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The effect of plasma lipids and lipid lowering interventions on bone mineral density: a Mendelian randomization study

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Abstract

Several epidemiological studies have reported a relationship between statin treatment and increased bone mineral density (BMD) and reduced fracture risk, but the mechanism underlying the purported relationship is unclear. We used Mendelian randomization (MR) to assess whether this relationship is explained by a specific effect in response to statin use, or by a general effect of lipid-lowering. We utilized 400 single nucleotide polymorphisms (SNPs) robustly associated with plasma lipid levels as exposure. The outcome results were obtained from a heel estimated BMD (eBMD) GWAS from the UK Biobank and DXA BMD at four body sites and fracture GWASs from the GEFOS consortium. We performed univariate and multivariable MR analyses of low-density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) and triglyceride levels on BMD and fracture. Univariate MR analyses suggested a causal effect of LDL-C on eBMD (β = -0.06; standard deviation change in eBMD per standard deviation change in LDL-C, 95% CI=-0.08 to -0.04; P = $4x10^{-6}$), total body BMD (β =-0.05, 95%CI=-0.08 to -0.01, P=$6x10^{-3}$) and potentially on lumbar spine BMD. Multivariable MR suggested that the effects of LDL-C on eBMD and total body BMD were independent of HDL-C and triglycerides. Sensitivity MR analyses suggested that the LDL-C results were robust to pleiotropy. MR analyses of LDL-C restricted to SNPs in the *HMGCR* region showed similar effects on eBMD (β = -0.083; -0.132 to -0.034; P = 0.001) to those excluding these SNPs (β= -0.063; -0.090 to -0.036; P = $8x10^{-6}$). Bidirectional MR analyses provided some evidence for a causal effect of eBMD on plasma LDL-C levels. Our results suggest that effects of statins on eBMD and total body BMD are at least partly due to their LDL-C lowering effect. Further studies are required to examine the potential role of modifying plasma lipid levels in treating osteoporosis.

Introduction

Statins, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are principal therapeutic agents in lowering blood cholesterol, especially, low density lipoprotein cholesterol (LDL-C). Several randomized controlled trials (RCTs) have reported increased bone mineral density (BMD) following statin administration (1)(2). These results could reflect a direct effect of statins on BMD, as suggested by findings from several *in vivo* studies that statins stimulate bone formation (3)(4)(5). An alternative possibility is that the relationship between statin use and BMD is at least partially mediated by an effect of LDL cholesterol (LDL-C) on bone metabolism (6)(7). For example, Parhami et al proposed that products of lipid and lipoprotein oxidation may contribute to the pathophysiology of osteoporosis (8). Consistent with this hypothesis, several observational epidemiological studies have documented a link between coronary heart disease and osteoporosis (9)(10). However, observational epidemiological studies are subject to confounding and reverse causality, making interpretation of such associations difficult and their meaning uncertain.

Mendelian randomization (MR) uses genetic variants as instrumental variables to estimate the causal effect of modifiable environmental exposures on medically relevant outcomes (11)(12). For example, we previously used this method to demonstrate a causal effect of greater fat mass on BMD in children (13). Recent MR studies have suggested a causal relationship between LDL-C lowering and bone (14)(15)(16). These studies, however, have not taken advantage of the full range of data on lipids and BMD available in the public domain (e.g. GWAS of DXA-scans based on BMD at different body sites). In order to obtain a more comprehensive understanding of the relationship between blood lipids and BMD, we performed a two sample MR study (17). We utilized summary GWAS data from the Global Lipids Genetics Consortium (18) to proxy lipid exposures (400 instruments), and summary GWAS data of ultrasound derived heel estimated BMD on 426,824 UK Biobank European participants (19), DXA-derived BMD (total body BMD, N=66,611; forearm BMD, N=8,143; femoral neck BMD, N=32,735; lumbar spine BMD, N=28,498) (20)(21) and fracture (GEFOS ALLFX fracture, N=264,973; UK Biobank fracture, N=426,795) (19)(22). To obtain estimates of the causal effect of blood lipids on BMD and fracture, we performed two sample inverse variance weighted (IVW) MR (23), and also a series of sensitivity analyses including MR Egger regression (24), weighted median MR analysis (25) and multivariable MR (26)(27) which may produce more robust causal estimates in the presence of horizontal genetic pleiotropy (28). To determine whether any relationship between blood lipids and eBMD might be mediated by

direct effects of statin use, we compared causal estimates obtained using SNPs in the *HMGCR* gene (i.e. whose product is the target of statin therapy) versus estimates using SNPs outside this gene. In addition, we conducted a reverse MR analysis to test whether there was any evidence for BMD causally affecting plasma lipids (29).

Methods

Two sample inverse variance weighted Mendelian randomization analysis of estimated BMD

We performed a series of two-sample MR analyses using summary results data from the Global Lipids Genetics Consortium (N = 331,368) (18) and the UK Biobank Study of eBMD (N = 426,824) (19). In total, 400 conditionally independent SNPs robustly associated with blood lipids ($P < 5 \times 10^{-8}$) were selected as instruments for the MR analyses (see Supplementary Table 1). Of these SNPs, 195 variants were associated with HDL-C, 147 were associated with LDL-C, and 163 were associated with triglycerides at genome-wide levels of significance (see Supplementary Table 2, 3 and 4). We refer to MR analyses involving all these variants as analyses using the "Complete Set" of SNPs. To obtain estimates of the causal effect of lipid fractions on eBMD, we performed two-sample IVW MR analysis (30) on each lipid fraction separately. Analyses were performed using the TwoSampleMR R package of MR-Base (31) (https://github.com/MRCIEU/TwoSampleMR).

Sensitivity analyses

Standard MR analyses rely on the validity of a number of core assumptions to produce accurate causal estimates of the exposure on the outcome (11)(30)(32). One of these assumptions states that genetic instruments (lipid SNPs) must only potentially be related to the outcome (i.e. eBMD) through their relationship with the exposure (lipid levels). Thus, if there are additional pleiotropic paths between the SNP and outcome that do not pass through lipid levels, then standard MR analyses may produce biased causal estimates of blood lipids on eBMD. We therefore applied three recent extensions of the basic IVW MR method, that can produce more accurate causal estimates, MR Egger regression (24), weighted median MR (25) and mode-based estimator (weighted mode approach) (33), given horizontal pleiotropy is common place.

In MR Egger regression, as long as the 'INSIDE assumption' (INstrument Strength is Independent of Direct Effect) assumption is met, the slope of the weighted regression line provides an asymptotically unbiased causal estimate of the exposure on the outcome that is free from the effects of horizontal pleiotropy (24). In general, MR Egger regression is potentially more robust to horizontal pleiotropy but has less statistical power compared to the IVW MR method. In addition, the intercept of the MR Egger regression line quantifies the amount of directional pleiotropy present in the data averaged across the genetic instruments.

We assessed the no measurement error (NOME) assumption in MR Egger regression using an adaptation of the I^2 statistic to the two-sample summary data MR context, which is referred to as I^2_{GX} and accounts for uncertainty in the SNP-exposure estimates. I^2_{GX} provides an estimate of the degree of regression dilution in the MR-Egger causal estimate (34).

We also used the weighted median MR and mode-based MR approaches as additional sensitivity analyses, which provide consistent causal estimates of the exposure on the outcome even when up to 50 of the information contributing to the analysis comes from genetic variants that exhibit pleiotropy (or even the majority of information in the case of the mode-based MR) (25)(33). Thus, MR Egger regression, weighted median MR and mode-based MR provide causal estimates of the exposure (lipids) on the outcome (eBMD) under different assumptions. If all approaches (i.e. IVW MR, MR Egger regression, weighted median MR and mode-based MR) provide similar estimates of the causal effect of lipids on eBMD, then we can be more confident that our findings are robust. All sensitivity analyses were performed using the MR-Base R package as described above (31). We further applied MR-PRESSO (35) as another sensitivity analysis, which attempts to reduce heterogeneity in the estimate of the causal effect by removing SNPs that contribute to the heterogeneity disproportionately more than expected. We conducted this analysis by using the MR-PRESSO R package (https://github.com/rondolab/MR-PRESSO). The number of distributions was set to 10000 and the threshold was set to 0.05.

When applying MR, we make an assumption that SNPs used to proxy lipids exert their primary association on lipids, and that any correlation with eBMD is a consequence of a causal effect of lipids on eBMD. But, if eBMD exerts a causal effect on lipids, then there is a possibility that some SNPs primarily associated with eBMD might also pass the genome-wide significant threshold in a GWAS of lipids with large sample size. These eBMD SNPs could then

misleadingly be applied as genetic instruments for lipids, when actually they should be applied as genetic instruments for eBMD. In other words, in very large GWAS it can be difficult to determine whether a SNP has its primary association with the exposure under study, or the outcome (36). This was particularly relevant to the current study as some of the eBMD associated SNPs were also robustly associated with lipids (i.e. reached the genome-wide significant threshold). We therefore applied MR Steiger filtering (37) as implemented in the TwoSampleMR R package (31) to test the causal direction of each of the 400 lipid associated SNPs on the hypothesized exposures (lipids) and outcome (eBMD). This approach infers the causal direction between phenotypes using a simple inequality. Given trait A causes trait B then one would expect that:

$$\sum_{i=1}^{M} cor(g_i, A)^2 > \sum_{i=1}^{M} cor(g_i, B)^2$$

because $cor(g_i, B)^2 = cor(A, B)^2 * cor(g_i, A)^2$, where "cor" denotes correlation, and the vector g contains a set of M SNPs that influence trait A. For any SNP that had a $cor(g,A)^2 < cor(g,B)^2$ (which means it showed evidence of primarily affecting eBMD rather than lipids), we removed those lipids SNPs and conducted IVW MR, MR Egger and weighted median MR using the remaining instruments ("Steiger filtered" set). The process of choosing validated instruments using Steiger filtering followed these steps:

1. Select lipid instruments from the main analysis (p-value threshold $5x10^{-8}$).
2. Classify instruments in each MR analysis based on Steiger filtering:
    - 'TRUE': evidence for causality in the expected direction i.e. lipids influence eBMD.
    - 'FALSE': evidence for causality in the reverse direction i.e. eBMD influences lipids. Instruments with 'FALSE' were removed from the sensitivity analysis.
    - 'NA': no result (due to missing effect allele frequencies in the outcome data or missing numbers of cases and controls for binary traits).

Individual Steiger filtering results can be found in Supplementary Table 2, 3 and 4.

Two sample Mendelian randomization analysis for estimated BMD controlled for possible confounders

To control for the possible introduction of confounding by including adiposity, height, smoking and alcohol intake related variants in our MR analyses, we cross referenced our LDL-C

instruments with the most up-to-date list of SNPs related to body mass index (BMI) (38), height (38), smoking (39) and alcohol intake (40). We excluded LDL-C SNPs that were in linkage disequilibrium with published GWAS variants associated with BMI, height, smoking and alcohol intake (r^2>0.5 in the 1000 Genome Europeans; Supplementary Table 3). We excluded ten BMI associated SNPs, 20 height associated SNPs and three SNPs associated with alcohol intake. No SNPs associated with smoking related traits were in LD with any LDL-C SNPs. We then conducted the IVW MR, MR Egger and weighted median MR analyses again using the remaining instruments.

Since men and women differ markedly in terms of their average BMD, we conducted a sex-specific MR analysis using male and female only eBMD as a sensitivity test (19). The same MR analyses were applied, including IVW MR, MR-Egger and WM MR approaches.

Two sample multivariable Mendelian randomization analysis of estimated BMD

Since many of the SNPs used in the previous MR analyses were associated with more than one lipid fraction, we applied multivariable MR (Figure 1) to identify the causal effect of HDL-C, LDL-C and triglycerides on eBMD, using a "weighted regression-based method" approach where the inverse-variance weights were applied to a multivariable regression model (26)(27). Multivariable MR has an advantage over univariate MR in that it accounts for the potential pleiotropic influence of other exposures included in the analysis (i.e. HDL-C, LDL-C and triglycerides). However, similar to IVW MR, multivariable MR relies on the assumption that the relationship between the instruments and the outcome is only mediated by the exposure variables tested in the analysis (i.e. LDL-C, HDL-C and triglycerides), which in the real world may not always be the case. We therefore fitted a multivariable MR model with an unconstrained intercept term, which has the effect of allowing for directional pleiotropy, similar to the situation in MR Egger regression (41). Since multivariable MR does not require each genetic instrument to be related to every exposure variable (merely that each SNP is a strong instrument for at least one exposure), we applied the method to the complete set of 400 lipid associated SNPs. We performed sensitivity analyses coding the direction of the SNPs as LDL-C increasing, HDL-increasing and then triglyceride-increasing to examine whether the direction of coding affected the multivariable MR Egger regression results.

Predicting the impact of lipid lowering pharmaceutical interventions on estimated BMD

Analogous to what has been done in several previous MR studies of lipids and coronary heart disease (42)(43)(44), we used a selection of genetic variants at the 3-Hydroxy-3-Methylglutaryl-CoA Reductase *(HMGCR)*, Niemann-Pick C1-Like 1 (*NPC1L1*) and Proprotein convertase subtilisin/kexin type 9 (*PCSK9*) genes to mimic the expected action of statins, ezetimibe and evolocumab, respectively on BMD. Since some of the SNPs within these genes were in incomplete linkage disequilibrium (LD), we used a likelihood-based two-sample MR approach that takes into account the correlation between genetic instruments when estimating the causal effect of lipid lowering drugs on eBMD (17). LD correlation estimates (r) between markers were obtained in CEU individuals using the LD matrix webserver (45). As a further sensitivity analysis, we repeated MR analyses using all SNPs outside the *HMGCR* region.

If statins causally affect bone mineral density via "direct effect" (i.e. independent of lipids), then we would expect to see significant causal estimates for MR analyses involving *HMGCR* SNPs, but not for analyses involving SNPs in the *NPC1L1* and *PCSK9* genes, nor the rest of the genome. In contrast, if the effect of the SNPs on eBMD were mediated through blood lipids, then we would expect to obtain significant causal estimates using lipid associated SNPs across the rest of the genome. We formally compared the different causal estimates obtained using SNPs in the different gene regions using heterogeneity tests (46).

Bidirectional Mendelian randomization of estimated BMD

Finally, in order to test the potential causal effect of BMD on blood lipids, we used summary results data from 1,103 conditionally independent genetic variants reported in a recent eBMD GWAS using 426,824 UK Biobank European individuals (19) as instrumental variables. Sentinel SNPs with a marginal P value smaller than $5x10^{-8}$ were selected from the Morris et al paper. LD clumping was conducted for the eBMD instruments with an r^2 threshold of 0.01. We then extracted summary results association data on these variants on LDL-C, HDL-C and triglycerides from the Global Lipids Genetics Consortium (18)(44). We performed IVW MR, weighted median MR and MR-Egger regression methods using the TwoSampleMR R package as described above (31).

When applying Bidirectional MR, we also applied the Steiger filtering analysis (37) to investigate the causal direction of each eBMD associated SNP on the hypothesized exposure (BMD) and outcomes (lipids). When the Steiger test showed evidence of primarily affecting lipids rather than eBMD, we removed these eBMD SNPs and conducted bidirectional MR using the remaining instruments.

Two sample Mendelian randomization analysis of DXA-derived BMD and fracture

Heel-ultrasound derived measures (eBMD) have previously been found to predict fracture risk as accurately as DXA-based measures (47), and to be moderately correlated with DXA derived BMD at the hip and spine (r = 0.4 to 0.6) (48)(49). However, in comparison to heel ultrasound-derived BMD, DXA-derived BMD is used more widely clinically to assess fracture risk. We therefore conducted comprehensive two sample MR analyses to estimate the causal relationship between the three lipid subtypes and four DXA-derived BMDs and fracture. The outcomes included total body BMD (20), forearm BMD (21), femoral neck BMD (21), lumbar spine BMD (21) measured by DXA and fracture from the GEFOS ALLFX study (22) and UK Biobank (19). Together with eBMD, we tested 6 outcomes in total, using a conservative Bonferroni corrected threshold ($\alpha=8.33x10^{-3}$, as 6 outcomes were assessed) to account for the multiple risk factors tested. The same MR analysis pipeline was applied for these analyses, including IVW MR, MR Egger and weighted median MR with and without Steiger filtering, multivariable MR and bi-directional MR.

Two sample Mendelian randomization analysis of total body BMD across lifespan

To understand whether the effects of LDL-C on BMD in adulthood were consistent throughout the lifespan, we conducted an MR analysis of LDL-C on total body BMD across five age groups: age 15 or less, 15 to 30, 30 to 40, 45 to 60 and 60 or more. The complete list of LDL-C instruments were used, while the total body BMD data were obtained from Medina-Gomez *et al.* (20). The MR IVW, MR Egger, weighted median MR and mode-based MR were conducted using the TwoSampleMR R package.

LD score regression for quantifying sample overlapping across studies

Sample overlap may bias the causal estimates derived from two sample MR when the study suffers weak instrument bias (50). Given the sample size of the lipids GWAS we used in this study, weak instrument bias is less likely to be an issue. We quantified the level of sample overlap between the GLGC consortium and GEFOS consortium (and UK Biobank). Bivariate LD score regression (51) was applied to the three lipid subtypes and seven bone phenotypes we analysed in this study. The bivariate LD score regression intercept is a function of the degree of sample overlap between the two studies. An intercept close to zero implies little evidence of sample overlap.

Results

Mendelian randomization estimates the causal effects of plasma lipids on estimated BMD

Table 1 presents results from the univariate MR analyses of plasma lipids and eBMD. Each effect represents the estimated causal change in standard deviations (SD) of eBMD per SD change in serum level of HDL-C, LDL-C or triglycerides. IVW MR, MR Egger, weighted median MR and mode-based MR using the complete set of SNPs all suggested a causal effect of increased LDL-C on reduced eBMD (IVW estimate: β = -0.060, 95%CI = -0.084 to -0.036, P = $4x10^{-6}$). The MR estimate after removing outliers identified by MR-PRESSO suggested a consistent negative effect of LDL-C on eBMD (MR-PRESSO estimate: β = -0.041, 95%CI = -0.055 to -0.027, P = $8x10^{-8}$)

Excluding SNPs related to BMI (β = -0.058, 95%CI = -0.082 to -0.034, P= $3x10^{-6}$, Supplementary Figure 1), height (β = -0.057, 95%CI = -0.082 to -0.032, P= $4x10^{-5}$), or alcohol intake (β = -0.060, 95%CI = -0.084 to -0.036, P= $4x10^{-6}$) in the MR analyses yielded similar estimates of the causal effect of increased LDL-C on reduced eBMD. The sex-specific MR suggested that LDL-C was strongly associated with both male and female eBMD (Supplementary Figure 2).

In contrast, univariate MR analyses revealed little evidence for a causal effect of HDL-C or triglycerides on eBMD (IVW HDL-C estimate: β = -0.016, 95%CI = -0.40 to 0.08, P = 0.2; IVW triglycerides estimate: β = 0.021, 95%CI = -0.49 to 0.07, P = 0.1). The directionality test using Steiger filtering showed that most of the lipid SNPs exerted their primary effect on lipids as opposed to eBMD. 146 SNPs showed evidence of a primary causal effect on LDL-C, 190

SNPs on HDL-C and 158 SNPs on triglycerides opposed to eBMD (Supplementary Table 2, 3 and 4). MR using the Steiger filtered set of SNPs also showed strong evidence of LDL-C causally influencing eBMD (IVW estimate: β = -0.058, 95%CI = -0.081 to -0.035, P = 8.9×10^{-7}) and little evidence that HDL-C or triglyceride levels causally influenced eBMD (IVW HDL-C estimate: β = -0.013, 95%CI = -0.31 to 0.05, P = 0.15; IVW triglycerides estimate, β = 0.015, 95%CI = -0.035 to 0.05, P = 0.13) (Table 1). Funnel plots and scatter plots for this sensitivity analysis are presented in Supplementary Figure 3.

The funnel plots presented in Figure 2 display MAF-corrected genetic associations for each of the individual SNPs on lipid levels (y-axis) plotted against their causal effect estimates (x-axis). Visual inspection of Figure 2 provided little indication for the existence of directional horizontal pleiotropy for LDL-C (panel A), but a suggestion of directional pleiotropy for HDL-C (panel B) and potentially for triglycerides (panel C). In particular, SNPs less strongly related to increased HDL-C tended to be associated with reduced eBMD. This interpretation was consistent with estimates of the intercepts from the MR Egger regression analyses (LDL-C: intercept = -0.001, P = 0.1; HDL-C: intercept = -0.004, P = 3×10^{-4}; Triglycerides: intercept = 0.002, P = 0.08). Figure 2 also illustrates the associations between the LDL-C (panel D) / HDL-C (panel E) / triglycerides (panel F) variants and eBMD in the form of scatter plots, with the MR Egger regression and IVW MR lines superimposed on the data points (the slopes representing the estimated causal effects). In Figure 2D, we observed some outlier SNPs, which may increase the magnitude of the causal estimates of the effect of LDL-C on eBMD obtained in the IVW and MR Egger regression analyses. The weighted median MR estimate, which is less influenced by the presence of outliers, was lower compared to IVW and MR Egger estimates (β= -0.028, 95%CI= -0.042 to -0.014, P=1×10^{-4}). In addition, the Cochrane Q test also suggested strong heterogeneity in estimates of the causal effect across the different LDL-C instruments (Q = 1605.24, P = 6.39×10^{-244}). Thus, although the overall effect of pleiotropy on the pooled results was likely to be small (as indicated by an MR Egger regression intercept close to zero), it is likely that many individual SNPs exhibited horizontal pleiotropy (which then tended to cancel out when the estimates were combined together in meta-analysis/Egger regression).

Assessment of the NO Measurement Error (NOME) assumption (34) with respect to the MR-Egger estimate gave unweighted I^2_{GX} = 0.995 and weighted I^2_{GX} = 0.995. This suggests a minor 0.5% attenuation of the causal estimate toward zero, as a consequence of uncertainty in the

SNP exposure estimates.

Multivariable IVW analysis provided additional evidence for a causal effect of LDL-C on eBMD (β = -0.055, 95%CI = -0.080 to -0.030, P = $2.8x10^{-5}$), independent of the effects of HDL-C and triglycerides. The causal effect estimate was comparable to estimates produced from the previous univariate IVW MR analyses (Figure 3). As shown in Supplementary Table 5, there was no independent association between HDL-C and eBMD (β = -0.020, 95%CI = -0.046 to -0.006, P = 0.124) and triglycerides on eBMD (β = 0.013, 95%CI = -0.028 to 0.043, P = 0.397), consistent with univariate MR findings (Figure 3). The direction the alleles were coded in the multivariable analyses did not materially affect the results (Supplementary Table 5).

Genetic prediction of the impact of lipid lowering interventions on estimated BMD

Table 2 displays estimates of the causal effect of LDL-C level on eBMD obtained using SNPs from genes whose proteins are targets for lipid lowering drugs. Results obtained using 5 SNPs in the region of the *HMGCR* gene (43) suggest that reducing the activity of *HMGCR* (i.e. mimicking the effect of statins) increases eBMD (β = -0.083, 95%CI = -0.132 to -0.034, P = 0.001). In contrast, results using 7 SNPs in the region of the *PCSK9* gene (43) plus rs11591147 (44), and 5 SNPs in the region of *NPC1L1* from Ference et al (42), suggested that genetically reducing the activity of *PCSK9* (mimicking Evolocumab) and *NPC1L1* (mimicking Ezetimibe) had no clear effect on eBMD (*PCSK9*: β = -0.007, 95%CI = -0.027 to 0.013, P = 0.486; *NPC1L1*: β = -0.004, 95%CI = -0.059 to 0.051, P = 0.887). Interestingly, there was some evidence of heterogeneity in causal effect estimates across the different SNPs in the *PCSK9* gene, with one SNP in particular providing evidence for a causal effect in the opposite direction to the majority of the other SNPs. The SNPs used to explore the effect of the lipid lowering drugs are listed in Supplementary Table 6.

Sensitivity analysis using non-HMGCR lipid lowering SNPs on estimated BMD

Table 2 and Supplementary Figure 4 display the causal estimates of LDL-C on eBMD excluding the *HMGCR* SNPs from the MR analyses. We found that the 140 LDL-C associated SNPs outside the *HMGCR, PCKS9* and *NPC1L1* regions still produced significant estimates

of a negative causal effect of LDL-C on eBMD (β = -0.063, 95%CI = -0.090 to -0.036, P = $8x10^{-6}$). Supplementary Table 7 shows heterogeneity test results comparing causal estimates obtained from different gene regions (LD correlation matrices between SNPs in *HMGCR*, *PCSK9* and *NPC1L1* are shown in Supplementary Table 8). We found that the causal effect estimates using SNPs in the *HMGCR* gene were not different from those generated from the rest of the genome (excluding *HMGCR*, *PCSK9* and *NPC1L1* SNPs) (Cochran Q = 0.487, P=0.485). This finding suggests that some of the effect of the SNPs on eBMD may be mediated through LDL-C (i.e. through mechanisms not involving HMGCR and statins). In addition, the confidence intervals surrounding the causal effect estimates obtained using SNPs in *PCSK9* and *NPC1L1* were wide and overlapped zero and were also different to the ones obtained using the *HMGCR* SNPs and the rest of the genome.

Bi-directional MR estimating the reverse causal effect of estimated BMD on plasma lipids

We also investigated the potential reverse causal effect of eBMD on blood lipids. After LD clumping, 574 out of 1,103 SNPs reported as robustly associated with eBMD from the UKBB study could be found in the blood lipids GWAS data (44). Where the exact eBMD variant was not available in the lipids GWAS a proxy variant was used instead (LD r2 > 0.8 with the leading SNP as the proxy SNP). The Steiger filtering analysis suggested that most of these SNPs exerted their primary effect on eBMD as opposed to lipid levels. 514 SNPs showed evidence of a primary causal effect on eBMD as opposed to LDL-C, 507 SNPs opposed to HDL-C and 515 opposed to triglycerides (last four columns in Supplementary Table 9). Supplementary Table 10 presents univariate MR results for the effect of these remaining eBMD associated SNPs on plasma lipids. IVW MR, weighted Median MR and MR-Egger regression results showed no strong evidence of eBMD causally influencing HDL-C or triglyceride levels although there was some evidence that eBMD might influence LDL-C. Interestingly, even after Steiger filtering and MR Egger regression, Cochrane Q statistics suggested the presence of considerable heterogeneity remaining in the analysis. The funnel plot and scatter plot for the bidirectional MR are presented in Supplementary Figure 5.

Mendelian randomization estimates of the causal effect of plasma lipids on DXA-derived BMD and fracture

We further investigated whether the effect of lipids on eBMD was similar to their effect on DXA-derived BMDs and fracture. Supplementary Table 11 and Supplementary Table 12 present univariate and multivariable MR estimates for the causal effect of the three lipid subtypes on DXA-derived BMD at four body sites and fracture. IVW MR, MR Egger, weighted median MR and mode-based MR using the complete set (and the Steiger filtered set) of SNPs suggested a causal effect of increased LDL-C on reduced total body BMD (IVW estimate: β = -0.047, 95%CI = -0.080 to -0.014, P = $6x10^{-3}$), which was similar to the multivariable MR estimate (β = -0.041, 95%CI= -0.072 to -0.011, P = $8.4x10^{-3}$). MR also showed a suggestive effect of LDL-C on lumbar spine BMD (univariable MR: β = -0.048, 95%CI= -0.095 to -0.002, P = 0.04; multivariable MR: β = -0.045, 95%CI= -0.096 to 0.006, P = 0.08). Multivariable MR also showed an effect of LDL-C on forearm BMD (β = -0.094, 95%CI= -0.160 to -0.027, P=$6.14x10^{-3}$) and a suggestive effect on fracture in UK Biobank (OR= 1.026, 95%CI=1.001 to 1.052, P=0.046). These findings were consistent with the results of MR analyses examining the relationship between LDL-C and eBMD. For HDL-C associations, univariate MR suggested strong effects of HDL-C on forearm BMD (β =-0.075, 95%CI=-0.131 to -0.019, P= $8.3x10^{-3}$) and fracture in UK Biobank (OR=1.035, 95%CI=1.013 to 1.057, P=$4x10^{-3}$). But after controlling the effect of LDL-C and triglycerides in a multivariable MR model, these effects were attenuated somewhat. Only a suggestive result was observed between HDL-C and fracture in UK Biobank (OR= 1.027, 95%CI= 1.002 to 1.053, P=0.035). Multivariable MR suggested a negative effect of HDL-C on total body BMD (β = -0.041, 95%CI= -0.070 to -0.011, P = 0.007), but this association was not supported in the univariate MR. For triglycerides, univariate MR suggested evidence of an effect of triglycerides on fracture in UK Biobank (OR=0.963, 95%CI=0.937 to 0.989, P=$7x10^{-3}$). The multivariable MR using fracture data from the ALLFX study supported these results (OR=0.946, 95%CI=0.9079 to 0.985), but the causal effect did not replicate using multivariable MR in the UK Biobank (OR= 0.978, P=0.137). There was no strong evidence for a relationship between HDL-C (or triglycerides) on femoral neck BMD or lumbar spine BMD. In general, the consistent relationship between LDL-C and BMD at multiple sites was not apparent for HDL-C and triglycerides (Supplementary Table 11 and 12). The bidirectional MR results suggested no strong consistent effect of DXA-based BMD and fracture on lipids (Supplementary Table 13).

Mendelian randomization estimates of the causal effect of LDL-C on total body BMD across lifespan

Figure 4 presents the MR results of LDL-C on total body BMD at 5 different age groups: age 15 or less, 15 to 30, 30 to 40, 45 to 60 and 60 or more. This MR analysis suggested that LDL-C showed a consistent negative effect on total body BMD across all age groups. Age group "age 15 or less" showed the strongest evidence for a causal effect (β = -0.064, 95%CI= -0.121 to -0.007, P = 0.028). Confidence intervals were wide across because of the stratification of individuals into different age groups.

Finally, Supplementary Table 14 shows the results of bivariate LD score regression analyses designed to assess the degree of sample overlap between GLGC consortium and GEFOS consortium (and UK Biobank). The intercepts of the 21 bivariate LD score regression analyses were close to zero (column "gcov_int"), suggesting little sample overlap across the groups.

Discussion

We performed an MR study to examine whether previous findings of an inverse observational association between serum cholesterol and BMD reflected a causal relationship. We found that eBMD increased by 0.064 SD per SD lower LDL-C, based on IVW MR analyses of the complete SNP set. Interestingly, MR analyses using five SNPs in the region of the *HMGCR* gene and using all the other known LDL-C related variants across the genome also produced strong estimates of the causal effect of LDL-C lowering on eBMD. Taken together, these observations suggest that gains in BMD following statins are at least partly due to a causal effect of lowering LDL-C.

Aside from studies of statins (1)(2)(3)(4)(5), some very recent MR studies have also suggested a negative causal effect of LDL-C level on BMD, although there are some key differences between their studies and ours, and we have conducted a more powerful and comprehensive MR study of lipids on BMD across five different body sites and fracture(14)(15)(16). Using the latent causal variable (LCV) approach, O'Connor and Price suggested a negative causal relationship between LDL-C and eBMD (14), however their LCV approach cannot take into account the possibility of bi-directional effects between two phenotypes (i.e. our study showed bi-directional associations for LDL-C on eBMD as well as on total body BMD). Cherny *et al.* applied univariable MR to summary results data of eBMD from the first release of the UK

Biobank and found a causal effect of LDL-C on eBMD (16). However, the complex pleiotropic relationships between SNPs influencing LDL-C, HDL-C and triglycerides were not modelled using multivariable MR. Also, their reverse MR analysis suggested no effect of eBMD on LDL-C, whilst our study indicated a possible effect. Finally, Li et al. found evidence of a bi-directional effect of LDL-C on total body BMD but found a unidirectional effect of LDL-C on eBMD using the first release of UK Biobank only (15), while our study using better powered exposure and outcome data suggested a bi-directional effect. Also, our study suggested that the effect of LDL-C on total body BMD was consistent across the lifespan (from adolescence to old age), which was not examined in Li et al. From a methodological point of view, our MR study also used more advanced methods such as mode-based MR as well as Steiger filtering and MR-PRESSO for instrument selection and, providing more robust evidence on causal relationships than previous work (15).

We were also keen to determine whether the causal relationship between LDL-C and BMD which we observed translated into an effect on fracture risk. Although little effect was observed for LDL-C in univariate analyses, MVMR suggested that decreased LDL-C led to a small reduction in fracture risk. In contrast, decreased HDL-C and triglycerides were related to lower and higher fracture risk respectively in univariate analyses, whereas in MVMR, HDL-C was unrelated to fracture risk, while decreased triglycerides was related to higher fracture risk using ALLFX data but showed little association in the UKBB. These findings are consistent with the reduction in BMD caused by LDL-C. However, they are difficult to explain in the case of HDL-C and triglycerides which were unrelated to BMD. Therefore, while our results provide some indication that lipids may affect fracture risk, the findings are generally too weak and inconsistent to draw firm conclusions.

Other cholesterol-lowering agents might lack the same tendency as statins to reduce BMD, which is further suggested by the results of our sensitivity analyses in which genetic instruments for other classes of cholesterol-lowering agents, ezetimibe and evolocumab, were unrelated to BMD. However, this finding contrasts with the effects of non-statin pathways as a whole on BMD, assessed by examining all LDL-C genetic instruments apart from *HMGCR*, which showed similar effects to instruments within the *HMGCR* region. Conceivably, ezetimibe and evolocumab might exert distinct, adverse effects on BMD, countering beneficial effects resulting from lowering of LDL-C levels.

A recent meta-analysis of 10 studies found that LDL-C levels were higher in patients with osteoporosis compared to controls (52). Such a relationship could conceivably contribute to the association between coronary vascular disease, for which high LDL-C levels are an important risk factor, and osteoporosis (9)(10). An inverse relationship between LDL-C and eBMD, suggested by our results, is consistent with evidence that lipids may contribute to the pathophysiology of osteoporosis through lipid oxidation (7). For example, oxidized lipids, especially oxidized LDL, characteristic of hyperlipidemia, may have direct adverse effects on cellular components of bone, inhibiting osteoblastic differentiation and bone formation, increasing adipogenesis of MSCs at the expense of their osteogenic differentiation, and inducing osteoclastic differentiation and bone resorption (8)(53)(54). In addition, oxidized lipids induce the expression of cytokines such as *MCP-1*, *M-CSF* and *IL-6* both *in vitro* and *in vivo*, thought to play a role in osteoporotic bone loss (55)(56).

A key assumption underlying the MR methodology is that the SNPs used as instruments are merely related to the outcome of interest via the exposure variable under study. This is invalidated by horizontal pleiotropy, whereby the genetic instrument for the exposure relates to the outcome via separate pathway to the exposure. There were several potential mechanisms for horizontal pleiotropy in the present study. For example, based on our previous finding that BMI is causally related to BMD (13), and the fact that LDL-C tends to be higher in obese individuals, it's conceivable that relationships which we observed between LDL-C and eBMD are affected by BMI. That said, whereas we observed an inverse relationship between LDL-C and eBMD, increased BMI has a positive effect on eBMD, suggesting these causal pathways would be in opposite directions. In addition, it's conceivable that some of the SNPs selected from the lipid GWAS affect eBMD independently of altered lipid levels. For example, a variant upstream of *ESR1* is very strongly associated with eBMD (and LDL-C). Likewise, a SNP in the *RSPO3* gene is strongly associated with eBMD, HDL-C and triglycerides (although not with LDL-C) (57). Furthermore, a given lipid-related SNP could conceivably affect BMD by influencing a different lipid class, since many of the SNPs are associated with multiple lipid subtypes (see Figure 1).

Despite these potential sources of horizontal pleiotropy, there was not strong evidence of directional pleiotropy within the set of 147 LDL-C instruments, as reflected by the estimate of the MR-Egger intercept (I=-0.002, p=0.09), and the finding that the causal estimate from MR-Egger was consistent with estimates obtained from the IVW MR and weighted median MR

methods. Moreover, we observed an almost equivalent effect of LDL-C on eBMD following exclusion of obesity-associated SNPs (β = -0.058, 95%CI = -0.082 to -0.034, P = $3x10^{-6}$). In addition, there was little evidence of a causal effect of HDL-C or triglycerides on eBMD, and in multivariable MR analysis the effect of LDL-C on eBMD was independent of these other lipid classes. Our findings contrast with a recent MR study based on a more restricted sample of UK Biobank that found evidence of an inverse effect of eBMD, as measured here, on HDL-C and other cardiovascular and type 2 diabetes mellitus risk factors (58).

A causal relationship between LDL-C and eBMD is consistent with estimates of the genetic correlation between blood lipids and eBMD (49) using Linkage Disequilibrium (LD) score regression (51)(59). However, unlike the present analyses, a genetic correlation does not imply causality or indicate a direction of effect. Interestingly, our bidirectional MR analyses provided some evidence for a causal effect of eBMD on LDL-C in all three sets of MR analyses (i.e. inverse variance, MR Egger and weighted median approaches), although large Cochrane Q statistics suggested the existence of uncontrolled genetic pleiotropy that may have contaminated the analyses. A putative causal pathway is consistent with several lines of evidence that the skeleton plays a role in regulating energy metabolism. For example, research involving mouse models has suggested that bone turnover, which is inversely related to BMD, influences insulin sensitivity and adiposity via osteocalcin (an osteoblast-specific protein) (60). Likewise, individuals with rare genetic mutations in *LRP5* which predispose to very high bone mass have markedly increased fat mass and reduced bone turnover, consistent with a causal influence of reduced osteocalcin on fat accumulation (61). Given the strong relationship between LDL-C levels and insulin sensitivity (62), our observation that eBMD has a causal effect on LDL-C is consistent with this apparent control of energy metabolism by the skeleton.

Our finding that the causal effect of LDL-C lowering on eBMD was most closely mirrored by forearm DXA results may reflect the fact that this lipid effect targets trabecular bone; like the heel used for eBMD measurements, the distal forearm site from which DXA measurements were obtained has a relatively high proportion of trabecular bone. In contrast, the femoral neck, where DXA showed little causal relationship with LDL-C, mainly comprises cortical bone. Although the lumbar spine is also rich in trabecular bone, DXA measures at this site showed a somewhat weaker causal relationship with LDL-C, compared to forearm BMD. This may reflect the fact that, contrary to the heel and distal radius, lumbar spine BMD is strongly

influenced by artefacts due to age-related degenerative changes, which may partly mask relationships with trabecular BMD.

Strengths and Limitations

One of the main strengths of our study was use of very large GWAS, which helps overcome power limitations of MR. In addition, application of two-sample MR avoids bias towards the observational association caused by weak instruments (50). A further strength of our study was the application of several analytical approaches to detect and correct for horizontal pleiotropy.

Conclusions

Having performed an MR study to examine the causal effect of lipid lowering on eBMD, we found evidence that lowering LDL-C improves eBMD, independently of HMGCR inhibition. Our results illustrate how MR can be used profitably to investigate clinical questions and drug interventions relevant to osteoporosis and bone health. Further studies are justified to explore the mechanisms by which lower LDL-C improves BMD, and to examine their potential role in treating osteoporosis, for example based on methods such as network MR (63).

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Figure legends

Figure 1. Directed acyclic graph illustrating multivariable MR analysis of the relationship between lipids levels and DXA BMD/eBMD. In total, 400 conditionally independent SNPs robustly associated with blood lipids ($p < 5 \times 10^{-8}$) were used in the multivariable MR analysis. Many of the SNPs act pleiotropically and affect more than one lipid fraction. In addition, estimating an intercept in the multivariable MR regression (rather than having it constrained to zero) is akin to allowing for the possibility of additional horizontal pleiotropy in MR Egger regression.

Figure 2. Results of the MR analysis of lipid levels on eBMD using the complete set of instruments. Funnel plots displaying instrument strength (y-axis) plotted against causal effect estimates (x-axis) for SNPs associated with LDL-C (Panel A), HDL-C (Panel B), and triglycerides (Panel C) and scatter plots displaying estimates of the association between each SNP and eBMD (y-axis) against estimates of the association between each SNP and lipid level, i.e. LDL-C (panel D), HDL-C (panel E) and triglycerides (panel F). The error bars on each of the points represent, 95% CI. SNPs in circles denote those associated with one lipid subtype ($P<5x10^{-8}$) but not the other two ($P>0.05$), whereas the remaining SNPs are denoted by triangles. For Panel A, B and C, the inverse-variance weighted MR, MR-Egger and weighted median MR causal effect estimates are represented by dotted, solid and double dotted lines respectively. For Panel D, E and F, the slope of the solid line represents the MR-Egger regression estimate of the causal effect of serum lipids on eBMD and the inverse-variance weighted estimate is represented by the slope of the dotted line. The y-intercept of the solid regression line is an estimate of the degree of directional pleiotropy in the dataset.

Figure 3. Forest plot comparing causal effect estimates of serum lipid levels on eBMD using univariate and multivariable MR. The analysis was conducted using the complete set of lipid associated SNPs Abbreviations: SD, standard deviation; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; TG: triglycerides; eBMD: estimated bone mineral density measured at the heel; IVW inverse variance weighted; Egger, MR-Egger regression; WM, weighted median Mendelian randomization; MultiMR, multivariable Mendelian randomization. Note: all outlier exclusion approaches lead to reduced standard errors.

Figure 4. Forest plot comparing causal effect estimates of LDL-C levels on total body BMD throughout the lifespan. The X-axis displays the magnitude of the causal effect of LDL-C on total body BMD, while the Y-axis represents total body BMD at 5 different time points: age 15 or less, 15 to 30, 30 to 40, 45 to 60 and 60 or more.

Table 1. Summary of the univariate Mendelian randomization estimates of the causal effect of plasma lipids on eBMD using different sets of SNPs as instruments. eBMD was the outcome for all analyses.

Exposure	SNP selection	Methods	N_SNPs	Beta	Standard error	P value
LDL-C	complete	IVW	147	-0.06	0.012	$4x10^{-6}$
	complete	WM	147	-0.028	0.007	$1x10^{-4}$
	complete	Egger	147	-0.04	0.018	0.03
	complete	MBE	147	-0.035	0.005	$1.1x10^{-9}$
	Steiger filtered	IVW	146	-0.058	0.012	$8.9x10^{-7}$
	Steiger filtered	WM	146	-0.027	0.007	$1.3x10^{-4}$
	Steiger filtered	Egger	146	-0.043	0.017	0.01
	Steiger filtered	MBE	146	-0.035	0.005	$1.1x10^{-9}$
	Outlier removed	MR-PRESSO	118	-0.041	0.007	$7.7x10^{-8}$
HDL-C	complete	IVW	195	-0.016	0.012	0.20
	complete	WM	195	0.009	0.005	0.06
	complete	Egger	195	0.023	0.016	0.10
	complete	MBE	195	0.007	0.004	0.09
	Steiger filtered	IVW	190	-0.013	0.009	0.15
	Steiger filtered	WM	190	0.010	0.005	0.03
	Steiger filtered	Egger	190	0.019	0.012	0.11
	Steiger filtered	MBE	190	0.006	0.003	0.05
	Outlier removed	MR-PRESSO	148	-0.009	0.006	0.12
Triglycerides	complete	IVW	163	0.021	0.014	0.10
	complete	WM	163	0.007	0.007	0.30
	complete	Egger	163	-0.004	0.02	0.80
	complete	MBE	163	0.006	0.006	0.30
	Steiger filtered	IVW	158	0.015	0.010	0.13
	Steiger filtered	WM	158	0.006	0.006	0.31
	Steiger filtered	Egger	158	0.006	0.014	0.69
	Steiger filtered	MBE	158	0.002	0.005	0.75
	Outlier removed	MR-PRESSO	128	0.006	0.006	0.29

Abbreviations: 'complete' refers to all SNPs associated with the particular lipid fraction, "Steiger filtered" refers to SNPs associated with the lipid fraction and pass Steiger filtering (31); "Outlier removed" refers to SNPs associated with the lipid fraction and pass MR-PRESSO outlier removal step (35); Beta represents the standard deviation change in BMD per standard deviation change in plasma lipid level; N_SNPs refers to the number of SNPs used as instrumental variables in the analysis; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; eBMD: estimated bone mineral density at the heel; IVW: inverse variance weighted Mendelian randomization; Egger: MR-Egger regression; WM: weighted median Mendelian randomization; MBE: weighted mode based Mendelian randomization .

Table 2. Instrumental variable estimates of the causal effect of LDL-C levels on eBMD.

Target gene (Drug)	# SNPs	Outcome	Beta	Standard error	P value	P_H
HMGCR (Statin)	5	eBMD	-0.083	0.025	0.001	0.818
NPC1L1/ (Ezetimibe)	5	eBMD	-0.004	0.027	0.887	0.906
PCSK9/ (Evolocumab)	7	eBMD	-0.007	0.0104	0.486	0.049
Other SNPs	140	eBMD	-0.063	0.014	$8x10^{-6}$	0

Estimates are shown using SNPs in the *HMGCR*, *NPC1L1* and *PCSK9* gene regions, and using all LDL associated SNPs outside these regions (Other SNPs). Pharmacological inhibitors of the HMGCR, NPC1L1 and PCSK9 proteins reduce LDL cholesterol levels in the blood. Analyses were performed using a likelihood-based approach that accounts for correlations between genetic variants as described in Burgess et al (19). Effects of *HMGCR* SNPs on LDL cholesterol were estimated using results from Ference et al (43); Effects of *PCSK9* SNPs on LDL-C were estimated using results from Ference et al (43) and the effect of rs11591147 was obtained from the Global Lipids Genetics Consortium (44); Effect of *NPC1L1* SNPs on LDL-C were estimated using results from Ference et al (42). The eBMD GWAS of 426,824 individuals was used to estimate the effect of the SNPs on eBMD. Abbreviations: *HMGCR*, 3-Hydroxy-3-Methylglutaryl-CoA Reductase gene; *NPC1L1*, Niemann-Pick C1-Like 1 gene; *PCSK9*, Proprotein convertase subtilisin/kexin type 9 gene. Beta represents the estimated causal standard deviation change in eBMD per standard deviation change in LDL-C; P_H is the heterogeneity test p-value for differences in the causal effect estimates across the SNPs within a gene using a likelihood ratio heterogeneity test (17).

Figure 1

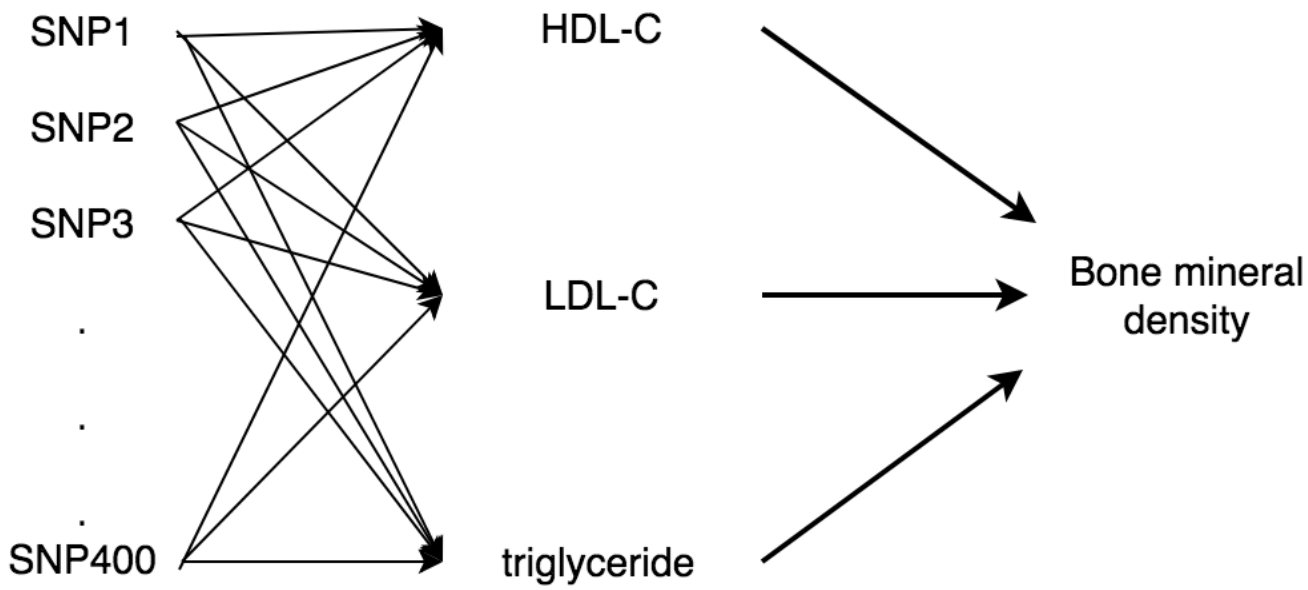

SNP1
SNP2
SNP3
.
.
.
SNP400
HDL-C
LDL-C
triglyceride
Bone mineral density

Figure 2

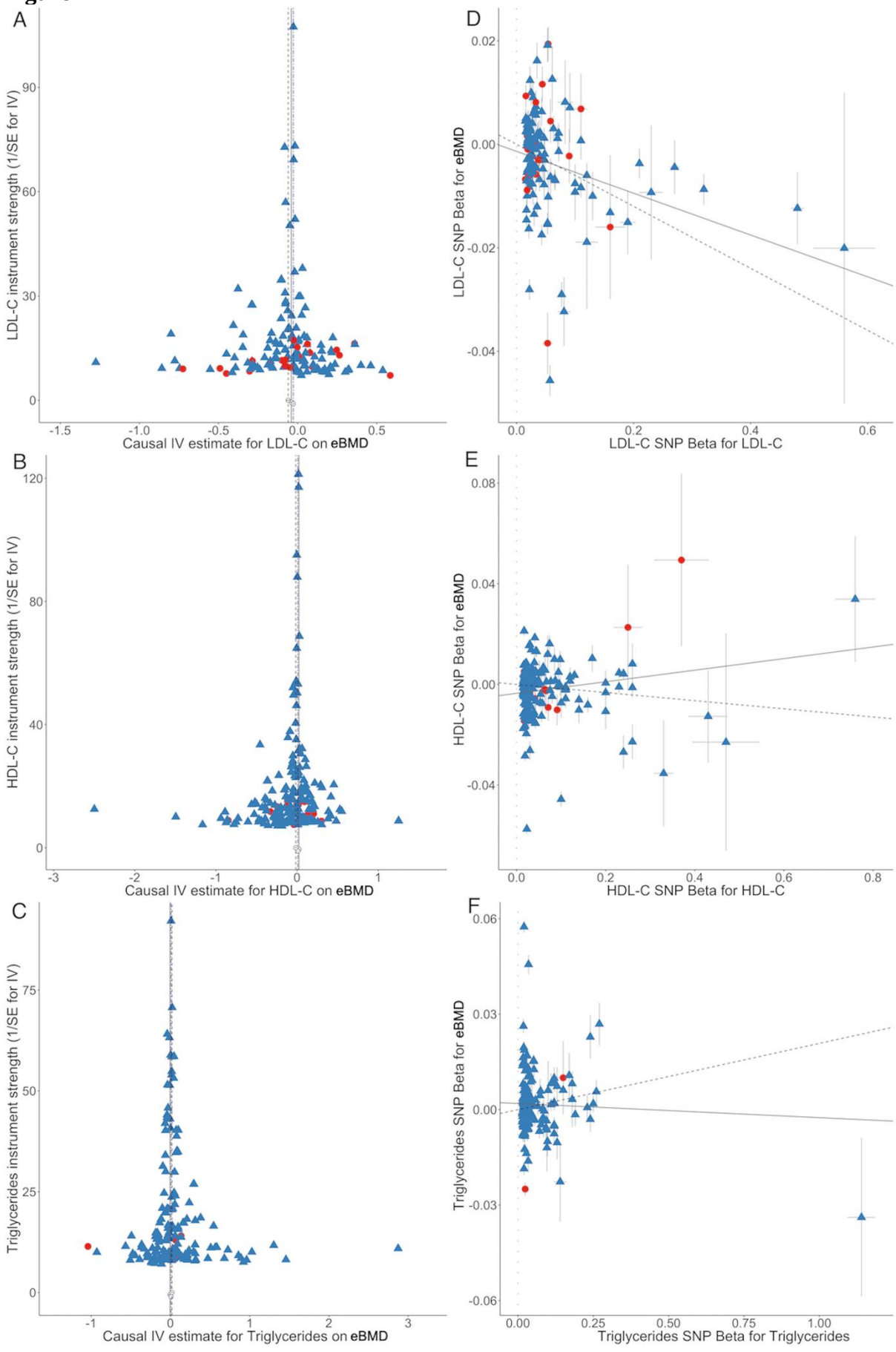

A
LDL-C instrument strength (1/SE for IV)
Causal IV estimate for LDL-C on eBMD
D
LDL-C SNP Beta for eBMD
LDL-C SNP Beta for LDL-C
B
HDL-C instrument strength (1/SE for IV)
Causal IV estimate for HDL-C on eBMD
E
HDL-C SNP Beta for eBMD
HDL-C SNP Beta for HDL-C
C
Triglycerides instrument strength (1/SE for IV)
Causal IV estimate for Triglycerides on eBMD
F
Triglycerides SNP Beta for eBMD
Triglycerides SNP Beta for Triglycerides

Figure 3

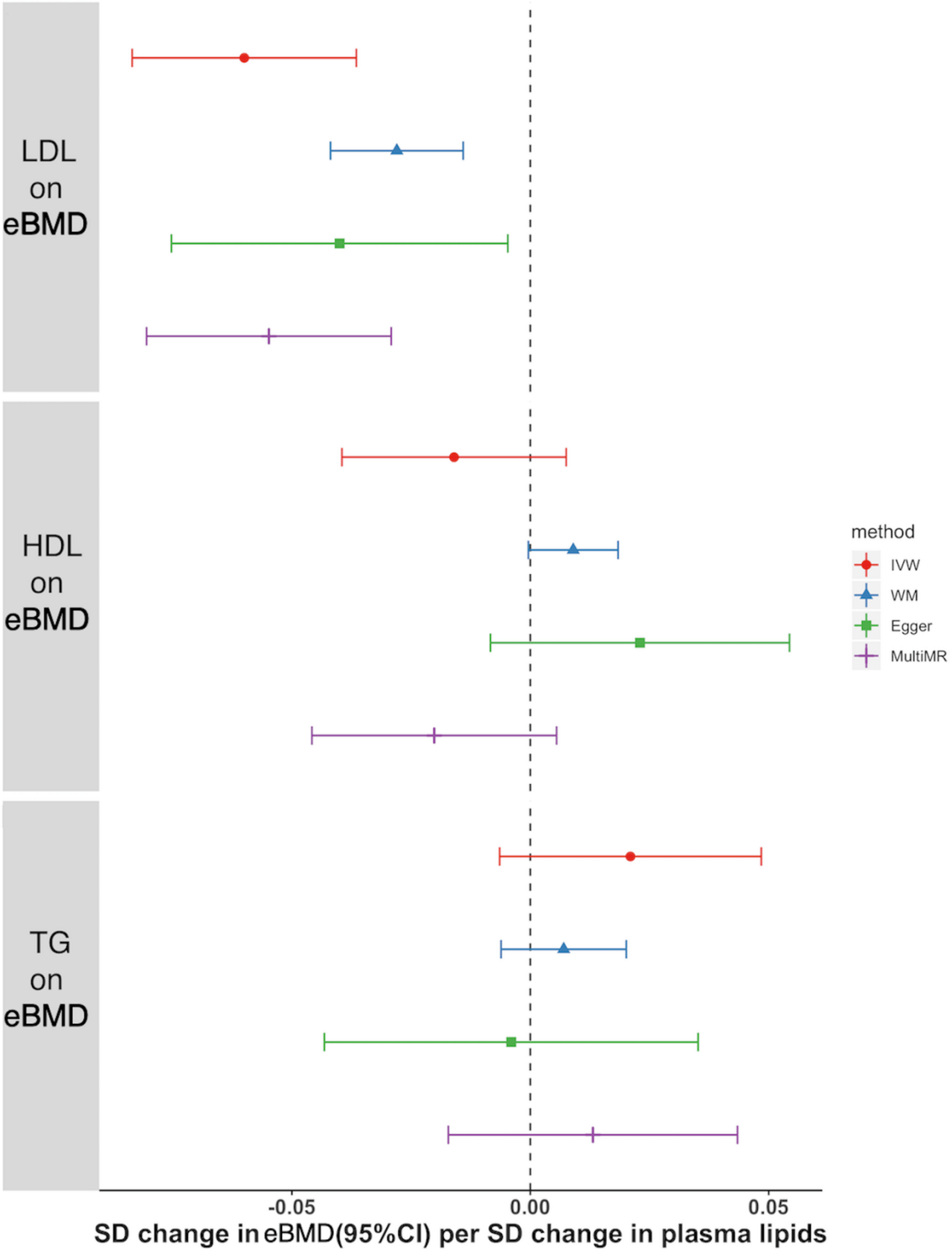
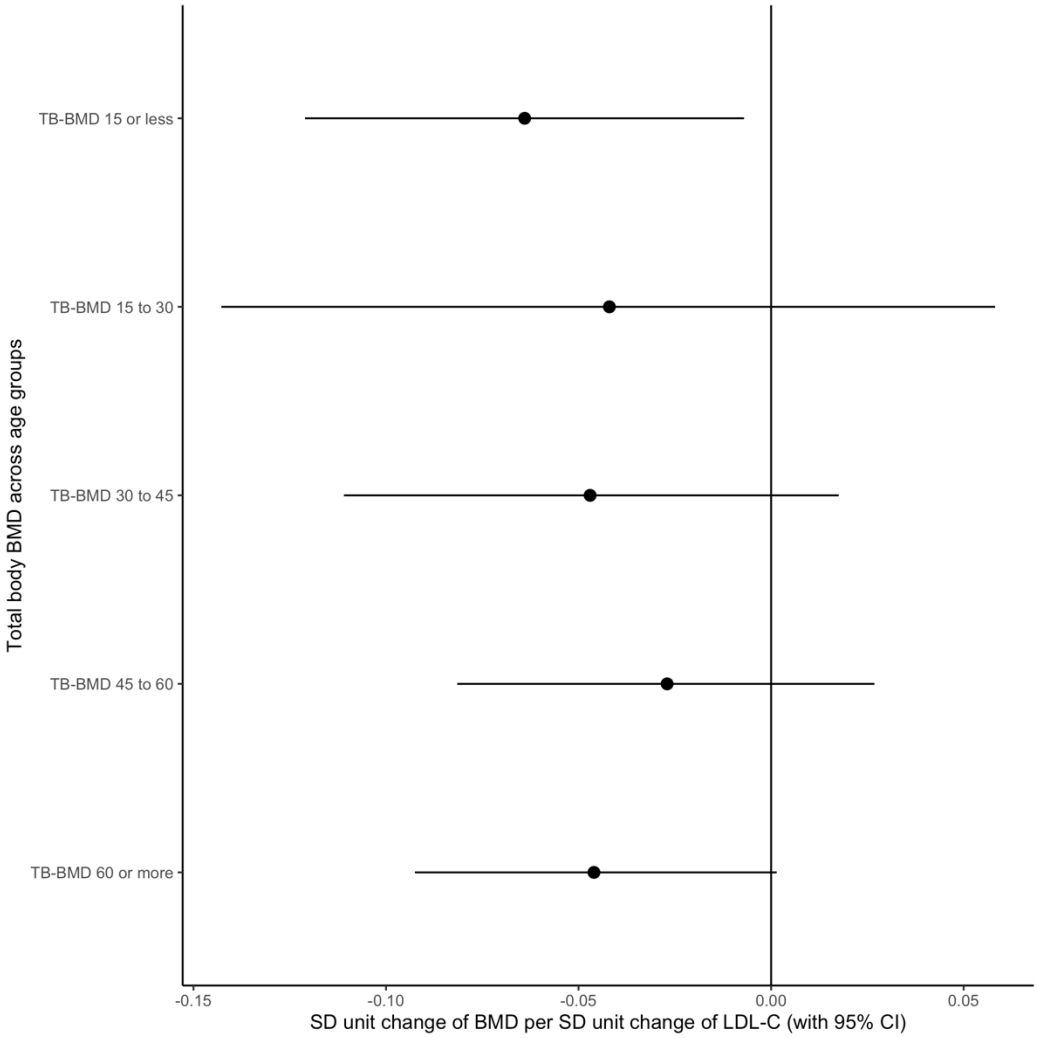

LDL
on
eBMD
HDL
on
eBMD
TG
on
eBMD
method
IVW
WM
Egger
MultiMR
-0.05
0.00
0.05
SD change in eBMD(95%CI) per SD change in plasma lipids

Figure 4


TB-BMD 15 or less
TB-BMD 15 to 30
TB-BMD 30 to 45
TB-BMD 45 to 60
TB-BMD 60 or more
Total body BMD across age groups
-0.15
-0.10
-0.05
0.00
0.05
SD unit change of BMD per SD unit change of LDL-C (with 95% CI)