Genetic predisposition to increased serum calcium, bone mineral density, and fracture risk in individuals with normal calcium levels: mendelian randomisation study

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ABSTRACT

OBJECTIVE
To determine if genetically increased serum calcium levels are associated with improved bone mineral density and a reduction in osteoporotic fractures.

DESIGN
Mendelian randomisation study.

SETTING
Cohorts used included: the UK Biobank cohort, providing genotypic and estimated bone mineral density data; 25 cohorts from UK, USA, Europe, and China, providing genotypic and fracture data; and 17 cohorts from Europe, providing genotypic and serum calcium data (summary level statistics).

PARTICIPANTS
A genome-wide association meta-analysis of serum calcium levels in up to 61 079 individuals was used to identify genetic determinants of serum calcium levels. The UK Biobank study was used to assess the association of genetic predisposition to increased serum calcium with estimated bone mineral density derived from heel ultrasound in 426 824 individuals who had, on average, calcium levels in the normal range. A fracture genome-wide association meta-analysis comprising 24 cohorts and the UK Biobank including a total of 76 549 cases and 470 164 controls, who, on average, also had calcium levels in the normal range was then performed.

RESULTS
A standard deviation increase in genetically derived serum calcium (0.13 mmol/L or 0.51 mg/dL) was not associated with increased estimated bone mineral density (0.003 g/cm^2, 95% confidence interval −0.059 to 0.066; P=0.92) or a reduced risk of fractures (odds ratio 1.01, 95% confidence interval 0.89 to 1.15; P=0.85) in inverse-variance weighted mendelian randomisation analyses. Sensitivity analyses did not provide evidence of pleiotropic effects.

CONCLUSIONS
Genetic predisposition to increased serum calcium levels in individuals with normal calcium levels is not associated with an increase in estimated bone mineral density and does not provide clinically relevant protection against fracture. Whether such predisposition mimics the effect of short term calcium supplementation is not known. Given that the same genetically derived increase in serum calcium is associated with an increased risk of coronary artery disease, widespread calcium supplementation in the general population could provide more risk than benefit.

WHAT IS ALREADY KNOWN ON THIS TOPIC
Calcium supplementation in the general population is common and often intended to reduce the risk of fracture

Calcium supplementation has been associated with an increased risk of coronary artery disease and its protective effects on bone health remain unclear

WHAT THIS STUDY ADDS
Genetic predisposition to increased serum calcium levels in individuals with normal calcium levels is not associated with an increase in estimated bone mineral density and does not provide clinically relevant protection against fracture

Given that the same genetically derived increase in serum calcium is associated with an increased risk of coronary artery disease, widespread calcium supplementation in the general population might provide more risk than benefit

Introduction
Fragility fractures are a large problem worldwide in both women and men, with an impact on quality of life and mortality.[1 2] Calcium supplementation is promoted and emphasised by prevention and treatment guidelines to reduce the risk of osteoporosis and fractures,[3-5] and is now common among the general adult population in high income countries.[6-8] For example, in the NHANES study, 53% of Americans used dietary calcium supplements and 43% reported daily use.[9]

Evidence, however, from multiple studies indicates that increased serum calcium, a short term consequence of calcium supplementation, is associated with an increased risk of cardiovascular disease and mortality.[10 11] A meta-analysis of serum calcium on incident risk of cardiovascular disease indicated that serum calcium was associated with an increased risk of cardiovascular disease.[12] Higher circulating calcium levels have also been found to be associated with an increased risk of stroke in observational studies.[13 14]

However, observational associations of serum calcium with cardiovascular disease can be susceptible to confounding, even after controlling for known risk factors of the disease. Consequently, randomised controlled trials of calcium supplement use were undertaken because the process of randomisation breaks the association with confounding variables. Although these randomised controlled trials did not prespecify cardiovascular disease as a primary outcome, several meta-analyses have provided conflicting evidence, and most of these studies relied on short term calcium supplementation.[10 15-19]

Another way to overcome confounding is by mendelian randomisation. Mendelian randomisation is an established genetic epidemiology method that uses natural genetic variation to strengthen causal inference by mimicking a lifelong randomised controlled trial.[20] Specifically, genetic variants are identified that are reproducibly associated with the risk factor and are then tested for their combined effect on the disease outcome. Since genetic variants are randomly assigned at conception, this method greatly decreases confounding. Further, since conception always precedes disease onset, such studies are not prone to reverse causation. Mendelian randomisation studies are less prone to regression dilution bias than observational studies because genotypes are measured with a high degree of precision. Lastly, mendelian randomisation provides an estimate of lifelong exposure. Nonetheless, mendelian randomisation studies are limited by potential bias owing to horizontal pleiotropy, where the genetic variant influences the outcome, independently of the exposure, among other limitations.[21]

Our recent mendelian randomisation study found that lifelong genetically predicted increased serum calcium levels were associated with a higher risk of coronary artery disease and myocardial infarction,[22] such that a one standard deviation increase in serum calcium (0.13 mmol/L or 0.51 mg/dL) was associated with an increased odds of coronary artery disease (odds ratio 1.25, 95% confidence interval 1.08 to 1.45, P=0.003), comparable with previous randomised controlled trial meta-analysis estimations.[12] Thus, given the risks of increased serum calcium and the high prevalence of calcium supplementation, it is important to understand the potential beneficial effects of calcium on skeletal health and fracture so that patients and their doctors can balance potential risks against potential benefits.

Clearly calcium is required for normal skeletal development and maintenance since net calcium excretion must be replaced.[5] Indeed, severe hypocalcemia due to deficient calcium or vitamin D intake, or both, leads to diminished bone density and increased risk of fracture, which is improved with increased calcium intake.[23-32] Yet, what remains unclear is whether additional calcium supplementation to an ordinary diet can lead to clinically relevant improvements in heel ultrasound bone mineral density and prevent fractures in the general adult population, who in general, have normal serum calcium and parathyroid hormone levels. Randomised controlled trial data supporting calcium supplementation to prevent fractures is inconsistent and even large trials, such as the Women's Health Initiative, have not shown any reduction in the risk of fracture with calcium plus vitamin D in community dwelling older women and men.[33-38] Calcium supplementation alone might even increase the risk of hip fracture, the most devastating type of fragility fracture.[38 39] Further, a recent randomised controlled trial using bisphosphonates to prevent bone fractures found profound beneficial effects without calcium supplementation,[40] a result also supported by another randomised controlled trial with bone mineral density as an outcome.[41]

Given the potential risks of calcium supplements and their widespread use, it is important to understand if increasing calcium results in a reduced risk of osteoporosis and fracture. We therefore assessed whether genetically predicted lifelong higher serum calcium levels were associated with bone mineral density and the risk of fracture by using mendelian randomisation. To do so, we identified the genetic determinants of serum calcium levels in 61 079 individuals and tested their effect on estimated bone mineral density (n=426 824) and the risk of fracture (76 549 cases and 470 164 controls).

Methods

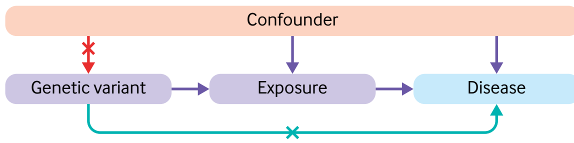
Study design and data sources

Selection of instrumental variables

Figure 1 shows that the causal interpretation of mendelian randomisation estimates relies on three assumptions. Firstly, the genetic variants, termed single nucleotide polymorphisms, must be associated with the risk factor of interest. Secondly, the genetic variant must not be associated with confounders (common causes of the risk factor and outcome association), which are not in the causal pathway between the risk factor and the outcome. Thirdly, the genetic variant is independent of the outcome conditional on the risk factor and confounders (that is, absence of horizontal pleiotropy). This means that the genetic variant's effect on the outcome should only be mediated by the risk factor and, thus, not have a direct effect on the outcome independent of the risk factor. We undertook a two sample mendelian randomisation approach to test the effect of increased serum calcium on bone mineral density and fracture.[42] Two sample mendelian randomisation identifies genetic variants to be associated with the exposure in one dataset and then tests the association of these variants with the outcome in a separate dataset. The advantage of this approach is that it allows for larger sample sizes, providing more precise estimates of effect of the exposure on the outcome. For an overview of the concepts and methods deployed in mendelian randomisation studies, we refer interested readers to a recent review by Holmes and colleagues.[43]

Associations between single nucleotide polymorphisms and serum calcium concentration

We obtained seven single nucleotide polymorphisms associated with total serum calcium concentrations at a genome-wide significant level of $P<5x10^{-8}$ from the largest serum calcium genome-wide association study meta-analysis to date.[44] Genome-wide significant associations between the single nucleotide polymorphism and serum calcium comply with the first assumption of mendelian randomisation (that is, association between instrument and exposure). The study consisted of a discovery cohort of 39 400 individuals of European descent from 17 population



Mendelian randomisation assumptions
1. Genetic variants are associated with exposure (calcium levels)
2. Genetic variants are not associated with potential confounders
3. Genetic variants are not associated with outcome (eg, fracture), except through the exposure (such as a lack of horizontal pleiotropic effects)

Fig 1 | Mendelian randomisation assumptions

based studies. Serum calcium concentrations were quantified by colorimetric assay in most cohorts.

Discovery analysis identified 14 genome-wide significant single nucleotide polymorphisms associated to serum calcium concentration, of which seven were replicated in a subsequent replication cohort of 21 676 individuals of European descent. The replicated single nucleotide polymorphisms were rs1801725 in *CASR*, rs1550532 in *DGKD*, rs780094 in *GCKR*, rs7336933 near *VWA8* and *DGKH*, rs10491003 near *GATA3*, rs7481584 in *CARS*, and rs1570669 near *CYP24A1* (table 1). In addition to the seven replicated single nucleotide polymorphisms, we included a genetic variant, rs17711722: *VKORC1L1* locus, which failed replication as defined by O'Seaghdha and colleagues (P>0.05 for replication),[44] although the single nucleotide polymorphism remained genome-wide significant after meta-analysis of their discovery and replication results. In addition, rs17711722: *VKORC1L1* locus displayed functional plausibility given that it encodes an enzyme involved in vitamin K physiology, which is associated with calcium homeostasis.[45,46]

Bone mineral density and fracture genome-wide association study

Bone mineral density is a clinically relevant measure used to diagnose osteoporosis and to risk-stratify for fracture. Estimated bone mineral density is derived from two heel ultrasound measures, velocity of sound, and broadband ultrasound attenuation, which is highly heritable (50% to 80%),[47] and is a strong predictor of risk of fracture.[48,49] To estimate the effect of serum calcium level on estimated bone mineral density and fracture we obtained summary statistics for the associations between the eight calcium modifying single nucleotide polymorphisms and estimated bone mineral density and fractures risk from our recent genome-wide association study on estimated bone mineral density consisting of 426 824 white British individuals from the UK Biobank.[50] In this well powered genome-wide association study, 518 genome-wide significant loci accounted for 20% of the total variance in estimated bone mineral density, using 13.7 million single nucleotide polymorphisms imputed to the Haplotype Reference Consortium panel.[51]

We performed an updated fracture fixed effect meta-analysis comprising a total of 24 cohorts from two recently published fracture genome-wide association studies, which included 23 cohorts from Genetic Factors for Osteoporosis consortium (GEFOS), EPIC-Norfolk study, and UK Biobank's full release.[50,52] The meta-analysis involved a total of 76 549 cases and 470 164 controls (table 2). Fracture cases in GEFOS and EPIC-Norfolk cohorts included adults who had any fractures confirmed by medical, radiological, or questionnaires with the exclusion of skull, fingers, and toes as well as high trauma fractures, when available. The details of case ascertainment of each cohort were described previously.[52] For UK Biobank, we included fracture cases reported from either hospital based fracture diagnosis according to ICD-10 (international classification of diseases, 10th revision) codes, or questionnaire based self reported fracture. Fractures located at skull, face, hands and feet, pathological, and atypical fractures; periprosthetic fractures were excluded. Detailed description of fracture ascertainment in UK Biobank was described previously.[50] Controls from all cohorts were defined as patients without a history of fracture. Approximately 69.5% of all fracture cases and 78.1% of all samples in the 24 cohorts were from UK Biobank, whereas GEFOS

Table 1 | Summary statistics for calcium estimated bone mineral density (eBMD) and fracture for single nucleotide polymorphisms (SNPs) influencing serum calcium

				Calcium serum GWAS			eBMD GWAS *			Fracture GWAS		
Nearby gene	Chr	Associated SNP	Calcium increasing allele	Allele freq	Effect (mmol/L)	P value	Allele freq	Effect (g/cm^2)	P value	Allele freq	Odds ratio (95% CI)	P value
DGKD	2	rs1550532	C	0.32	0.0045	8x10^{-11}	0.32	0.0022	0.50	0.32	1.004 (0.992 to 1.016)	0.51
CASR	3	rs1801725	T	0.15	0.0178	9x10^{-86}	0.13	0.0046	0.077	0.13	0.993 (0.976 to 1.011)	0.45
GATA3	10	rs10491003	T	0.09	0.0068	5x10^{-9}	0.09	−0.0002	0.77	0.09	1.013 (0.993 to 1.034)	0.21
CARS	11	rs7481584	G	0.72	0.0045	1x10^{-10}	0.72	−0.0067	0.001	0.71	1.003 (0.990 to 1.016)	0.62
DGKH, *VWA8*	13	rs7336933	G	0.85	0.0055	9x10^{-10}	0.85	0.00098	0.83	0.85	1.018 (1.000 to 1.033)	0.05
CYP24A1	20	rs1570669	G	0.34	0.0045	9x10^{-12}	0.34	−0.0045	0.016	0.34	0.993 (0.981 to 1.005)	0.25
VKORC1L1	7	rs17711722	T	0.47	0.00375	8x10^{-9}	0.44	0.0043	0.0180	0.45	0.998 (0.986 to 1.010)	0.68

*Mean (SD) eBMD is 0.54 g/cm^2 (0.12 g/cm^2)
GWAS=genome-wide association study

Table 2 | Cohort descriptions

Source	Study design	Country of origin	Ethnicity	Mean (SD) age	Study type	Assessment method	Fracture	Non-fracture	Total
Estimated bone mineral density cohort									
UK Biobank	Cohort	UK	Mixed (white British subset used for analysis)	56.6 (8.1)	Cohort	Heel quantitative ultrasound (heel BMD)	NA	NA	426 824
Fracture cohort									
Age, Gene/Environment Susceptibility Reykjavik Study	Cohort	Iceland	Northern European	76.4 (5.5)	Population based	Medical and radiographic records	1458	1727	3185
Anglo-Australasian Osteoporosis Genetics Consortium	Population cohort, and case control for fracture cases	Australia	North western European	69.6 (8.6)	Population based, clinical based	Questionnaire, radiography	685	1113	1798
B-vitamins for the PRevention Of Osteoporotic Fractures	Intervention study	Netherlands	North western European	74.3 (6.5)	General population	History of fractures before baseline: questionnaire. Incident fractures: self report, validated at GP or hospital	715	1483	2198
Cardiovascular Health Study	Cohort	US	European American	73.2 (5.9)	Population based	Self report of incident fracture of the hip, leg, arm, or vertebra	519	2742	3261
DeCODE Genetics Study	Cross sectional	Iceland	North western European	60.7 (13.9)	Population based, clinical based	Medical records, radiographic documentation, questionnaire	1836	14 560	16 396
Estonian Genome Center University of Tartu-I	Cohort	Estonia	Northern European	55.4 (20.2)	Population based	Medical records, questionnaire	217	4296	4513
Estonian Genome Center University of Tartu-II	Cohort	Estonia	Northern European	40.3 (16.1)	Population based	Medical records, questionnaire	71	1717	1788
Erasmus Rucphen Family	Cohort	Netherlands	North western European	48.8 (14.6)	Family based isolate	Interview	260	1342	1602
Framingham Heart Study	Cohort	US	European American	64.7 (11.2)	Population and family based	Medical records, questionnaire	1520	2782	4302
Gothenburg Osteoporosis and Obesity Determinants Study	Cohort	Sweden	Northern European	18.9 (0.6)	Population based	Radiographic document	273	597	870
Health Aging and Body Composition	Cohort	US	European American	73.8 (2.9)	Population based	Radiographic	308	1353	1661
Hong Kong Southern Chinese	Case control	China	Southern Chinese	48.9 (15.6)	Population based, clinic based	Medical records, radiographic, and questionnaire	79	627	706
MrOS	Cohort	US	European American	73.9 (5.9)	Clinic based	Questionnaire, radiographic documentation	918	3555	4473
The PROpective Study of Pravastatin in the Elderly at Risk	Cohort, randomised controlled trial	Netherlands, UK, Ireland	North western European	75.4 (3.4)	Clinic based	Medical records	426	4816	5242
Rotterdam Study I	Cohort	Netherlands	North western European	69.4 (9.0)	Population based	Medical records, questionnaire	2163	3574	5737
Rotterdam Study II	Cohort	Netherlands	North western European	63.8 (7.1)	Population based	Medical records, questionnaire	932	1220	2152
Rotterdam Study III	Cohort	Netherlands	North western European	56.1 (5.4)	Population based	Medical records, questionnaire	505	2421	2926
Study of Osteoporotic Fractures	Cohort	US	European American	71.5 (5.2)	Clinic based	Questionnaire, radiographic documentation	1611	1698	3309
TwinsUK	Cohort	UK	North western European	49.9 (13.6)	Population based, family based	Medical records, radiographic, and questionnaire	839	4111	4950
Women's Genome Health Study	Cohort	US	European American	54.1 (7.1)	Population based	Questionnaire	1832	20 498	22 330
Women's Health Initiative Clinical Trial	Quasi case/control	US	European American	69.0 (6.4)	Population based	Medical records	1058	647	1705
Women's Health Initiative Observational Study	Quasi case/control	US	European American	69.0 (6.5)	Population based	Medical records	1603	989	2592
CV risk in Young Finns Study	Cohort	Finland	Northern European	38.0 (5.0)	Population based	Medical records	611	975	1586
European Prospective Investigation into Cancer, Norfolk study	Cohort	UK	North western European	59.1 (9.3)	Population based	Medical records	2926	17 710	20 636
UK Biobank	Cohort	UK	Mixed (white British subset used for analysis)	56.8 (8.0)	Cohort	Questionnaire, based on answering yes to the question "Have you fractured/broken any bones in the last 5 years?" at either baseline or first follow up	53 184	373 611	426 795

cohorts represented 26.7% of cases and 18.2% of all samples and EPIC-Norfolk provided 2.8% of all cases and 3.8% of all samples. Table 2 shows the mean age of the GEFOS and EPIC-Norfolk cohorts.

Linear regression involving UK Biobank's fracture cohort was performed using BOLT-LMM,[53] an algorithm that allows for large scale mixed model association testing. Our genome-wide association study analysis included adjustment for sex, age, assessment centre, genotyping array, and first 20 principle components. Before meta-analysis with GEFOS and EPIC-Norfolk, and owing to the use of BOLT-LMM,[53 54] the effect size estimate and standard error of each genetic variant (single nucleotide polymorphism) from UK Biobank's genome-wide association study analysis was transformed to odds ratios by using the following approximation: log odds ratio=$\beta/(\mu^*(1-\mu))$, where μ is the case fraction in UK Biobank's fracture cohort.[52]

The UK Biobank full release fracture genome-wide association study was performed on its white British subpopulation, which included 53 184 fracture cases and 373 611 controls. The mean age of the UK Biobank fracture cohort is 56.8±8.0 (table 2). The study cohorts involved in the serum calcium genome-wide association study were mostly of European descent.[44] The estimated bone mineral density genome-wide association study cohort consisted of all white British patients from the UK.[55] Fracture cohorts were predominantly of European descent: Europe (91.6%), North America (8.0%), Australia (0.3%), and East Asia (0.1%).[50]

We used METAL to perform fixed effects meta-analysis of these results. Individual genome-wide association studies were corrected by genomic control,[56] and a total of 8 818 767 autosomal single nucleotide polymorphisms were included in the meta-analysis. The average genomic inflation λ of 24 cohorts was 1.025 and was adjusted accordingly when performing the meta-analysis. Finally, summary statistics from the eight calcium associated single nucleotide polymorphisms (table 1) were extracted from the fracture meta-analysis described above.

Single nucleotide polymorphism validation and pleiotropy assessment

We next undertook sensitivity analysis to understand if any of the single nucleotide polymorphisms might violate assumptions of mendelian randomisation.

Linkage disequilibrium

Single nucleotide polymorphisms used for mendelian randomization analysis are assumed to be independent of each other.[21] Given that the eight identified calcium associated single nucleotide polymorphisms are located on different chromosomes, they would segregate independently of each other and, hence, are not in linkage disequilibrium.

Pleiotropy

To avoid a biased estimation of the effect of serum calcium (risk factor) on either the risk of estimated bone mineral density or fracture (outcomes), the genetic variants (instruments) used in the mendelian randomisation analysis should only affect the outcome only through serum calcium. Thus, we evaluated potential associations of our selected calcium associated single nucleotide polymorphisms with known determinants of bone mineral density and fracture by searching the selected single nucleotide polymorphisms in Phenoscanner, a database of genome-wide association study results.[57] We further assessed whether each of the eight single nucleotide polymorphisms were expression quantitative trait loci for genes that could be associated with known determinants of bone mineral density and fracture by using the GTEX database.[50 58] Although direct pleiotropic effects that influence the outcome independently of the risk factor violate mendelian randomisation assumptions (red arrow in fig 1), vertical pleiotropy does not. Vertical pleiotropy is defined as the association of a single nucleotide polymorphism with more than one phenotype in the same biological pathway.[43] For example, genetically lowered calcium at *CASR* could lead to increased parathyroid hormone,[59] which itself might influence bone mineral density and fracture. But since calcium directly influences parathyroid hormone, which influences the outcome, this is not a violation of mendelian randomisation assumptions.

Comparison of genetically derived effects with pharmacological effects of calcium supplementation

We modelled the effect of a one standard deviation genetically derived increase in serum calcium on estimated bone mineral density and fracture. We compared this one standard deviation increase with the magnitude of increase in serum calcium levels after calcium supplementation. To do so, we compared data from a recent randomized crossover trial of calcium supplementation.[60]

Mendelian randomisation

Individual mendelian randomisation estimates from the seven independent serum calcium associated single nucleotide polymorphisms were calculated by using the Wald method.[61] We meta-analysed individual mendelian randomisation estimates by using both inverse-variance weighted and a random effects models using R and the package MendelianRandomization and RStudio.[62-64] The estimated associations of genetically predicted serum calcium with estimated bone mineral density and odds of fracture were expressed with respect to one standard deviation increase in serum calcium levels, which is equivalent to 0.51 mg/dL or 0.13 mmol/L. This standard deviation equivalence was derived from serum calcium's pooled variance calculation involving the 30 cohorts reported in O'Seaghdha and colleagues and included in the serum calcium genome-wide association study.[44]

Sensitivity analyses

To explore potential pleiotropic effects, we carried out three sensitivity meta-analyses: simple and weighted median and mendelian-randomisation-Egger regression methods using the R package MendelianRandomization.[63] Simple and weighted median meta-analyses provide estimations that are robust to the inclusion of up to 50% invalid instruments in a mendelian randomisation analysis.[65] The intercept estimate from mendelian-randomisation-Egger regression analysis provides a useful estimation of directional horizontal pleiotropy, that is, the magnitude and direction of the effect of the single nucleotide polymorphisms on the outcome not mediated through the exposure.[66]

In addition to the primary analysis, we performed two additional sensitivity analyses. Firstly, we repeated our analysis including rs17711722 near *VKORC1L1*. rs17711722 is associated with serum calcium levels at a genome-wide significant level, but it was not included in the main findings because it did not meet replication criteria.[44] Lastly, we performed an additional analysis by excluding rs1801725 *(CASR)*, which contributed the most weight in the inverse-variance weighted primary meta-analysis (*CASR* single nucleotide polymorphism explained 0.49% of the serum calcium variance). This single nucleotide polymorphism is in *LD* with single nucleotide polymorphism rs73186030, which has been associated with parathyroid hormone levels.[59] The rationale was to test whether, in its absence, the estimated calcium on bone mineral density and on fracture effects were similar to those from primary analyses or if they were mostly driven by *CASR*.

In addition, to assess whether Asian ancestry influenced our fracture mendelian randomisation results, we performed the fracture genome-wide association study meta-analysis removing the HKOS (Southern Chinese) cohort and repeated the fracture mendelian randomisation.

Patient and public involvement
No patients or member of the general public were directly involved in the design, recruitment, or conduct of the study. After publication, dissemination of the results will be sought across different countries involving respective patient organisations, the general public, and other stakeholders; typically, across social media, scientific meetings, and media interviews.

Results
Single nucleotide polymorphism selection
Table 1 shows that the single nucleotide polymorphisms in eight loci previously identified to be associated with serum calcium were rs1801725 in *CASR* ($P=9x10^{-86}$), rs1550532 in *DGKD* ($P=8x10^{-11}$), rs780094 in *GCKR* ($P=1x10^{-10}$), rs7336933 near *VWA8* and *DGKH* ($P=9x10^{-10}$), rs10491003 nearby *GATA3* ($P=5x10^{-9}$), rs7481584 in *CARS* ($P=1x10^{-10}$), rs1570669 near *CYP24A1* ($P=9x10^{-12}$), and rs17711722 near *VKORC1L1* ($P=8x10^{-9}$). The selected single nucleotide polymorphisms collectively explained 0.77% of the variance in total serum calcium levels, which is sufficient to influence the risk of coronary artery disease.[22]

Known biology at associated loci
Among these single nucleotide polymorphisms, rs1801725 (*CASR*) and rs1570669 (*CYP24A1*) are located nearby genes whose functions are involved in calcium homeostasis.[22] The most strongly associated calcium locus includes *CASR*, a calcium sensing receptor. *CASR* encodes a protein whose main function is to capture small changes in circulating calcium concentrations and consequently modify parathyroid hormone secretion and renal cation handling.[67] *CYP24A1* encodes an enzyme that plays a role in calcium homeostasis and the metabolism of the active form of vitamin D.[68] The diacylglycerol kinase genes *DGKD* and *DGKH* genes have recently been implicated in calcium signaling.[69] *GATA3* and the *CARS* locus are reportedly associated with hypocalcemia in the hypoparathyroidism, sensorineural deafness, and renal dysplasia (hypoparathyroidism, deafness, and renal dysplasia syndrome) and Beckwith-Wiedemann syndromes, respectively.[70] The remaining single nucleotide polymorphism, rs17711722, used for sensitivity analysis is located in *VKORC1L1*, is also associated with calcium homeostasis.[45] Thus, all loci associated with calcium levels contained genes with plausible biological effects on calcium levels.

Pleiotropy evaluation
Single nucleotide polymorphisms rs1801725 in *CASR*, rs7336933 near *VWA8* and *DGKH*, rs10491003 nearby *GATA3*, rs7481584 in *CARS*, and rs1570669 near *CYP24A1* were not associated with any phenotypes other than serum calcium in the Phenoscanner and MRBase databases. Besides its association with calcium levels, rs1550532 in *DGKD* showed evidence of association with bilirubin levels.[71-73] Bilirubin, however, is not associated with bone mineral density or with fracture.[74] Single nucleotide polymorphism rs17711722 (*VKORC1L1*) showed genome-wide level associations with corneal structure and central corneal thickness,[75] yet these phenotypes are not known to be related to a calcium-independent effect on estimated bone mineral density and fracture. Single nucleotide polymorphism rs780094 in *GCKR* had genome-wide level associations with triglycerides levels, cholesterol, waist circumference, and several other lipid-related phenotypes.[73 76-78] Waist circumference is highly associated with weight and BMI, which are known determinants of lower extremity bone density and potentially heel bone mineral density.[79] Thus, the highly pleiotropic nature of rs780094 in *GCKR* represents a calcium-independent effect on our outcomes of interest and was removed from all subsequent analyses.

Association of calcium levels modifying single nucleotide polymorphisms with estimated risk of bone mineral density and fracture
Table 1 shows that the summary statistics for the association between seven calcium increasing single nucleotide polymorphisms and estimated bone mineral density and fracture odds were directly

Table 3 | Mendelian randomisation results for effect of serum calcium on estimated bone mineral density (eBMD) and fracture risk

	Main analysis				Sensitivity Analyses							
	6 serum calcium associated SNPs				Including rs17711722 (VKORC1L1)				Excluding rs1801725 (CASR)			
Method	Mean eBMD (95% CI)	P value	Fracture odds ratio (95% CI)	P value	Mean eBMD (95% CI)	P value	Fracture odds ratio (95% CI)	P value	Mean eBMD	P value	Fracture odds ratio (95% CI)	P value
IVW	0.003 (−0.059 to 0.066)	0.92−	1.01 (0.89 to 1.15)	0.85	0.011 (−0.05 to 0.073)	0.72−	1.01 (0.90 to 1.13)	0.91	−0.049 (−0.144 to 0.047)	0.32−	1.12 (0.92 to 1.36)	0.25
Simple median	0.009 (−0.052 to 0.070)	−0.76	1.11 (0.93 to 1.33)	0.24	0.023 (−0.035 to 0.081)	0.44−	1.10 (0.91 to 1.32)	0.32	−0.004 (−0.093 to 0.085)	0.93−	1.13 (0.89 to 1.42)	0.32
Weighted median	0.030 (−0.006 to 0.067)	−0.10	0.99 (0.89 to 1.11)	0.91	0.031 (−0.005 to 0.068)	0.09−	0.98 (0.88 to 1.10)	0.76	−0.009 (−0.093 to 0.076)	0.84−	1.12 (0.90 to 1.41)	0.31
MR-Egger intercept	−0.003 (−0.006 to 0.001)	−0.11	1.00 (1.00 to 1.01)	0.39	−0.001 (−0.005 to 0.002)	0.49−	1.00 (1.00 to 1.01)	0.52	−0.005 (−0.019 to 0.008)	0.45−	0.98 (0.96 to 1.01)	0.14

Results are expressed per one standard deviation (0.51 mg/dL or 0.13 mmol/L) increase in serum calcium concentration. Estimated bone mineral density (eBMD) is expressed in g/cm^2.
IVW=inverse-variance weighted meta-analysis

obtained without the use of proxy single nucleotide polymorphisms from their respective studies.[55] None of the seven calcium single nucleotide polymorphisms had genome-wide significant associations with either estimated bone mineral density or odds of fracture (all P>0.08).

Comparison of genetically derived effects with pharmacological effects of calcium supplementation

A previous crossover randomised controlled trial showed that 500 mg of calcium citrate in a fasting state lead to a maximal increase in total serum calcium levels by approximately 0.07 mmol/L, four hours after administration.[60] The mendelian randomisation analyses here represent a change in total serum calcium of one standard deviation, which is 0.13 mmol/L. Therefore, the effects of total serum calcium presented include the anticipated effects of calcium supplementation.

Mendelian randomisation analysis: serum calcium on estimated bone mineral density

Table 3 and figure 2 show that when performing mendelian randomisation analyses, a one standard deviation (that is, 0.51 mg/dL or 0.13 mmol/L) increase in serum calcium concentration was not associated with a clinically relevant change in estimated bone mineral density (change per standard deviation increase in serum calcium 0.003 g/cm^2, 95% confidence interval −0.059 to 0.066; P=0.92). The mean and standard deviation of estimated bone mineral density are 0.54 g/cm^2 and 0.12 g/cm^2, respectively.

Figure 2 lists the individual level randomisation estimates of the single nucleotide polymorphisms used in the inverse variance weighted analysis. Mendelian randomisation estimates as determined by rs7481584 (*CARS* −0.19 g/cm^2, 95% confidence interval −0.30 to −0.08; P=0.001) and rs1570669 (*CYP24A1* −0.13 g/cm^2, −0.24 to −0.02; P=0.02) showed a statistically significant decrease in estimated bone mineral density per standard deviation increase in serum calcium. However, only the former remained statistically significant after Bonferroni correction for multiple hypothesis testing involving six tests, that is, $0.05/6=8.3 \times 10^{-3}$. Mendelian randomisation estimates as determined by the remaining single nucleotide polymorphisms showed a lack of association between a standard deviation increase in serum calcium and estimated bone mineral density.

Table 3 shows that the sensitivity meta-analyses with six single nucleotide polymorphisms involving simple median (0.009 g/cm^2, 95% confidence interval −0.052 to 0.070; P=0.76) and weighted median estimation (0.030 g/cm^2, −0.006 to 0.067; P=0.10) supported the inverse-variance weighted primary analysis. The mendelian-randomisation-Egger regression intercept, which provides an approximate estimation of directional pleiotropic effects on estimated bone mineral density through pathways independent of serum calcium, showed no significant evidence for such effects (−0.003 g/cm^2, −0.006 to 0.001; P=0.11).

Table 3 shows that the inclusion of an additional serum calcium increasing single nucleotide polymorphism (rs17711722, *VKORC1L1*) to the primary analysis also indicated that a one standard deviation increase in serum calcium was not associated with clinically relevant change in estimated bone mineral density of 0.011 g/cm^2 (95% confidence interval −0.050 to 0.073; P=0.72).

To assess the degree to which our primary mendelian randomisation inverse-variance weighted estimate would change by removing the single nucleotide polymorphism that provided most weight to the inverse-variance weighted analysis, we ran an additional sensitivity analysis excluding rs1801725 (*CASR*). Results were, as expected, less precise but did not differ materially from the results of the primary analyses (eg, inverse-variance weighted estimate −0.049 g/cm^2, 95% confidence interval −0.144 to 0.047; P=0.32; table 3).

Mendelian randomisation analysis: serum calcium association with fracture

We estimated the effect of a genetically average increased serum calcium on odds of fracture by implementing a random effects model and inverse-variance weighted method, which included six calcium-increasing alleles described in table 1. Figure 3 and table 3 show that a one standard deviation increase in serum calcium concentration was not associated with odds of fracture (odds ratio 1.01, 95% confidence interval 0.89 to 1.15; P=0.85).

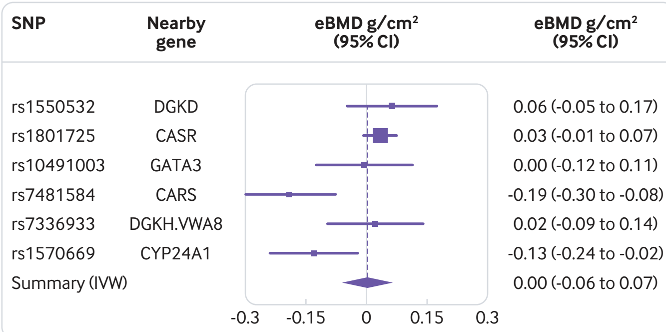


Fig 2 | Serum calcium effects on estimated bone mineral density (eBMD). Two sample mendelian randomisation: individual and inverse-variance weighted (IVW) results

Figure 3 shows the estimates from individual level single nucleotide polymorphism mendelian randomisation analysis. Mendelian randomisation estimates after Bonferroni correction for multiple hypothesis testing involving six tests did not show a change in odds of fracture per one standard deviation increase in serum calcium.

Table 3 shows that sensitivity meta-analyses with six single nucleotide polymorphisms involving simple median (odds ratio 1.11, 95% confidence interval 0.93 to 1.33; P=0.24) and weighted median estimation (0.99, 0.89 to 1.11; P=0.91) supported inverse-variance weighted primary analysis results. Mendelian-randomisation-Egger intercept regression results (1.00, 1.00 to 1.01; P=0.39) provided no evidence of directional pleiotropic effects on fracture odds through pathways independent of serum calcium.

The inclusion of an additional serum calcium increasing single nucleotide polymorphism (rs17711722, VKORC*1L1*) to our primary analysis with one standard deviation increase in serum calcium (inverse-variance weighted odds ratio 1.01, 95% confidence interval 0.90 to 1.13; P=0.91).

We also assessed the degree to which our primary mendelian randomisation inverse-variance weighted estimate would change by removing the single nucleotide polymorphism that provided highest weight to the inverse-variance weighted analysis, that is, rs1801725 (*CASR*). Again, the results were less precise, but were not materially different from the primary inverse-variance weighted estimate (inverse-variance weighted odds ratio 1.12, 95% confidence interval 0.92 to 1.36; P=0.25).

Finally, to assess whether the presence of a cohort of Asian ancestry (HKOS) in our fracture genome-wide association study could affect our fracture mendelian randomisation results, we performed the fracture genome-wide association study meta-analysis after removing the HKOS (Southern Chinese) cohort and repeated the fracture mendelian randomisation analysis. As observed in table 4 and figure 4, our instrumental variables' summary statistics and mendelian randomisation results were virtually identical to those obtained in our primary analysis. The genomic inflation factor (λ) without and with the inclusion of HKOS cohort remained unchanged at 1.025. Therefore, inclusion of the HKOS cohort of Southern Chinese ancestry did not affect our results.

Discussion

This mendelian randomisation study showed that a standard deviation increase in lifelong serum calcium levels was not associated with increased estimated bone mineral density or reduced risk of fracture in individuals with normal calcium levels. The magnitude of a one standard deviation increase in genetically predicted serum calcium includes the increase in serum calcium that would be anticipated after calcium supplementation.[60 80] Assuming a linear effect between calcium levels and the studied outcomes, this suggests that widespread efforts to use calcium supplements in the general population for long periods of time are unlikely to have any substantial effect on bone health outcomes. Further, we have recently shown that genetically determined increase in serum calcium derived from the same instruments (that is, single nucleotide polymorphisms) is associated with a clinically relevant increase in the risk of coronary artery disease.[22] Thus, the cardiovascular risks of

Table 4 | Summary statistics for fracture single nucleotide polymorphisms (SNPs) influencing serum calcium including and excluding HKOS cohort of Asian descent

				Fracture GWAS			Fracture GWAS excluding HKOS cohort		
Nearby gene	Chr	Associated SNP	Calcium increasing allele	Allele freq	Odds ratio (95% CI)	P value	Allele freq	Odds ratio (95% CI)	P value
DGKD	2	rs1550532	C	0.32	1.004 (0.992 to 1.016)	0.51	0.32	1.004 (0.992 to 1.017)	0.53
CASR	3	rs1801725	T	0.13	0.993 (0.976 to 1.011)	0.45	0.13	0.993 (0.976 to 1.011)	0.44
GATA3	10	rs10491003	T	0.09	1.013 (0.993 to 1.034)	0.21	0.09	1.013 (0.993 to 1.034)	0.21
CARS	11	rs7481584	G	0.71	1.003 (0.990 to 1.016)	0.62	0.71	1.004 (0.991 to 1.017)	0.59
DGKH, VWA8	13	rs7336933	G	0.85	1.018 (1.000 to 1.033)	0.05	0.85	1.017 (1.000 to 1.033)	0.05
CYP24A1	20	rs1570669	G	0.34	0.993 (0.981 to 1.005)	0.25	0.34	0.993 (0.980 to 1.005)	0.24
VKORC1L1	7	rs17711722	T	0.45	0.998 (0.986 to 1.010)	0.68	0.45	0.998 (0.986 to 1.010)	0.71

GWAS=genome-wide association study

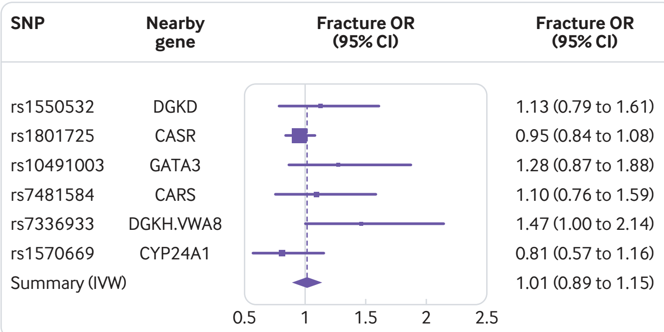


Fig 3 | Serum calcium effects on odds of fracture. Two sample mendelian randomisation: individual and inverse variance weighted (IVW) results

increasing serum calcium in the general population are unlikely to be offset by beneficial effects on bone density and fracture.

Calcium is vital to many biological processes, and its serum concentration is tightly regulated. Net calcium excretion must be replaced, but the amount of calcium needed is debated. What is not well understood is whether increases in serum calcium amongst individuals who have a normal varied diet and normal calcium levels lead to a decrease in the risk of fracture. As outlined above, there is conflicting observational epidemiological evidence that calcium supplementation does not reduce the risk of fracture, yet such studies could be prone to bias since the individuals most likely to use calcium supplements are those more likely to be at a higher risk of fracture.[81] The mendelian randomisation approach employed here overcomes this potential confounding by relying on the random assignment of alleles at conception, thereby preventing associations with such potentially confounding factors.

One way to improve the quality of evidence in medical research is to employ the principles of triangulation of different sources of evidence. If results are consistent across different types of study designs, and these different types of designs have different sources of potential bias, then the results can be combined in the framework of triangulation to provide a higher standard of evidence.[82 83] Of importance, our recent mendelian randomisation analysis that instrumented lactase persistence in adults as a surrogate for consumption of calcium-bearing dairy products found evidence of no protective effect of sustained dairy intake on the risk of fracture,[52 84] which is supported by our present results. Further, our mendelian randomisation findings are consistent with those of the aforementioned observational studies, numerous randomised controlled trials, and randomised controlled trial meta-analysis.[33-38]

Strengths of this study include the large sample size for estimated bone mineral density and fracture.[50 52] Further, multiple sensitivity analyses, including removing the single nucleotide polymorphisms most strongly associated with calcium levels, led to similar findings. Despite multiple sensitivity analyses, we did not identify pleiotropic effects of the single nucleotide polymorphisms on the skeletal outcomes, independent of serum calcium, as a potential source of bias. Of relevance, the serum calcium genome-wide association study from where we obtained serum calcium instruments was composed of population based cohorts that, on average, are normocalcemic, thus our reference population is normocalcemic adults. Hence, our study conclusions are applicable to normocalcemic adult populations similar to those in the general population, not to subpopulations, for example, with extremely low calcium level, which could benefit from calcium supplementation.

Limitations

These findings cannot provide insight into the effects of hypocalcemia and its correction on estimated bone mineral density and the risk of fractures. We have assumed a linear effect between calcium levels and the studied outcomes and tested these effects on individuals from the general population, who on average, have normal serum calcium levels. Thus, these findings can only provide insight into the effect of further increases in serum calcium levels in eucalcemic individuals. Most individuals studied for bone mineral density and fracture outcomes did not have osteoporosis as defined by bone mineral density measurement. Thus, the effects of genetically increased calcium in such individuals should be tested separately.

Most randomised controlled trials for prevention of fracture have used calcium and vitamin D supplements in conjunction with fracture preventive therapies and it is not clear whether giving such drugs in the absence of calcium supplements would provide the same protective benefits as were shown in these randomised controlled trials. Nonetheless, many randomised controlled trials for fracture prevention have given calcium and vitamin D supplements in the control arm of the study. Further, a recent randomised controlled trial of zoledronate showed marked reductions in the risk of fracture without the use of calcium.[40]

Regarding study populations, there is no overlap between the fracture genome-wide association study and the estimated bone mineral density genome-wide association study. Fracture genome-wide association

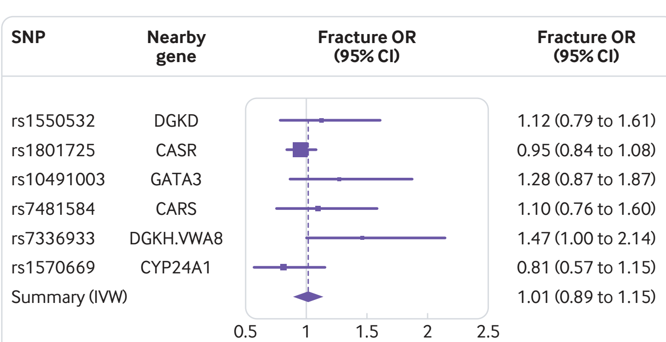


Fig 4 | Serum calcium effects on odds of fracture excluding cohort with Asian descent: sensitivity analysis. Two sample mendelian randomisation: individual and inverse variance weighted (IVW) results

study overlapped in a 2.8% with the calcium genome-wide association study population. However, we do not expect a substantial impact given the low degree of overlap.[85] Confounding by ancestry, also known as population stratification, can bias mendelian randomisation studies. A method to overcome such confounding is to limit the study to people of the same ancestry. Although most individuals in the fracture study were of European ancestry, ~1% of individuals were of Asian descent. However, removal of this Asian cohort did not impact on our results.

Our study provides insights into serum calcium levels and not tissue level concentrations. While these different compartments of calcium homeostasis might have different effects on the risk of fracture, calcium supplementation acts on the skeleton by first influencing serum calcium. Thus, the results presented here can provide insight into the expected effects of calcium supplementation by serum calcium. Further, the mendelian randomisation estimate for fracture, a binary outcome, was expressed as an odds ratio, which is a non-collapsible measure, yet this estimator still provides a valid test of the null hypothesis.[86] Canalization, which is the sum of compensatory feedback mechanisms returning a physiological system to homeostasis, can bias mendelian randomisation results towards the null. However, it is plausible the same mechanisms which maintain calcium homeostasis would act in a similar fashion on serum calcium raised by supplementation and by genetic effects. Further, the genetic predisposition to increased serum calcium used in our study is of sufficient biological and clinical relevance because of its association with increased risk of coronary artery disease.[22]

Conclusions

A genetic predisposition to increased serum calcium, amongst individuals with normal calcium levels, was not associated with increased estimated bone mineral density or decreased risk of fracture. The degree to which lifelong genetically derived increased serum calcium mimics the effect of long term calcium supplementation is not known. Since genetically elevated serum calcium is strongly associated with an increased risk of coronary artery disease, widespread calcium supplementation in the general population does not appear to have a favourable risk-benefit profile.

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Ethical approval: No separate ethical approval was required due to the use of publicly available summary data.

Data sharing: No additional data are available.

The manuscript 's guarantor (AC) affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned ((and, if relevant, registered)) have been explained.

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