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Lowering of circulating sclerostin may increase risk of atherosclerosis and its risk factors: evidence from a genome-wide association meta-analysis followed by Mendelian randomization

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ABSTRACT

Background

We aimed to establish the causal effects of lowering sclerostin, target of the anti-osteoporosis drug romosozumab, on atherosclerosis and its risk factors.

Methods

A genome-wide association study meta-analysis was performed of circulating sclerostin levels in 33,961 European individuals. Mendelian randomization (MR) was used to examine causal effects of sclerostin lowering on 15 atherosclerosis-related diseases and risk factors.

Results

18 conditionally independent variants were associated with circulating sclerostin. Of these, one *cis* signal in SOST and three *trans* signals in *B4GALNT3*, *RIN3* and *SERPINA1* regions showed directionally opposite signals for sclerostin levels and estimated bone mineral density. These four variants were selected as genetic instruments. MR using the *cis*-SNPs suggested lower sclerostin increased risk of hypertension, type 2 diabetes (T2DM) (OR=1.26; 95%CI=1.08 to 1.48) and myocardial infarction (MI) (OR=1.31, 95% CI=1.183 to 1.45); sclerostin lowering was also suggested to increase extent of coronary artery calcification (CAC) (β=0.74, 95%CI=0.33 to 1.15), levels of apoB (β=0.07; 95%CI=0.04 to 0.10) and triglycerides (β=0.18; 95%CI=0.13 to 0.24), and to reduce HDL-C levels (β=-0.14; 95%CI=-0.17 to -0.10). MR using both *cis* and *trans* instruments also suggested that lower sclerostin increased hypertension risk (odds ratio [OR]=1.09, 95%CI=1.04 to 1.15), but otherwise had attenuated effects.

Conclusions

This study provides genetic evidence to suggest that lower levels of sclerostin may increase risk of hypertension, T2DM, MI, and extent of CAC, and lead to an atherogenic lipid profile. Taken together, these findings underscore the requirement for strategies to mitigate potential adverse effects of romosozumab treatment on atherosclerosis and its related risk factors.

Key words: sclerostin, genome-wide association study, drug safety, atherosclerosis diseases and risk factors, Mendelian randomization

Introduction

Inhibition of sclerostin is a therapeutic approach to increasing bone mineral density (BMD) and lowering fracture risk in patients with osteoporosis. However, two phase III trials of romosozumab, a first-in-class monoclonal antibody that inhibits sclerostin, reported higher numbers of cardiovascular serious adverse events in the romosozumab treated group as compared to the comparator*(1,2)*. However, a similar imbalance of cardiovascular disease (CVD) was not seen in another study comparing romosozumab to placebo*(3)*. Possibly, these different results reflect a beneficial effect of bisphosphonate treatment on risk of CVD. For example, zoledronate, a bisphosphonate, has been found to decrease all-cause mortality, to which reduced CVD mortality may contribute*(4)*. However, a beneficial effect on mortality was not borne out in a meta-analysis of drug trials of zoledronate and other bisphosphonates*(5)*. The role of sclerostin in the vasculature is unknown, though some studies have shown that its inhibition may promote vascular calcification, which could increase the risk of CVD*(6)*. Given these concerns regarding CVD safety, marketing authorization for romosozumab indicates previous myocardial infarction (MI) or stroke as contraindications, underlying the urgent need to understand the causal role of sclerostin lowering on CVD outcomes, thereby providing physicians and patients with more credible information when balancing the risks and benefits of treatment.

Mendelian randomization (MR) uses genetic variants as proxies for an exposure to estimate the causal effect of a modifiable risk factor on a disease*(7)*, which minimises the bias from confounders or reverse causality. In a recent MR study using BMD-associated variants in the *SOST* region as a proxy for lower sclerostin levels, Bovijn *et al.* found genetic evidence consistent with a potential adverse effect of sclerostin lowering on CVD-related events*(8)*. However, some weaknesses of this study were discussed, e.g. the *SOST* single nucleotide polymorphisms (SNPs) used in this analysis are >30kb downstream of the target gene. Another MR study using sclerostin gene expression in arterial and heart tissue as the exposure suggested little evidence of a causal effect of sclerostin expression on risk of MI or stroke*(9)*.

An alternative approach to instrument selection is to use SNPs identified from a well-powered genome-wide association study (GWAS) of circulating sclerostin. In an earlier GWAS of sclerostin levels we identified three *trans*-acting genetic variants associated with sclerostin, including a top variant in the *B4GALNT3* region. However, we only observed marginal genetic associations in the *cis*-*SOST* region and had limited power to examine causal relationships with

extra-skeletal phenotypes*(10)*. Therefore, a more powerful GWAS of circulating sclerostin is needed to identify stronger genetic predictors, including those in the *cis*-acting region. A further consideration is that a bidirectional causal pathway appears to exist between sclerostin and BMD, whereby reduced sclerostin levels cause an increase in BMD, whereas higher BMD increases sclerostin levels, possibly reflecting a feedback pathway*(10)*. Therefore, findings from a sclerostin GWAS are potentially subject to mis-specification of the primary phenotype(11), with genetic signals being detected which are primarily related to BMD rather than sclerostin. In order to mitigate against this, we aimed to implement a SNP selection strategy intended to identify SNPs with directionally opposite associations with sclerostin levels and BMD.

The goal of the present study was to examine potential safety concerns of sclerostin lowering on atherosclerosis and its risk factors using an MR approach, based on a set of instruments derived from an updated GWAS meta-analysis of circulating sclerostin. To enable sufficient power to examine causal effects on extra-skeletal phenotypes, we aimed to identify genetic predictors of sclerostin with good instrument strength, incorporating both *cis*- and *trans*-acting variants, having assembled a sample over three times the size of our previous GWAS study*(10)*.

Materials and Methods

Summary of study design

Figure 1 illustrates the design and participants of this study. First, we conducted a GWAS meta-analysis and post-GWAS follow-up analyses of circulating sclerostin in 33,961 European individuals from nine cohorts(12–20) (details of the cohorts and the characteristics of QC, imputation and GWAS analysis of each cohort were presented in **Supplementary Note 1**). Second, we conducted MR analyses of circulating sclerostin using genetic instruments from both *cis* and *trans* regions (**Supplementary Table 1**), and from the *cis* region only (**Supplementary Table 2**). The outcomes are 15 atherosclerosis related diseases and risk factors (**Supplementary Table 3**). Bi-directional MR was conducted for the 15 atherosclerosis related diseases and risk factors using genetic instruments shown in **Supplementary Table 4**.

GWAS Meta-analysis of sclerostin

Sclerostin measures in the nine cohorts were standardized to standard deviation (SD) units. Each cohort ran a GWAS across all imputed or sequenced variants. Age and sex and the first 10 principal components (PCs) were included as covariates in all models (except INTERVANL and LURIC). Details of the GWAS model, imputation panel and covariates of each cohort are provided in **Supplementary Note 1**. We standardized the genomic coordinates to be reported on the NCBI build 37 (hg19), and alleles on the forward strand. Summary level quality control was conducted for each cohort separately using EasyQC; only individuals with European ancestry and genetic variants with MAF >1% were selected for the meta-analysis. Meta-analysis (using a fixed-effect model implemented in METAL*(21)*) was restricted to variants with a minimal sample size >10,000 individuals, MAF >1%, and high imputation quality score (R^2 >0.8 for variants imputed in MaCH*(22)* and INFO >0.8 for variants imputed in IMPUTE*(23)* (n=11,680,861 variants). Meta-analysed P value lower than 5×10^{-8} was used as a threshold to define genome-wide significant associations. A random effects model meta-analysis was also conducted using GWAMA version 2.2.2*(24)*. Heterogeneity was assessed using the I^2 statistic and Cochran's Q test. The genetic effect estimate of SNP is presented in terms of the SD unit change in sclerostin levels, scaled from the difference in sclerostin level per effect allele.

Conditional analysis and genetic fine mapping

We carried out an approximate conditional and joint genome-wide association analysis (GCTA-COJO) to detect multiple independent association signals at each of the sclerostin locus*(25)*. SNPs with high collinearity (Correlation $r^2 > 0.9$) were ignored, and those situated

more than 10 Mb away were assumed to be in complete linkage equilibrium (LD). A reference sample of 8,890 unrelated individuals of ALSPAC mothers was used to model patterns of LD between variants. Conditionally independent variants with $P<5\times10^{-8}$ were annotated to the physically closest gene list in dbSNP (https://www.ncbi.nlm.nih.gov/SNP/).

Functional mapping and annotation of sclerostin genetic association signals

Genetic colocalization of gene expression quantitative trait loci (eQTLs) and the sclerostin signals

We investigated whether the SNPs influencing serum sclerostin level were driven by *cis*-acting effects on transcription by evaluating the overlap between the sclerostin-associated SNPs and eQTLs within 500kb of the gene identified, using data derived from all tissue types from GTEx v8*(26)*. Where eQTLs overlapped with sclerostin-associated SNPs, we used genetic colocalization analysis*(27)* to estimate the posterior probability (PP) of each genomic locus containing a single variant affecting both circulating sclerostin and gene expression levels in different tissues.

We used Functional Mapping and Annotation of Genome-Wide Association Studies (FUMA) *(28)*, an integrative web-based platform (http://fuma.ctglab.nl), containing information from 18 biological data repositories and tools, to characterise the genetic association signals of sclerostin as well as gene-set enrichment using the STARNET web app*(29)* (more details in **Supplementary Note 2**).

LD score regression analyses

Estimation of SNP heritability and genetic correlation using LD score regression

To estimate the amount of genomic inflation in the data due to residual population stratification, cryptic relatedness, and other latent sources of bias, we used LD score regression*(30)*. We further quantified the overall SNP-based heritability with LD score regression using a subset of 1.2 million HapMap SNPs (SNPs in the major histocompatibility complex [MHC] region were removed due to complex LD structure). To estimate the genetic correlation between reduced sclerostin level and 12 atherosclerosis-related diseases and risk factors and two bone phenotypes, we used a platform based on LD score regression as implemented in the online web utility LD Hub*(31)*. Heritability estimate for small vessel disease was out-of-bounds and therefore this data was removed.

Mendelian randomization

Selection of genetic predictors for sclerostin

From the 18 conditionally independent sclerostin variants identified (**Supplementary Table 1A**), we selected valid genetic predictors of sclerostin for the MR using three further criteria: (i) only selected those genetic variants showed single SNP MR evidence of sclerostin on BMD estimated using ultrasound in heel (eBMD, data from UK Biobank; single SNP MR P value of sclerostin on eBMD<0.001; **Supplementary Table 1B**); (ii) the sclerostin reducing alleles of the genetic variants were associated with increased BMD level (i.e., these variants showed a negative Wald ratio for sclerostin on BMD). The final set of four genetic variants after applying these two additional criteria are listed in **Supplementary Table 1C**. The analysis using these four variants is noted as the *cis* and *trans* analysis.

Due to the greater relevance of the *cis*-acting variants, we conducted a sensitivity analysis using genetic variants restricted to *cis*-acting variants (defined as ±500 kb genomic region from the leading *SOST* SNP) (noted as the *cis*-only analysis). Of the 41 SNPs associated with circulating sclerostin (at a regional-wide association threshold<1×10^{-6}) in the *SOST* region (±500 kb genomic region from rs66838809), LD clumping identified five correlated SNPs with LD r^2<0.8 (**Supplementary Table 2**). Such an LD r^2 threshold was used here to avoid multi-collinearity caused by SNPs in very high LD. These correlated instruments were used in a generalised IVW approach that considered LD among instruments in the MR model (more details in later section).

Outcome selection

We selected eight atherosclerosis-related diseases and seven atherosclerosis-related risk factors as primary outcomes. This list comprised two endpoints related to ischaemic heart disease (coronary artery disease (CAD)*(32)* and MI*(33)*), four stroke endpoints (ischemic stroke, cardioembolic stroke, large vessel disease, small vessel disease)*(34)*, two measures of arterial calcification [CAC*(35)*, abdominal aortic calcification (AAC)*(36)*], hypertension, T2DM*(37)*, and five lipid/lipoprotein risk factors [low density lipoprotein (LDL-C), high density lipoprotein (HDL-C), triglycerides, apolipoprotein A-I (apoA-I), and apolipoprotein B (apoB)]*(38)*. Detailed information, including sample size, Mesh term and consortium name for the outcomes are listed in **Supplementary Table 3**. After applying PhenoSpD*(39)*, which takes into account the correlation between the 15 atherosclerosis-related diseases and risk factors, the number of independent tests was 9.7 (Bonferroni corrected threshold=5.15×10^{-3}).

Mendelian randomization of sclerostin on atherosclerosis-related phenotypes

For the *cis* and *trans* analysis, we applied a set of two-sample MR approaches [inverse variance weighted (IVW), MR-Egger, weighted median, single mode estimator and weighted mode estimator]*(40)* to estimate the effect of circulating sclerostin on the 15 atherosclerosis-related diseases and risk factors. Although we had a small number of relevant variants available for this analysis, we still used the MR-Egger intercept term as an indicator of potential directional pleiotropy. Heterogeneity analysis of the instruments was conducted using Cochran's Q test.

For the *cis*-only analysis, we applied a generalised IVW MR model followed by generalised Egger regression to account for LD structure between correlated SNPs in the *SOST* region and to boost statistical power*(41)*. The generalised Egger regression intercept term was used as an indicator of potential directional pleiotropy. For the *cis*, *trans*, and *cis*-only analyses, the above-mentioned Bonferroni corrected P-value threshold of 5.0×10^{-3} was used to control for multiple testing.

Bidirectional Mendelian randomization analysis of atherosclerosis-related phenotypes on sclerostin

To investigate the possibility of reverse causality between atherosclerosis-related diseases and risk factors and circulating sclerostin level, we used genetic variants associated with 15 atherosclerosis-related diseases and risk factors as genetic predictors (small vessel disease data has no valid genetic predictors, therefore, we were not able to perform bidirectional MR for this trait; for other genetic predictors, the genetic association data were extracted from relevant GWAS listed in **Supplementary Table 4A**). We applied IVW, MR-Egger, weighted median, single mode estimator and weighted mode estimator)*(40)*. In addition, due to correlation between lipids and lipoproteins, we further applied a multivariable MR model*(42)* to estimate the independent effect of each lipid and lipoprotein on sclerostin (instruments listed in **Supplementary Table 4B** and **4C**). To further validate the directionality of the analysis, we conducted Steiger filtering analysis of the four selected sclerostin instruments on the 15 atherosclerosis-related diseases and risk factors.

All MR analyses were conducted using the MendelianRandomization R package and TwoSampleMR R package (github.com/MRCIEU/TwoSampleMR v0.5.6). The strength of the

genetic predictors of sclerostin and the 15 atherosclerosis-related diseases and risk factors were estimated using F-statistics.

Results

Genome-wide association signals of circulating sclerostin

GWAS results of circulating sclerostin were available in 33,961 European ancestry participants from a meta-analysis of nine cohorts (**Supplementary Note 1**). **Supplementary Figures 1** and **2** show the Manhattan and QQ plots of association results from the fixed-effects meta-analysis of sclerostin, respectively. Little evidence of inflation in the test statistics was found (genomic inflation factor λ=1.082; LD score regression intercept =1.023). Therefore, no genomic control correction was applied to the meta-analysis results. Single trait LD score regression results showed that common variants included in the GWAS meta-analysis explained 15.4% of the phenotypic variance of circulating sclerostin (SNP-based heritability h^2=0.154, P=3.01×10^{-13}; all valid variants across the genome were used to estimate the heritability).

After applying conditional analysis using GCTA-COJO, 18 conditionally independent variants within 15 genomic loci were associated with circulating sclerostin (**Table 1**). The strongest signal, rs215223, was close to the *B4GALNT3* gene (β of A allele=-0.136, SE=0.008, P=2.44×10^{-73}, effect allele frequency=0.405, variance explained by the variant=0.89%) (**Figure 2A**). One *cis*-acting variant in the *SOST* region, rs66838809, showed a strong association with sclerostin (β of A allele=-0.088, SE=0.015, P=1.45×10^{-9}, effect allele frequency=0.079, variance explained by the variant=0.11%; **Figure 2B**). Another variant, rs28929474, in the *SERPINA1* gene region, was associated with circulating sclerostin (β of T allele=0.173, SE=0.027, P=1.1×10^{-10}, effect allele frequency=0.021, variance explained by the variant=0.12%; **Figure 2C**). This missense variant constitutes the *PiZ* allele, causing alpha-1 anti-trypsin (α1AT) deficiency in homozygous cases*(43)*. The variant, rs7143806, in the *RIN3* gene region, was also associated with sclerostin (β of A allele=0.053, SE=0.010, P=3.35×10^{-8}, effect allele frequency=0.181, variance explained by the variant=0.08%; **Figure 2D**). The gene was reported to be associated with lower limb BMD*(44)*. The other 12 genomic loci were listed in **Table 1**. Results of the random effects meta-analysis were similar to those of the fixed-effect meta-analysis (**Supplementary Table 5A**). The degree of heterogeneity was low across studies for most of the identified genetic variants (**Table 1**). However, we observed evidence for heterogeneity for the *B4GLANT3* variant, rs215223. We found that this variant showed a robust negative and significant effect on sclerostin in all cohorts, however the genetic effect estimate was particularly large in the 4D cohort, comprising individuals with end-stage CKD (**Supplementary Table 5B**). A possible explanation for this finding is that sclerostin levels are

known to be elevated in patients with CKD, presumably reflecting a contribution of renal clearance to circulating levels(45). Nonetheless, this source of variation is unlikely to limit the validity of using *B4GALNT3* to instrument sclerostin levels in the general population, from which other participating cohorts were recruited.

Genetic colocalization analysis of sclerostin association signals with gene expression

For the 18 sclerostin associated variants, we identified four variants [rs215223 (in the *B4GALNT3* region), rs28929474 (in the *SERPINA1* region), rs66838809 (in the *SOST* region) and rs7143806 (in the *RIN3* region)] where sclerostin-increasing alleles were associated with lower eBMD (at P<0.001) (**Supplementary Table 1C** and **Supplementary Figure 3**). The genetic colocalization analysis for these four variants suggested that the expression of *B4GALNT3* and *SOST* genes showed strong evidence of colocalization with circulating sclerostin levels (colocalization probability=99% and 98%, respectively; **Supplementary Table 6A**). The *SERPINA1* and *RIN3* signal showed weaker evidence of colocalization with sclerostin (**Supplementary Figure 4** and **Supplementary Table 6A**). We also confirmed that the *SOST* SNP was associated with altered *SOST* expression in iliac crest bone tissue (**Supplementary Table 6B**). More details of the other bioinformatics function follow-up could be found in **Supplementary Note 2** and **Supplementary Table 5A** and **7**.

Genetic correlation between sclerostin levels and atherosclerosis-related traits

As expected, genetic correlation analysis between circulating sclerostin using genetic variants across the whole genome revealed a relationship between lower sclerostin and higher eBMD and, to a lesser extent, lower fracture risk (**Supplementary Table 8**). These analyses also showed a genetic overlap of lower sclerostin with increased hypertension risk (r_g=0.134, P=3.10×10^{-3}; **Table 2**), but not with any other atherosclerosis-related diseases or risk factors (**Supplementary Figure 5**).

Selection of genetic instruments for circulating sclerostin

We considered the *SOST cis* variant and *B4GALNT3, SERPINA1* and *RIN3 trans* variants identified above as possible instruments for MR analyses of the effect of lower sclerostin levels on atherosclerosis risk. The remaining 14 variants identified in our GWAS did not fit with our selection criteria and were therefore excluded from further analysis (**Supplementary Figure 3**). For all four SNPs, as expected, the alleles associated with lower circulating sclerostin levels were associated with increased eBMD and reduced fracture risk **(Supplementary Figure 6)**

and provided strong instrument strength (F-statistic=89.8). To examine possible pleiotropic effects, a phenome-wide association analysis of these four variants was performed, which suggested that the *B4GLANT3*, *RIN3* and *SOST* variants were additionally associated with lean body mass. Given the strong inter-relationships between bone mass and lean body mass, lean mass is potentially on the same biological pathway as sclerostin lowering effects on bone mass, representing vertical pleiotropy and not violating the 3rd MR assumption. The RIN3 variant was also related to HbA1c, endometriosis and breast cancer. The SERPINA1 variant was relatively pleiotropic, being associated with a range of traits including sex hormone-binding globulin levels, total testosterone, cholelithiasis, COPD, CAD and prostate cancer (**Supplementary Table 9A**).

We also examined potential pleiotropy by conducting a proteome-wide association scan of the four genetic variants. Sclerostin variants within *SOST* were not associated with any other proteins. In contrast, the *RIN3* region was associated with one other protein, and variants within *B4GLANT3* and *SERPINA1* regions were associated with an additional 16 and 58 proteins respectively (**Supplementary Table 9B**). In view of findings from phenome-wide and proteome-wide association studies suggesting *trans* SNPs for sclerostin have a strong possibility of pleiotropy, we elected to base our main analyses on a *SOST cis* instrument. We included five correlated variants (rs66838809, rs1107747, rs4793023, rs80107551, rs76449013), which together had acceptable instrument strength (conditional F-statistic 27.7) (**Supplementary Table 2**).

Effects of lower sclerostin on risk of atherosclerosis-related diseases and risk factors

We used the *cis*-acting *SOST* instrument to evaluate causal effects of lower sclerostin levels on 15 atherosclerosis-related diseases and risk factors (Bonferroni-corrected threshold=5.15×10^{-3}). The IVW analysis identified potential adverse effects of lower sclerostin on increased risk of hypertension (OR=1.08, 95% CI=1.01 to 1.15, P=0.03), T2DM (OR=1.26, 95% CI=1.08 to 1.48, P=0.004) and MI (OR=1.31, 95% CI=1.183 to 1.45, P=2.17×10^{-7}; **Table 2, Supplementary Figure 7**). Genetically predicted lower sclerostin was associated with higher levels of CAC (β=0.74; 95% CI=0.33 to 1.15; P=4.27×10^{-4}) and showed effects on four of the five lipids and/or lipoproteins in the *cis*-only analysis (**Table 2, Supplementary Figure 8A**). Results of the MR Egger analyses are shown in **Supplementary Table 10A**. We observed modest LD between the *cis SOST* variant (rs4793023) and the top-associated variant with

mRNA *CD300LG* expression (rs72836567; LD r^2=0.22 in the 1000 Genomes EUR population)*(26)*, where *CD300LG* is known to be strongly associated with lipid measures*(46)*. A sensitivity analysis excluding rs4793023 suggested that decreased sclerostin levels reduced HDL-C levels and increased triglycerides levels, whereas the MR effects on other lipids/lipoproteins were attenuated after this adjustment (**Supplementary Table 10B**). Heterogeneity analysis of MR estimates of each genetic instrument suggested little evidence of heterogeneity across the five genetic instruments (Cochran's Q test P>0.05; **Supplementary Table 10A**). In contrast, we observed little evidence of a causal effect of lower sclerostin on AAC, CAD, stroke (and its subtypes) and LDL-C.

We also examined causal effects of sclerostin using a *cis+trans* genetic instrument which also included *B4GALNT3, SERPINA1* and *RIN3* SNPs. The MR effects on stroke were estimated using two variants in the *B4GLANT3* and *RIN3* regions, where the genetic association information of the other two variants were missed in the stroke outcome datasets. Lower circulating sclerostin was associated with an increased risk of hypertension (OR per SD decrease in sclerostin=1.09, 95%CI=1.04 to 1.15, $P=7.93\times10^{-4}$), whereas the effects are generally attenuated for other outcomes in the cis+trans analyses (**Supplementary Figure 7** and **8**). Sensitivity analyses suggested little evidence of horizontal pleiotropy (Egger regression intercept=-0.003, P=0.27) or heterogeneity (Cochran's Q=2.85, P=0.42; **Supplementary Table 9C**). In contrast, little evidence for a causal effect of lower sclerostin on any other atherosclerosis-related disease or risk factor was identified (**Supplementary Table 9C** and **9D**).

Effects of atherosclerosis-related diseases and risk factors on circulating sclerostin

We further conducted bidirectional MR to evaluate the potential reverse causality of 15 atherosclerosis-related diseases and risk factors on circulating sclerostin (instruments listed in **Supplementary Table 4**). A marginal positive relationship for liability of T2DM on sclerostin was observed (β=0.02, SD change in sclerostin per unit increase of risk score of T2DM, 95%CI=0.001 to 0.045, P=0.04; **Supplementary Table 11A**). ApoB was negatively associated with sclerostin levels (β=-0.03, 95%CI=-0.01 to -0.06, $P=3.67\times10^{-3}$). However, the multivariable MR including apoB, LDL-C and triglycerides in the same model suggested that increased apoB levels increased sclerostin levels (β=0.03, 95%CI=0.001 to 0.07, P=0.041; **Supplementary Figure 8B** and **Supplementary Table 11B**). No other atherosclerosis-related

disease or risk factor showed a reverse effect on sclerostin (**Supplementary Table 11A**). Sensitivity analyses provided little evidence to suggest directional pleiotropy or heterogeneity of the causal estimates (**Supplementary Table 11A**). The Steiger filtering analysis further confirmed that the sclerostin instruments were likely to first change the sclerostin level and then influence the atherosclerosis outcomes as a causal consequence (**Supplementary Table 11C**).

DISCUSSION

We have presented findings from an updated GWAS meta-analysis of circulating sclerostin, which identified 18 sclerostin-associated variants, of which four in the *SOST*, *B4GALNT3, RIN3* and *SERPINA1* genes provided useful genetic instruments for determining the causal effects of lower sclerostin levels on atherosclerosis-related diseases and risk factors based on inverse relationships between sclerostin levels and BMD. Lower sclerostin levels showed similar causal effects on hypertension risk using both *cis*-only and combined *cis* and *trans* instruments, without evidence of reverse causality. *cis*-only analyses suggested additional causal effects of lower sclerostin levels on atherosclerosis-related diseases and risk factors, and in particular that lower levels of sclerostin increases risk of MI as a consequence of greater CAC. However, whereas the *cis* instrument suggested a causal effect of sclerostin lowering on CAC and MI, there was no equivalent effect on AAC or stroke.

These findings are in part consistent with two previous phase three trials with the sclerostin inhibitor, romosozumab, which found an increased event rate for myocardial infarction in those randomised to active treatment in postmenopausal women(1) and in men(2). That said, these trials also found an increased signal for stroke, whereas MR analyses in the present study were null with respect to stroke. One potential explanation for this apparent discrepancy is that our analyses for stroke had limited power, with both *cis* only and *cis+trans* analyses based on only two SNPs as the remaining SNPs were missing in the outcome GWAS dataset. Alternatively, suggestions of increased stroke risk in these two trials may have been spurious due to chance fluctuations in low absolute event rates , and equivalent findings were not observed in a third phase three trial(1).

Cis instruments are more likely to directly link with biology, which aligns with our finding that *cis*-only analyses identified more extra-skeletal effects of sclerostin. *Trans* instruments are, by their nature, more likely to be pleiotropic, which was supported by findings from phenome- and proteome-wide analyses suggesting all three *Trans* instruments selected had a high potential for pleiotropy . Additionally, *cis* variants may be better predictors ofsclerostin levels in tissues responsible for mediating biological effects. Based on eQTL data using bone tissue, the *cis* signal is predicted to alter expression and hence local levels of sclerostin in bone cells. Osteocytes, embedded within bone and constituting approximately 80% of bone cells, are the primary source of sclerostin, which then circulates locally through canaliculi to modulate the

activity of other bone cells, including osteoblasts, leading to changes in bone mass and strength*(47)*. Accordingly, the *cis* signal is expected to alter circulating levels of sclerostin through exchange between bone tissue and the circulation. In contrast, we previously hypothesised that the *trans* signal, *B4GALNT3*, replicated in the present study, primarily influences circulating sclerostin levels by affecting plasma clearance due to altered protein glycosylation*(10)*. Hence, any changes in tissue sclerostin levels resulting from the *B4GALNT3 trans* signal are likely to be secondary to altered circulating levels, rather than local production. Therefore, by its nature, the B4GALNT3 *trans* signal is expected to produce smaller changes in tissue sclerostin levels compared to a *cis SOST* signal, leading to a weaker effect on eBMD.

That the *SOST cis* signal is likely to produce greater increases in tissue sclerostin levels compared to *trans* signals, provides an explanation as towhy the *cis*-only analyses predicted more extra-skeletal effects of sclerostin lowering compared to the *cis+trans* analyses. Sclerostin is also expressed in vascular tissues including at sites of vascular calcification*(48)*, suggesting any effects of sclerostin on vascular tissues may also involve local sclerostin expression. Such an effect is likely mediated by sclerostin's well recognised action as a WNT inhibitor*(49)*, given the contribution of WNT signalling to the development of atherosclerosis*(50)*.

Pharmacokinetic studies suggest that romosozumab is largely retained within the circulation*(51)*, in-keeping with the relatively large size of a monoclonal antibody. That said, the pharmacological action of romosozumab, involving neutralisation of sclerostin activity in bone tissue, depends on the antibody penetrating skeletal tissue after systemic administration, which is likely to involve convection or endocytosis/pinocytosis via endothelial cells*(52)*. To the extent that effects of romosozumab on CVD risk also involve local tissue penetration, a *cis* instrument reflecting tissue levels of sclerostin may be more likely to predict effects of romosozumab on CVD risk than a *trans* instrument more closely linked to systemic levels.

There have also been several previous observational studies examining associations between circulating sclerostin and atherosclerosis related diseases and risk factors. Our recent observational study found observational associations in the opposite direction to those causal effects predicted by our MR analyses*(53)*, particularly in analyses restricted to the *cis* instrument. Interestingly, directionally opposite effects have also been observed in the case of eBMD and atherosclerosis risk, with a protective effect found in an observational analysis but

a harmful effect predicted by MR analyses*(53)*. The latter finding also raises the possibility that any effect of sclerostin lowering on atherosclerosis risk might be an indirect consequence of increased BMD, as opposed to a specific effect of sclerostin. However, arguing against this suggestion, there is little evidence that other therapeutic agents for osteoporosis acting to increase BMD affect atherosclerosis risk, apart from strontium ranelate for which the European Medicines Agency issued a warning, restricting use in those with a high risk of CVD*(54)*.

Two previous studies have used MR approaches to examine causal effects of sclerostin lowering on atherosclerosis and related risk factors. Bovijn *et al.* reported that two conditionally independent *SOST* SNPs, selected on the basis of their association with eBMD, predicted higher risk of MI and/or coronary revascularization, major cardiovascular events, hypertension, and T2DM*(8)*. Our MR finding on MI, using the *cis*-only instrument for circulating sclerostin, is consistent with these observations. In contrast, Holdsworth *et al.* found no association between gene expression level of *SOST* in tibial artery/heart tissue and risk of CVD, using three cis *SOST* eQTLs as instruments*(9)*. Despite the distinct methods used to proxy sclerostin lowering, our *cis* instrument is in strong LD with those used in these other studies. Indeed, our *cis* instrument shares an identical SNP with the Holdsworth study (see **Supplementary Table 12**). In terms of explanations for the differences observed, eQTL data from Holdsworth et al. was based on tibial artery/heart tissues, whereas circulating sclerostin as measured in the present study is mainly derived from bone, so different findings likely reflect distinct genetic regulatory mechanisms in between different tissues. Given the known relationship between bone and glucose metabolism*(55)*, the potential adverse effect of lower levels of sclerostin on T2DM also need further investigation in future RCTs.

In terms of other *trans*-acting pathways, we have identified two new *trans* signals for sclerostin, *RIN3* and *SERPINA1*. Previous GWASs have identified *RIN3* in association with lower limb and total BMD in children*(44)*, and Paget's disease of bone*(56)*. Homozygosity of *SERPINA1* underlies deficiency of α1AT, a glycoprotein mostly produced by the liver, which serves to protect lung tissue from tissue damage caused by proteases released from neutrophils. The loss of function allele was associated with higher sclerostin levels, and the mechanisms underlying this genetic association are unclear. α1AT deficiency causes early-onset chronic obstructive pulmonary disease (COPD)*(57)*, however we are not aware of any previous findings relating

α1AT to BMD or risk of osteoporosis. Given the lack of evidence of colocalization, it is also possible that a different gene was responsible for the genetic signal identified at this locus.

In terms of strengths, the present study had sufficient sample size to clearly detect a *cis (SOST)* signal, and our genetic instrument successfully accounted for bidirectional effects between sclerostin and BMD, by removing *trans* SNPs with the same direction of effect on sclerostin and eBMD. Our MR of sclerostin effects on atherosclerosis-related diseases and risk factors used circulating level of sclerostin as the exposure, which may predict adverse effects from sclerostin antibody inhibition more accurately than previous studies using BMD or *SOST* arterial expression as exposures. Finally, since genetic predictors in the *cis*- and/or *trans*-acting regions may yield different causal estimates on outcomes, we considered these separately. In terms of weaknesses, though postmenopausal women are the main target group for osteoporosis treatments such as romososumab, we were only able to examine predicted effects of sclerostin lowering in males and females combined, due to the lack of availability of sex-specific sclerostin GWAS dataset. In addition, the different cohorts used distinct methods to measure sclerostin, with the over half providing sclerostin measures through the SomaLogic platform, while the other half used a specific ELISA. However, despite these methodological differences, there was little evidence of heterogeneity of genetic associations between cohorts.

In conclusion, our updated GWAS meta-analysis of circulating sclerostin now identified a robust *cis* (*SOST*) signal, replicated our previous *B4GALNT3* signal, and identified new *trans* signals in the *RIN3* and *SERPINA1* genes. Genetically predicted lower sclerostin levels were found to associate with higher risk of hypertension, MI and T2DM, increased CAC, increased apoB and TG levels and reduced HDL-C levels. To the extent that genetically predicted lower lifelong exposure to sclerostin shares consequences with pharmacological inhibition over 12 months, our results underscore the requirement for strategies to mitigate potential adverse effects of romosozumab treatment on atherosclerosis and its related risk factors.

FIGURE LEGENDS

Figure 1. Summary of the design and results of the current study. This study included four major components: (1) meta-analysis of genome-wide association study of circulating sclerostin; (2) signal genetic trait analysis and functional annotation of the top sclersotin signals; (3) Mendelian randomizaiton and genetic correlation analysis of sclerostin on 15 atherosclerosis-related diseases and risk factors traits; (4) bidirectional Mendelian randomization analysis of 15 atherosclerosis-related diseases and risk factors on sclerostin.

Figure 2. Regional plot for the *B4GLANT3* (A), *SOST* (B), *SERPINA1* (C) and *RIN3* (D) regions. For each subplot, the upper part presented the genetic association information of variants on sclerostin within each of the four regions. The purple dot is the top association signal in each region. The dot in red to green are those variants in LD with the top signal. The middle part presented genes within each of the region. Genes in red are those genes that were mapped to the genetic association signals within this region. Genes in blue are those protein-coding genes that were not mapped to any of the genetic association signals. Genes in black are those non-protein coding genes that were not mapped to any genetic signals. The bottom part was the chromatin states of the genetic association signals. Different colors refer to different regulation elements. Detailed description of the regulation elements listed in **Supplementary Table 13**. For subplot A, Two SNPs were presented, rs215226 and rs215223. Rs215226 was the top hit for the previous GWAS meta-analysis of sclerostin. We highlighted it in the regional plot to show that it is in perfect LD with the top hit of the current GWAS meta-analysis, rs215233.

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Table 1. Meta-analysis results for loci that reached genome-wide significance ($P < 5 \times 10^{-8}$).

Locus	SNP	EA	OA	EAF	GENE	Cis/trans	BETA	SE	P	Q	Q P	I^2	R^2
chr1 50566286	rs61781020	A	G	0.049	*FAF1*	*Trans*	0.101	0.018	2.57×10^{-8}	17.825	0.058	0.439	0.10%
chr2 229236796	rs4973180	T	C	0.821	*PID1*	*Trans*	0.059	0.010	1.04×10^{-9}	6.691	0.754	0	0.10%
chr5 56813327	rs11960484	A	G	0.351	*MAP3K1*	*Trans*	-0.049	0.008	1.40×10^{-10}	14.823	0.139	0.325	0.11%
chr5 115994797	rs34498262	A	G	0.391	*LVRN*	*Trans*	0.065	0.008	1.41×10^{-17}	10.403	0.406	0.039	0.20%
chr5 116013119	rs17138656	A	G	0.124	*LVRN*	*Trans*	0.096	0.011	3.16×10^{-17}	14.273	0.161	0.299	0.20%
chr6 45189983	rs75523462	T	G	0.950	*SUPT3H*	*Trans*	0.104	0.017	1.31×10^{-9}	7.424	0.685	0	0.10%
chr6 133044782	rs34366581	T	G	0.326	*LINC00326*	*Trans*	0.047	0.008	2.80×10^{-9}	13.204	0.212	0.243	0.10%
chr8 119000461	rs11995824	C	G	0.454	*TNFRSF11B*	*Trans*	0.100	0.007	5.62×10^{-41}	16.222	0.093	0.384	0.49%
chr10 122342063	rs6585816	T	G	0.209	/	*Trans*	0.056	0.009	7.84×10^{-10}	6.121	0.805	0	0.10%
chr12 481093	rs215223	A	G	0.405	*B4GALNT3*	*Trans*	-0.136	0.008	2.44×10^{-73}	83.297	1.13×10^{-13}	0.880	0.89%
chr13 42378009	rs9594738	T	C	0.482	*TNFSF11*	*Trans*	-0.056	0.007	6.48×10^{-14}	9.217	0.512	0	0.15%
chr13 42513606	rs34136735	T	C	0.052	*TNFSF11*	*Trans*	0.171	0.017	5.69×10^{-23}	17.750	0.059	0.437	0.29%
chr13 42532378	rs665632	T	C	0.813	*TNFSF11*	*Trans*	0.082	0.010	4.82×10^{-17}	8.502	0.484	0	0.20%
chr14 92637384	rs7143806	A	G	0.181	*RIN3*	*Trans*	0.053	0.010	3.35×10^{-8}	13.360	0.204	0.251	0.08%
chr14 94378610	rs28929474	T	C	0.021	*SERPINA1*	*Trans*	0.173	0.027	1.10×10^{-10}	5.342	0.867	0	0.12%
chr17 43721253	rs66838809	A	G	0.079	*SOST*	*Cis*	-0.088	0.015	1.45×10^{-9}	13.369	0.147	0.327	0.11%
chr18 62390996	rs2957124	A	G	0.421	*TNFRSF11A*	*Trans*	-0.057	0.008	5.97×10^{-14}	11.286	0.257	0.203	0.16%
chr20 11231094	rs13042961	T	C	0.955	*JAG1*	*Trans*	0.126	0.019	4.65×10^{-11}	16.398	0.037	0.512	0.14%

Note: Locus (chromosome and position of the SNP), EA (effect allele), OA (other allele), EAF (effect allele frequency), GENE (nearest gene to the sclerostin associated SNP); Cis/trans (the associated SNP is close to the SOST region [noted as cis] or far away from this region [noted as trans]); BETA (SD change in serum sclerostin per effect allele), SE (standard error) and P (p-value)). Heterogeneity test (Q (Cochran's Q statistics), Q_P (Cochran's Q P value), I^2 (I^2 statistics)). R^2 is the variance explained by each of the top sclerostin variants.

Table 2. Mendelian randomization and genetic correlation analysis results of the effect of lower sclerostin levels on atherosclerosis and related risk factors.

Exposure	Outcome	Model	N SNPs	OR	LCI	UCI	P
Lower sclerostin levels	Type 2 diabetes	*Cis*-only MR	5	1.26	1.08	1.48	3.66×10^{-3}
Lower sclerostin levels	Myocardial infarction	*Cis*-only MR	5	1.31	1.18	1.45	2.17×10^{-7}
Lower sclerostin levels	Coronary artery disease	*Cis*-only MR	5	1.05	0.93	1.17	0.435
Lower sclerostin levels	Hypertension	*Cis*-only MR	5	1.08	1.01	1.15	0.030
Lower sclerostin levels	Ischemic stroke	*Cis*-only MR	2	0.93	0.56	1.52	0.763
Lower sclerostin levels	Cardioembolic stroke	*Cis*-only MR	2	0.88	0.33	2.34	0.797
Lower sclerostin levels	Large vessel disease	*Cis*-only MR	2	0.83	0.29	2.39	0.731
Lower sclerostin levels	Small vessel disease	*Cis*-only MR	2	0.57	0.19	1.72	0.318

Exposure	Outcome	Model	N SNPs	BETA	SE	P
Lower sclerostin levels	Coronary artery calcification	*Cis*-only MR	5	0.740	0.210	4.27×10^{-4}
Lower sclerostin levels	Aortic calcification	*Cis*-only MR	5	0.124	0.101	0.221
Lower sclerostin levels	Low density lipoprotein	*Cis*-only MR	5	0.013	0.016	0.429
Lower sclerostin levels	High density lipoprotein	*Cis*-only MR	5	-0.135	0.018	2.03×10^{-14}
Lower sclerostin levels	Triglyceride	*Cis*-only MR	5	0.184	0.026	2.82×10^{-12}
Lower sclerostin levels	Apolipoprotein A-I	*Cis*-only MR	5	-0.088	0.016	6.92×10^{-8}
Lower sclerostin levels	Apolipoprotein B	*Cis*-only MR	5	0.071	0.017	1.94×10^{-5}

Trait 1	Trait 2	Model	N SNPs	r_g	SE_r_g	P_r_g
Lower sclerostin levels	Coronary artery calcification	Genetic correlation	All SNPs	0.007	0.083	0.933
Lower sclerostin levels	Hypertension	Genetic correlation	All SNPs	0.134	0.045	3.10×10^{-3} **
Lower sclerostin levels	Type 2 diabetes	Genetic correlation	All SNPs	-0.041	0.073	0.573

Note: Model refers to which statistical method/model was applied. For *cis*-only analysis, inverse variance weighted MR method was used. N_snps means the number of genetic variants been included as predictors for sclerostin. Estimate, SE and P (and r_g, SE_r_g, P_r_g) are the association estimates, standard error and P value of the MR (or the genetic correlation analysis). OR, LCI and UCI are the odds ratio and 95% confidence interval of the MR estimates using *cis*-acting variants, which refers to odds ratio of disease risk per standard deviation unit lowering of sclerostin levels, and is not applicable for the genetic correlation analysis. Importantly, the Mendelian randomization and genetic correlation analyses have different assumptions therefore the effect estimate is not directly comparable. We listed them in the same table to compare the direction of effects and the P value estimates across the two approaches.

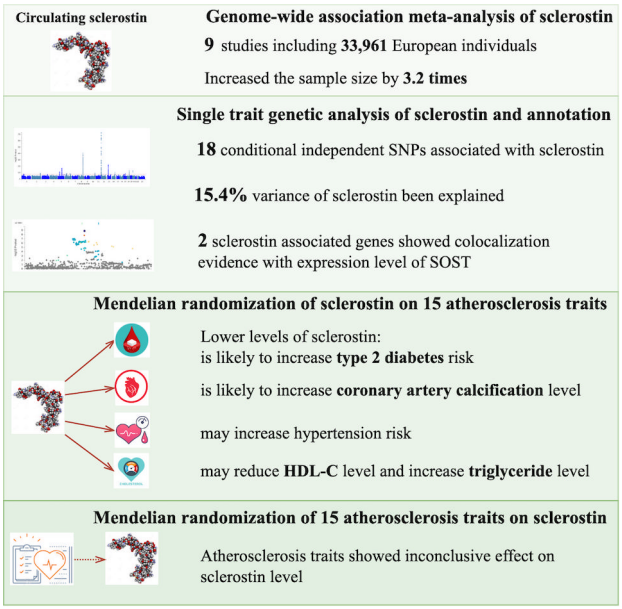


Figure1.DAG.v2.tiff

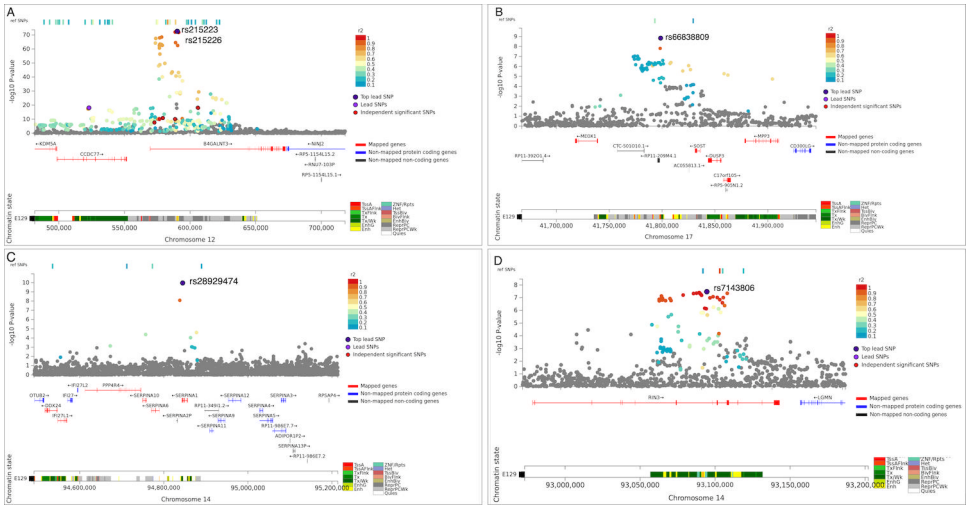
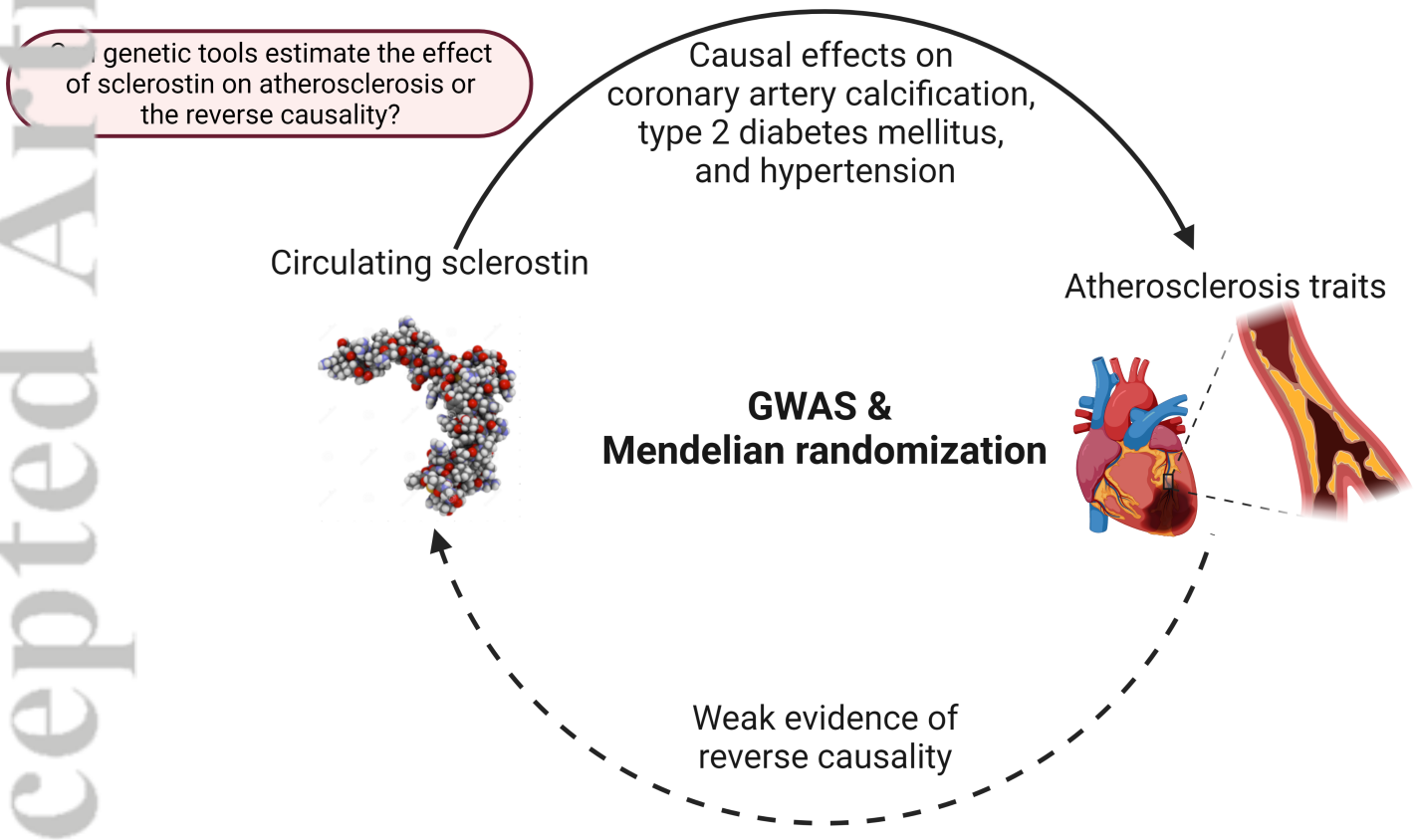


Figure2.regional-plots.tiff

Bidirectional Relationships Between Sclerostin and Atherosclerosis Traits



Zheng J, Wheeler E, Pietzner M, et al. Lowering of circulating sclerostin may increase risk of atherosclerosis and its risk fac[illegible]idence from a GWAS followed by Mendelian randomization. Arthritis Rheumatol 2023.

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