population from the NHANES III, and southern Chinese from the HKOS. The consistent results across the two cohorts supported that LDL-C was negatively associated with BMD irrespective of ethnicities and living styles. Secondly, independent genetic instruments selected for the MR analyses were obtained from large-scale GWAS with stringent thresholds for quality-control and association analysis. The strength of combined genetic instruments in each MR analysis was assessed by the F-statistic, which ranged from 3,213.67 to 26,183.12. The high F-statistic indicated a lower chance of weak instrument bias. We had adequate statistical power to detect a causal effect (over 80%) for 11 out of the 13 main MR analyses conducted (Table 1), assuming the causal beta coefficient is the same as

the beta estimate obtained from epidemiological observational studies for continuous outcome. Thirdly, in evaluating the causality of blood LDL-C level on TB-BMD and eBMD, two-sample MR analysis was adopted and there was no overlap between the samples of blood lipids and BMD. Therefore, over-fitting of the findings was avoided. The estimated effects derived by MR approach was similar to that derived from the epidemiological observation analysis conducted in two cohorts. Consistent results were also observed in various sensitivity analyses, suggesting that the evidence was robust and confounding factors were unlikely to explain the observed associations.

This study also has limitations. In the epidemiological observation study, BMD at femoral neck and lumbar spine were investigated as these sites were known to be prone to fracture. Whereas, TB-BMD was investigated in MR analysis as the GWAS meta-analysis(19) contains the largest sample for DXA-derived BMD to date and it is closely correlated with BMD at lumbar spine and femoral neck ($r>0.9$)(19). Meanwhile, eBMD was examined as an alternative BMD phenotype with a large GWAS dataset of over 140,000 participants. The large sample size of the TB-BMD and eBMD datasets enable our study to have sufficient statistical power to detect genuine causal effects. MR does not require prior understanding on the functions of the genetic instruments and how they influence the risk factors. It is possible that the genetic instruments may have an indirect effect on the outcome via a pathway that does not involve the risk factor of interest (horizontal pleiotropy). Our sensitivity analyses showed no evidence for unbalanced horizontal pleiotropy, though it

cannot be ruled out unequivocally. Regarding the MR analysis of eBMD on CAD, both datasets included participants from UK Biobank and there were likely overlapping samples. With causal association detected, bias would be present in the direction of the confounded association and the net bias would rely on the degree of overlap which could not be accounted for(59).

Conclusion

In conclusion, the current study provided strong evidence that genetic predisposition to lower blood LDL-C levels was associated with increase in both TB-BMD and eBMD. TB-BMD also had a negative causal role on LDL-C level, suggesting a positive feedback loop between bone and lipid metabolism. MR analysis using the genetic proxies of statin therapy demonstrated that statins' LDL-C-lowering effects could improve BMD. Null causal association was observed for LDL-C on fracture. Insufficient evidence was available to support the causation of BMD on CAD.

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