Estimating dose-response relationships for vitamin D with coronary heart disease, stroke, and all-cause mortality: observational and Mendelian randomisation analyses

Emerging Risk Factors Collaboration/EPIC-CVD/Vitamin D Studies Collaboration

Summary

Background Randomised trials of vitamin D supplementation for cardiovascular disease and all-cause mortality have generally reported null findings. However, generalisability of results to individuals with low vitamin D status is unclear. We aimed to characterise dose-response relationships between 25-hydroxyvitamin D (25[OH]D) concentrations and risk of coronary heart disease, stroke, and all-cause mortality in observational and Mendelian randomisation frameworks.

Methods Observational analyses were undertaken using data from 33 prospective studies comprising 500 962 individuals with no known history of coronary heart disease or stroke at baseline. Mendelian randomisation analyses were performed in four population-based cohort studies (UK Biobank, EPIC-CVD, and two Copenhagen population-based studies) comprising 386 406 middle-aged individuals of European ancestries, including 33 546 people who developed coronary heart disease, 18 166 people who had a stroke, and 27 885 people who died. Primary outcomes were coronary heart disease, defined as fatal ischaemic heart disease (International Classification of Diseases 10th revision code I20-I25) or non-fatal myocardial infarction (I21-I23); stroke, defined as any cerebrovascular disease (I60-I69); and all-cause mortality.

Findings Observational analyses suggested inverse associations between incident coronary heart disease, stroke, and all-cause mortality outcomes with 25(OH)D concentration at low 25(OH)D concentrations. In population-wide genetic analyses, there were no associations of genetically predicted 25(OH)D with coronary heart disease (odds ratio [OR] per 10 nmol/L higher genetically-predicted 25(OH)D concentration 0·98, 95% CI 0·95–1·01), stroke (1·01, [0·97–1·05]), or all-cause mortality (0·99, 0·95–1·02). Null findings were also observed in genetic analyses for cause-specific mortality outcomes, and in stratified genetic analyses for all outcomes at all observed levels of 25(OH)D concentrations.

Interpretation Stratified Mendelian randomisation analyses suggest a lack of causal relationship for 25(OH)D concentrations with both cardiovascular and mortality outcomes for individuals at all levels of 25(OH)D. Our findings suggest that substantial reductions in mortality and cardiovascular morbidity due to long-term low-dose vitamin D supplementation are unlikely even if targeted at individuals with low vitamin D status.

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Introduction

Vitamin D is an essential nutrient obtained from sunlight, dietary intake, and supplementation.[1] Observational epidemiological studies have consistently found that low concentrations of circulating 25-hydroxyvitamin D (25[OH]D), a metabolite used as a clinical indicator of vitamin D status, are associated with an increased risk of cardiovascular disease and all-cause mortality, as well as other chronic diseases.[2,3] However, several large randomised trials of vitamin D supplementation have reported null results,[4–6] casting doubt on the observational evidence. However, as trials have typically recruited participants irrespective of baseline 25(OH)D concentration, they have had limited power to test supplementation effects in subgroups with low 25(OH)D concentrations.[7]

An efficient approach for assessing the potential causal effect of vitamin D supplementation is Mendelian randomisation. Mendelian randomisation uses genetic variants specifically related to a particular exposure to compare genetically defined population subgroups with different average levels of the exposure. The independent segregation of alleles at conception means that these genetically defined subgroups should not differ systematically with respect to confounding variables, creating a natural experiment analogous to a randomised trial.[8] Therefore, Mendelian randomisation analyses can provide more reliable insights into causal relationships

Research in context

Evidence before this study

We searched for randomised trials investigating the effects of vitamin D supplementation on cardiovascular disease or all-cause mortality published in any language from inception up until April 16, 2021, in PubMed, Scientific Citation Index Expanded, and Embase using the search terms listed in appendix 3 p 5. An example search is ("Cardiovascular Diseases"[MeSH] OR "Cardiovascular Disease" OR "All-cause Mortality" OR "Mortality" OR "Survival"). However, no study had combined the following key features required to achieve reliable estimates of the effect of vitamin D supplementation on cardiovascular disease or mortality in vitamin D deficient individuals: availability of individual-participant data on large sample sizes (eg, >1000 individuals with 25-hydroxyvitamin D [25(OH)D] concentrations of less than 25 nmol/L) with moderate numbers of cardiovascular disease or all-cause mortality events (eg, > 100 events). In summary, large randomised controlled trials have not been able to show beneficial effects of vitamin D supplementation on risk of coronary heart disease, stroke, or mortality.

Added value of this study

We characterised dose-response relationships for 25(OH)D, a metabolite used as a clinical indicator of vitamin D status, with cardiovascular disease and mortality in the largest such epidemiological analysis done to date, using statistical methods tailored to the investigation of non-linear risk factor-disease relationships. Our observational analyses indicate threshold relationships between 25(OH)D concentrations and risk of both cardiovascular disease and all-cause mortality. However, genetic analyses did not support a causal effect of 25(OH)D concentrations on cardiovascular disease or mortality at any level of 25(OH)D concentrations.

Implications of all the available evidence

Taken together, these findings suggest that long-term low-dose vitamin D supplementation is unlikely to reduce cardiovascular disease or mortality risk even in individuals with low 25(OH)D levels.

between risk factors and disease outcomes than conventional observational analyses. Previous Mendelian randomisation analyses have reported null associations of genetically predicted 25(OH)D concentrations with coronary heart disease[9,10] and ischaemic stroke.[11,12] An inverse association has been observed between genetically predicted 25(OH)D and all-cause mortality.[13] Null findings have been observed for several further outcomes, including other cardiovascular diseases and cancers.[14]

Most previous Mendelian randomisation analyses assumed a linear dose-response relationship between genetically predicted 25(OH)D and cardiovascular disease. However, some observational analyses have reported non-linear associations,[15,16] suggesting methods that assume linearity might not provide an accurate picture of the dose-response relationship. Several non-linear Mendelian randomisation analyses have been performed (appendix 3 p 7). However, such analyses can be severely biased if genetic effects on the exposure vary in the population.[17] This report is a replacement version of a previous publication that was affected by this bias and since retracted.[18] In this study, we performed the largest observational analysis to date to characterise the shape of association between 25(OH)D concentrations and cardiovascular disease outcomes in an individual participant data meta-analysis of 33 prospective studies. We then did stratified Mendelian randomisation analyses, using the doubly ranked method,[19] which is robust to variation in the genetic effects on the exposure, to assess evidence for potential causal effects of 25(OH)D concentrations on risk of major cardiovascular disease outcomes including coronary heart disease and stroke, all-cause mortality, and cause-specific mortality for

population subgroups with different average 25(OH)D concentrations.

Methods

Study design and participants

We undertook observational analyses using data from UK Biobank, the European Prospective Investigation into Cancer and Nutrition Cardiovascular Disease study (EPIC-CVD), and 31 studies from the Vitamin D Studies Collaboration (VitDSC). Genetic analyses were done using data from UK Biobank, EPIC-CVD, and two Copenhagen population-based studies. Only baseline measurements of 25(OH)D were used in analyses.

UK Biobank is a prospective cohort study of around 500 000 people aged 40 to 69 years at baseline, recruited in 2006–10 from the UK and followed up for a median of 10·9 years (IQR 10·1–11·7).[20] For observational analyses, we analysed 384 711 individuals with a valid 25(OH)D measurement and without previous known cardiovascular disease at baseline. For genetic analyses, we included data on 333 002 unrelated individuals of European ancestries with a valid 25(OH)D measurement and genetic data that passed quality control steps described previously.[21] EPIC-CVD is a case-cohort study derived from a cohort of over 500 000 individuals recruited at 23 centres across 10 European countries.[22,23] Participants were followed up for a median of 9·5 years (IQR 6·1–12·6). We analysed 26 336 individuals with a valid 25(OH)D measurement and without previous known cardiovascular disease at baseline in observational analyses, and 22 142 individuals of European ancestry in genetic analyses with a valid 25(OH)D measurement and genetic data that passed quality control steps. VitDSC comprises 89 915 participants from 31 mostly population-based, prospective studies across

11 countries. We analysed individual participant data on 25(OH)D concentrations, conventional cardiovascular risk factors, and major incident cardiovascular morbidity and mortality for 67 992 individuals without previously known cardiovascular disease. The Copenhagen City Heart Study (CCHS) and Copenhagen General Population Study (CGPS) are prospective cohort studies in the Danish population.[24,25] CCHS was initiated in 1976 and participants were followed up periodically until 2018. Median follow-up was 21·4 years (IQR 12·3–32·6). CGPS was initiated in 2003 and has a median follow-up of 8·8 years (IQR 8·1–13·6). For genetic analyses, we analysed a total of 31 262 individuals from both studies with genetic data and a 25(OH)D measurement. For all studies, written informed consent was obtained from participants and approval was obtained from relevant ethics committees.

Procedures

Concentrations of 25(OH)D in blood were measured using the Liaison immunoassay analyser (DiaSorin; Saluggia, Italy) in the UK Biobank and Copenhagen studies, and liquid chromatography-tandem mass spectrometry in the EPIC-CVD study. In VitDSC, concentrations were measured by radioimmunoassay, direct chromatographic approaches, or other immuno-assays (appendix 3 p 8). Measurements were seasonally adjusted in each study to correspond to a measurement taken in autumn by subtracting the study-specific mean 25(OH)D concentration for the season the measurement was taken in and then adding the study-specific mean 25(OH)D concentration for autumn measurements. In EPIC-CVD, centre-specific means were used rather than study-specific means.

To minimise potential bias due to horizontal pleiotropy, we considered genetic variants from four gene regions previously shown to be strongly associated with 25(OH)D and implicated in the transport, metabolism, and synthesis of vitamin D:[26] *GC*, *DHCR7*, *CYP2R1*, and *CYP24A1*. The *GC* gene encodes vitamin D binding protein. The *DHCR7* gene product converts 7-dehydrocholesterol to cholesterol, reducing 7-dehydrocholesterol available for conversion to previtamin D_3 by solar radiation. The *CYP2R1* gene encodes vitamin D 25-hydroxylase, a regulator of 25(OH)D synthesis through 25-hydroxylation of vitamin D in the liver. The *CYP24A1* gene product inactivates the active form of vitamin D (1α25(OH)$_2$D). To maximise the variance explained by the genetic instrument, we considered available variants at each genetic locus and selected variants associated with 25(OH)D concentrations using a stepwise selection method (appendix 3 p 2). For UK Biobank and EPIC-CVD, 21 variants were included in the analysis (appendix 3 p 10). For the Copenhagen studies, because of limited availability of genetic measurements, analyses were restricted to three variants: two from the *CYP2R1* locus (rs12794714 and rs117913124) and one from the *DHCR7* locus (rs7944926).

We also considered a score based on 71 genetic variants from across the genome (referred to as a genome-wide score) previously shown to be associated with 25(OH)D concentrations at a genome-wide level of statistical significance.[27]

Outcomes

Outcomes were classified using International Classification of Diseases, Tenth Revision (ICD-10) codes. Primary outcomes were coronary heart disease, defined as fatal ischaemic heart disease (ICD-10 code I20–I25) or non-fatal myocardial infarction (I21–I23); stroke, defined as any cerebrovascular disease (I60–I69); and all-cause mortality. We performed secondary analyses for cause-specific mortality divided into cardiovascular mortality, cancer mortality, or non-cardiovascular non-cancer mortality using ICD-10 codes (appendix 3 p 9). Prespecified observational analyses included incident events only. We performed supplementary genetic analyses restricted to incident coronary heart disease and stroke events, and separating ischaemic stroke (I63–I64) and haemorrhagic stroke (I60–I61).

Statistical analysis

Observational associations were assessed by inverse-variance weighted random-effects meta-analysis of study-specific hazard ratios (HRs), calculated using Cox proportional hazards regression models stratified by sex and, as appropriate, centre or trial group. Primary analyses were adjusted for conventional risk factors, namely age at blood draw for 25(OH)D measurement, calendar month of blood draw, smoking status (current *vs* other), total cholesterol, HDL cholesterol, systolic blood pressure, known history of diabetes, and BMI; all measured at study baseline.

The primary dose-response analyses assessed the shape of association between 25(OH)D and outcomes by meta-analysis of fractional polynomials adjusted for the conventional risk factors. Supplementary analyses combined study-specific HRs by tenths of 25(OH)D and plotted the pooled HRs against the pooled mean 25(OH)D within each tenth.

We calculated a genetic risk score weighted by the conditional associations of the genetic variants with 25(OH)D concentration in UK Biobank (appendix 3 p 10). Mendelian randomisation estimates were calculated using the ratio method by dividing the genetic association with the outcome by the genetic association with 25(OH)D concentration and scaling the estimate to a 10 nmol/L difference in genetically predicted 25(OH)D concentration. Genetic associations were estimated using logistic regression for disease outcomes and using linear regression for 25(OH)D concentrations. All regression models were adjusted for age at baseline, sex, centre (for UK Biobank and EPIC-CVD), and ten genetic principal components of ancestry. We assessed specificity of the genetic risk score by testing its associations with a range of cardiovascular risk factors in the UK Biobank study.

	UK Biobank (n=333 002)	EPIC-CVD (n=22 142)	Copenhagen studies (n=31 262)
Age at baseline, years	57·1 (8·1)	54·8 (9·4)	57·5 (12·9)
Sex			
Female	177 733 (53·4)	11 426 (51·6)	17 311 (55·4)
Male	155 269 (46·6)	10 716 (48·4)	13 951 (44·6)
25(OH)D concentration, nmol/L	54·5 (19·6)	46·9 (16·4)	53·8 (25·9)
Coronary heart disease events	22 363 (6·7%)	5942 (26·8%)	5241 (16·8%)
Stroke events	10 489 (3·1%)	5478 (24·7%)	2199 (7·0%)
Deaths	20 340 (6·1%)	N/A*	7545 (24·1%)
BMI (kg/m²)	27·3 (4·8)	26·7 (4·3)	25·9 (4·2)
SBP (mm Hg)	137·5 (18·6)	137·4 (21·3)	140·1 (21·0)
Smoking†			
Current	34 085 (10·2%)	6867 (31·0%)	8387 (26·9%)
Other	298 940 (89·8%)	15 275 (69·0%)	22 784 (73·1%)
Diabetes			
Known history	15 822 (4·8%)	1234 (5·6%)	1244 (4·0%)
No known history	317 203 (95·2%)	20 908 (94·4%)	30 018 (96·0%)

Data are mean (SD) for continuous variables or N (%) for categorical variables. 25(OH)D concentrations are season-shifted to correspond to a measurement taken in autumn. 25(OH)D=25-hydroxyvitamin D. CHD=coronary heart disease. SBP=systolic blood pressure. *EPIC-CVD was specifically designed as a case-cohort study of cardiovascular disease outcomes, and therefore does not contribute to the analysis of non-cardiovascular disease or all-cause mortality outcomes. †91 participants had missing data in the Copenhagen studies.

Table 1: Baseline characteristics

In addition to analyses conducted in the overall study sample to estimate population-averaged causal effects, we also conducted stratified analyses in strata of the population constructed using the doubly ranked stratification method,[19] a statistical method for constructing strata of the population with different average levels of the exposure such that stratum membership is independent of the genetic instrument. Stratification on 25(OH)D levels directly would induce collider bias, meaning that the distribution of the genetic instrument would vary between strata, and the instrumental variable assumptions would be violated in the strata. A previously proposed method that stratifies on residual values of 25(OH)D (ie, the non-genetic component of 25[OH]D) requires the genetic effect on 25(OH)D concentrations to be linear and homogeneous in the population to provide unbiased estimates; an assumption that is generally implausible and is violated in this case.[17] We calculated Mendelian randomisation estimates for ten equally sized strata using the ratio method with the GRS as an instrumental variable, and combined stratum-specific estimates across studies using fixed-effect meta-analysis. All estimates from the doubly ranked method were averaged across 100 iterations of the method (appendix 3 p 3). All statistical analyses were done in R version 4.0.5, Stata/SE 15.1, or BOLT-LMM version 2.3.4.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

386 406 participants from the four studies were included in genetic analyses (table 1), including 33 546 people who had coronary heart disease, 18 166 people who had a stroke, and 27 885 people who died, and 500 962 participants were included in observational analyses (appendix 3 pp 11–13). Mean age of participants included in the genetic analysis ranged from 54·8 years (SD 9·4) to 57·5 years (12·9), with similar numbers of men and women in each study, and the mean season-shifted 25(OH)D concentrations (corresponding to an autumn measurement) were 54·5 nmol/L (SD 19·6) in UK Biobank, 46·9 nmol/L (16·4) in EPIC-CVD, and 53·8 nmol/L (25·9) in the Copenhagen studies. Mean 25(OH)D estimates did not notably differ by assay type (appendix 3 p 21). The focused genetic risk score explained 4·7% of the variance in 25(OH)D concentrations in UK Biobank study, 5·8% in EPIC-CVD, and 1·8% in the Copenhagen studies. This genetic risk score was not associated with a range of cardiovascular risk factors in UK Biobank, except for BMI and HDL cholesterol, although these associations were small (appendix 3 p 22). The genome-wide score was strongly associated with LDL cholesterol and triglycerides (appendix 3 p 23), and so Mendelian randomisation estimates using this score are unreliable.

Observational associations had a similar non-linear shape for all outcomes (figure 1; appendix 3 p 24): at low concentrations of 25(OH)D, there was an inverse association with all outcomes, whereas at higher concentrations of 25(OH)D, the association was null for cardiovascular mortality and weakly positive for other mortality outcomes. For coronary heart disease and stroke, there was no strong association with 25(OH)D concentrations above 50 nmol/L, but a progressively steeper association was observed below this threshold. For all-cause mortality, in comparison to other outcomes, the strength of the inverse association at lower 25(OH)D concentrations was stronger and began at a higher 25(OH)D concentration.

The shapes of the observational associations in the three primary data sources were broadly similar (appendix 3 p 25). Dose-response findings were also similar in supplementary analyses that combined study-specific HRs by deciles of 25(OH)D or according to the four 25(OH)D categories (appendix 3 pp 15, 26).

In overall Mendelian randomisation analyses (that is, population-averaged estimates across the full range of the 25[OH]D concentration distribution), there was no association between genetically predicted 25(OH)D and coronary heart disease (odds ratio [OR] 0·98 [95% CI 0·95–1·01]; p=0·18), stroke (OR 1·01 [0·97–1·05]; p=0·61), or all-cause mortality (OR 0·99 [0·95–1·02]; p=0·39; figure 2; appendix 3 p 16). However, there was some evidence of an overall inverse association with all-cause mortality in the Copenhagen studies (OR 0·89 [0·80–0·99]; p=0·030; appendix 3 p 16). In stratified analyses (ie, stratum-averaged estimates for strata of the population
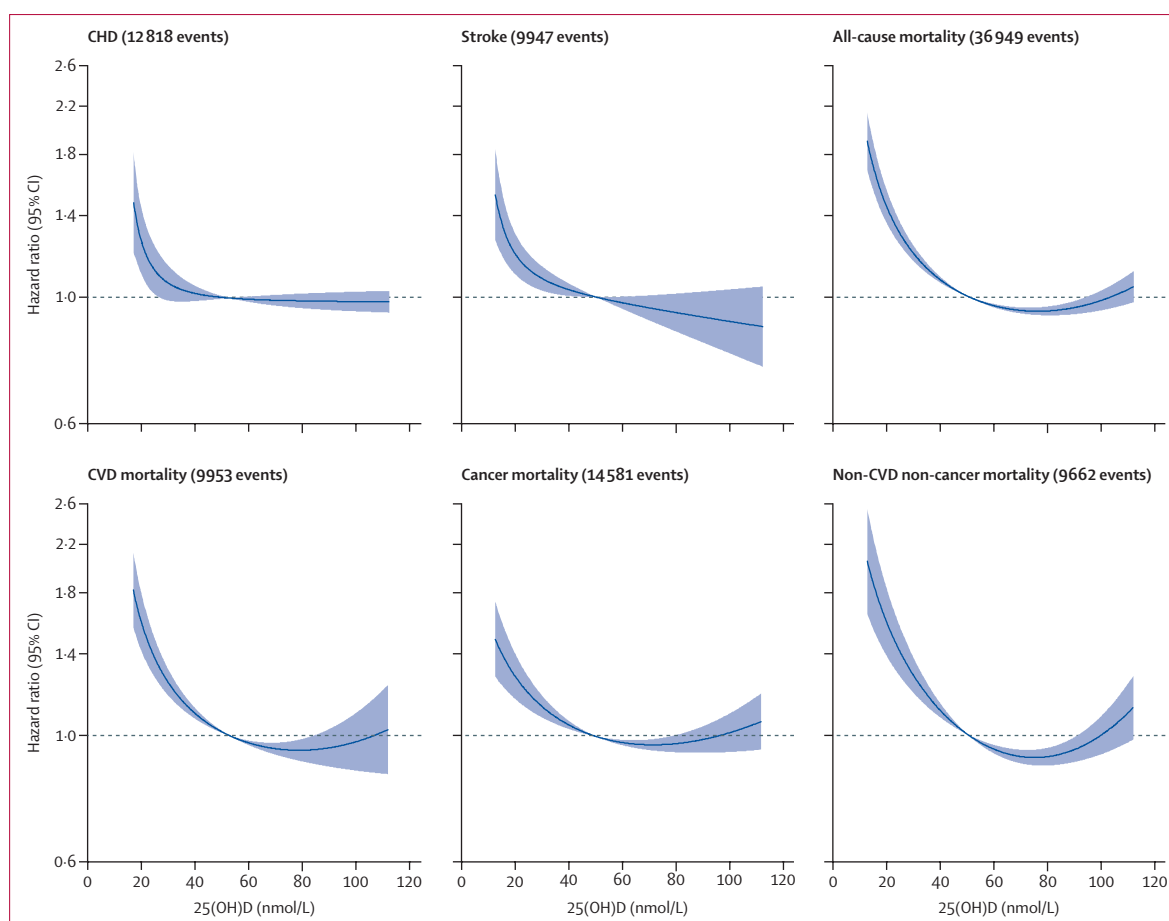
Figure 1: **Observational associations of 25(OH)D concentration with outcomes**
Reference value is 50 nmol/L, which is close to the mean concentration of 25(OH)D in most contributing studies. The shaded area represents the 95% CI for the dose-response curve. 25(OH)D=25-hydroxyvitamin D. CHD=coronary heart disease. CVD=cardiovascular disease.

with different average 25[OH]D concentrations), there were no clear associations between genetically-predicted 25(OH)D and the main outcomes in any stratum, and no discernible trends in the stratum-specific estimates (figure 2). The distributions of 25(OH)D concentrations in strata are provided in table 2.

Similar results were observed for supplementary analyses that considered incident stroke outcomes only and ischaemic stroke only (appendix 3 p 17), and incident coronary heart disease outcomes only (appendix 3 p 18). Estimates using the pleiotropic genome-wide score are presented in appendix 3 (p 19). The precision of estimates varied between strata, because genetic associations with 25(OH)D were much stronger in individuals with high 25(OH)D concentrations than in individuals with low 25(OH)D concentrations (appendix 3 p 27). In UK Biobank, genetic associations with 25(OH)D were 5·0-times stronger in the highest decile than the lowest decile; in EPIC-CVD, this ratio was 4·6, and in the Copenhagen studies, this ratio was 9·7.

Mendelian randomisation estimates for cause-specific mortality in UK Biobank and the Copenhagen studies are presented in figure 2 (study-specific estimates in appendix 3 p 20). In overall analyses, there was no association between genetically-predicted 25(OH)D and cardiovascular mortality (OR 1·01, 95% CI 0·95–1·08, p=0·71), cancer mortality (0·98, 0·93–1·02, p=0·29), or non-cardiovascular non-cancer mortality (1·00, 0·94–1·06, p=0·99). Similarly, null findings were obtained for mortality outcomes in all stratum-specific analyses.

A comparison between estimates from the doubly ranked method and the residual method for primary outcomes in the UK Biobank is presented in figure 3 (appendix 3 p 28 for mortality outcomes). Estimates differ substantially at low levels of 25(OH)D, with the residual method giving estimates in the protective direction, with confidence intervals that exclude the null for stroke and all-cause mortality, and the doubly-ranked method giving estimates in the harmful direction for primary outcomes, although compatible with the null in all cases.

Discussion

In observational analyses, we found evidence for non-linear dose-response relationships of 25(OH)D

concentrations with cardiovascular disease and mortality outcomes. However, population-averaged estimates from our genetic analyses suggest that interventions to increase 25(OH)D concentrations are unlikely to translate into substantial risk reductions for cardiovascular disease or all-cause mortality in the population overall. Similarly, genetic analyses conducted in individuals with low 25(OH)D concentrations provided no evidence supporting

a causal relationship between 25(OH)D concentrations and cardiovascular or mortality outcomes. Our results suggest that substantial reductions in mortality and cardiovascular morbidity due to long-term low-dose vitamin D supplementation are unlikely even from trials targeted at individuals with low vitamin D status.

The majority of large prior trials for cardiovascular disease and mortality were conducted in broadly selected groups of the population, so had limited reliability to assess evidence for causality in individuals with low 25(OH)D concentrations. Most previous Mendelian randomisation analyses did not consider estimates for strata of the population defined according to baseline 25(OH)D concentrations, and hence have not considered the shape of the causal relationship between 25(OH)D concentrations and cardiovascular disease or all-cause mortality. Previous non-linear Mendelian randomisation investigations conducted by ourselves[18] and by others[28] used the residual stratification method, which assumes that the effect of the genetic instrument on the exposure is linear and homogeneous in the population. These analyses suggested a protective causal effect of 25(OH)D at low 25(OH)D concentrations, as has been suggested for respiratory tract infections.[29] However, the residual method cannot reliably detect when the constant genetic effect assumption is violated,[19] and when it is violated, the method can produce biased results that reflect observational confounded associations rather than causal relationships.[17]

	UK Biobank	EPIC-CVD	Copenhagen studies
Stratum 1 (lowest)	28·6 (22·7–38·0)	25·3 (19·5–34·2)	25·3 (18·3–36·2)
Stratum 2	35·7 (27·0–45·0)	31·9 (24·1–40·1)	33·6 (23·1–44·8)
Stratum 3	41·1 (31·9–51·0)	36·4 (28·5–45·1)	40·1 (29·1–51·7)
Stratum 4	46·0 (36·1–56·5)	40·3 (32·2–49·3)	46·0 (34·7–57·9)
Stratum 5	50·7 (40·2–61·8)	44·1 (35·7–53·4)	51·6 (39·9–64·1)
Stratum 6	55·5 (44·3–67·4)	47·9 (38·7–57·2)	57·4 (44·7–70·7)
Stratum 7	60·7 (48·6–73·3)	51·8 (41·9–61·6)	63·7 (50·0–78·1)
Stratum 8	66·5 (53·3–80·2)	56·3 (45·7–67·6)	71·1 (55·8–87·5)
Stratum 9	73·8 (59·0–89·5)	62·2 (49·3–75·4)	81·1 (62·9–100·8)
Stratum 10 (highest)	86·2 (67·1–98·6)	72·7 (56·2–83·0)	100·0 (73·5–116·4)

Mean season-shifted 25(OH)D concentration in each stratum (nmol/L); with 10th and 90th percentiles of the distribution shown in parentheses (20th and 80th percentiles for the lowest and highest strata).

Table 2: Distribution of 25(OH)D concentrations in strata by study
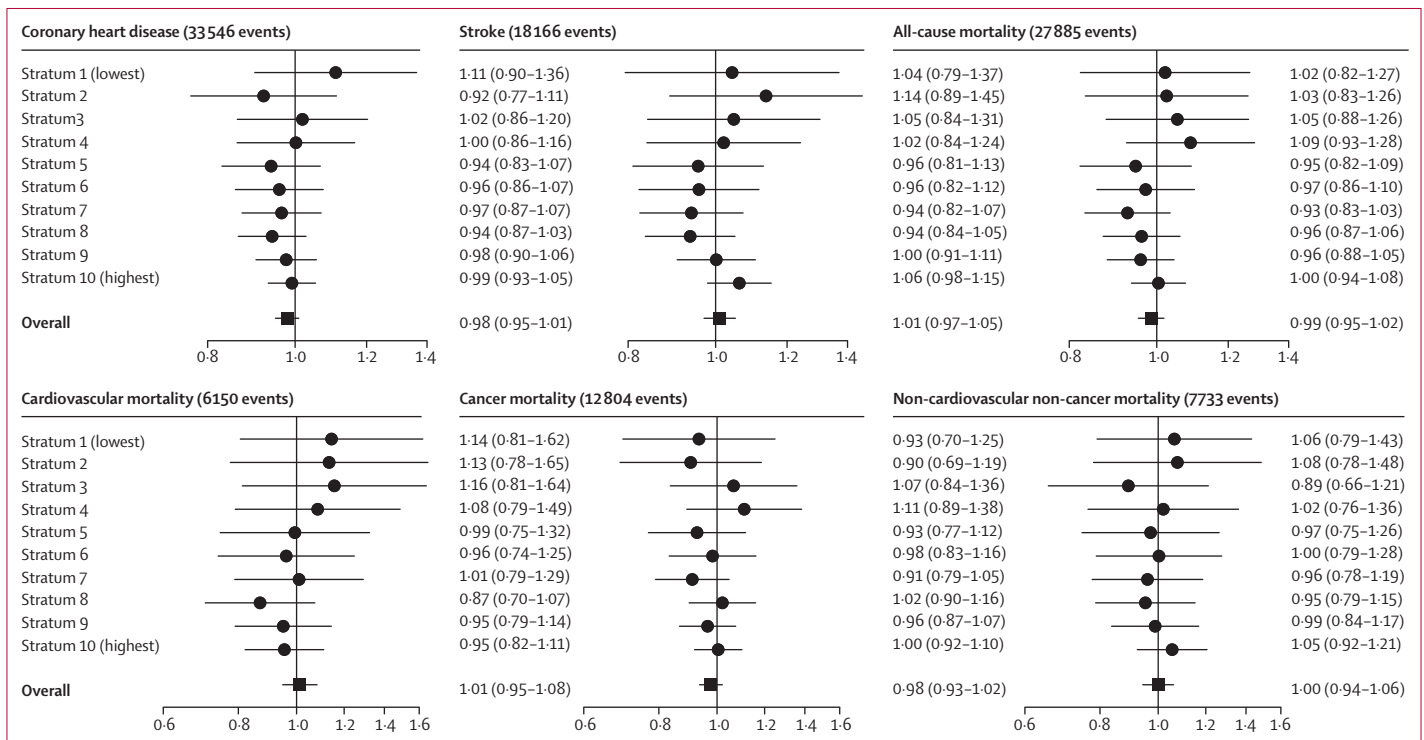
Figure 2: Mendelian randomisation estimates in overall population and strata of residual 25(OH)D concentrations
Estimates (95% CIs) represent odds ratios per 10 nmol/L increase in genetically predicted concentration of 25(OH)D in strata of the population defined by residual concentration of 25(OH)D.

We have several reasons for greater trust in the updated null results from the doubly ranked method over the previous results from the residual stratification method. First, genetic associations with the exposure varies strongly between strata, violating the assumption required by the residual method. There are several plausible reasons why genetic associations with 25(OH)D may be smaller in individuals with lower 25(OH)D concentrations: if genetic variants act via biological mechanisms relating to 25(OH)D synthesis or metabolism, then we may expect smaller magnitudes of effect in individuals with low levels of 25(OH)D synthesis and metabolism. Genetic variants may have similar proportional effects on 25(OH)D concentrations, rather than constant additive effects. Second, in the UK Biobank dataset, significant associations with confounders have been observed in 25(OH)D strata defined by the residual method.[17] This provides empirical evidence that the Mendelian randomisation assumptions are violated in strata defined by the residual method, even if they are not violated in the overall population. Third, a sensitivity analysis for the residual method log-transforming 25(OH)D concentrations before stratification provided substantially attenuated estimates in low 25(OH)D strata.[17] Log-transformation reduces the difference between genetic associations with 25(OH)D at the top and bottom of the distribution of 25(OH)D concentrations, and so should reduce bias due to violation of the constant genetic effect assumption. Fourth, theoretical investigations have shown greater bias in estimates from the residual method compared with the doubly ranked method in a wide range of simulated scenarios when the constant genetic effect assumption does not hold.[17,19] Finally, there were discrepancies in results from the residual method in our previous analysis;[18] namely, the overall estimate for cardiovascular mortality was positive (but non-significant), even though all stratum-specific estimates were negative.[30] While this is possible for observational associations (Simpson's paradox), it is a logical impossibility if all estimates have a causal interpretation. Hence, we have strong empirical and methodological reasons to doubt our previously published finding supporting a protective effect of 25(OH)D in low vitamin D status individuals.[18] However, all statistical methods, particularly those for inferring causal relationships, make untestable assumptions, which cannot be empirically verified. Hence, even for the doubly ranked method, there remains intrinsic uncertainty in the validity of results: in the validity of the genetic variants as instrumental variables, in the assumptions required for non-linear Mendelian randomisation, and so on.

Our revised investigation has several strengths. The Mendelian randomisation design means that estimates are less susceptible to bias from confounding and reverse causation than those from conventional observational analyses. Our focused genetic instrument for vitamin D
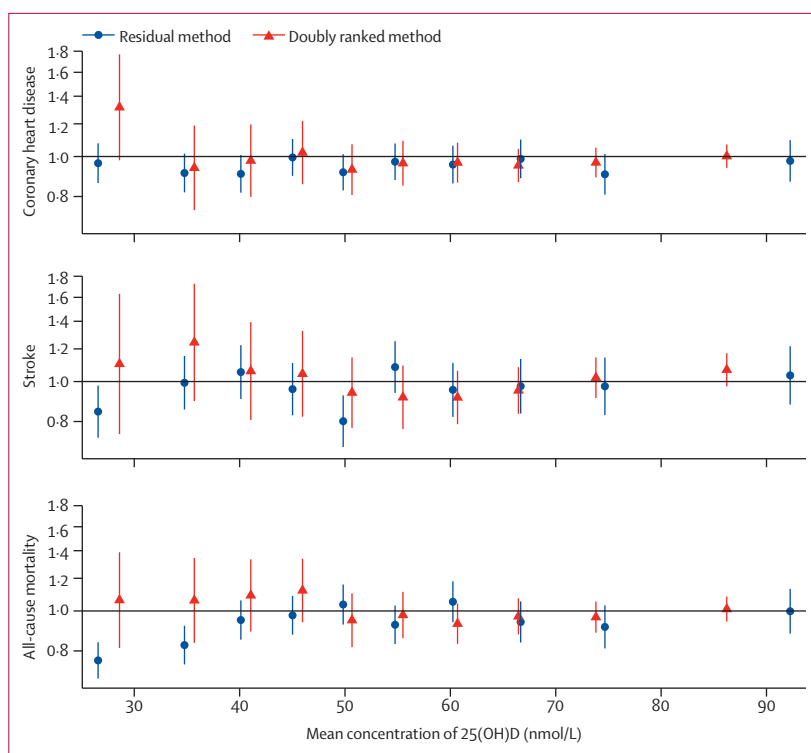
Figure 3: **Stratified Mendelian randomisation estimates for primary outcomes in UK Biobank from residual and doubly ranked methods.**
Estimates (95% CIs) represent odds ratios per 10 nmol/L increase in genetically predicted vitamin D concentration in strata of the population defined by residual concentration of 25(OH)D. Estimates from the residual method (unreliable when the genetic effect on the exposure vary in the population) are shown as blue circles. Estimates from the doubly ranked method (more reliable when the genetic effect on the exposure vary in the population) are shown as red triangles.

afforded strong statistical power and biological specificity, minimising the potential for bias due to horizontal pleiotropy arising from use of variants that do not have specific effects on vitamin D pathways. The focused score was not associated with major cardiovascular risk factors, providing empirical evidence to support the Mendelian randomisation assumptions. While the genetic instrument only explained a limited proportion of the variance in 25(OH)D levels, vitamin D supplementation in a randomised controlled trial would only explain a limited proportion of the variance in 25(OH)D levels. In the VITAL trial, supplementation only explained 26% of the variance in 25(OH)D levels.[4] While the limited proportion of variance explained affects the power to detect a causal effect, it does not preclude causal inferences either in a trial or a Mendelian randomisation investigation.

However, there are also potential limitations. First, the Mendelian randomisation assumptions state that the only causal pathway from the genetic variants to the outcome is via 25(OH)D concentrations. Although our variants are all from gene regions specifically relevant to vitamin D biology, variants in the *CYP24A1* gene region are known to associate with circulating calcium levels. Second, although weaker than the assumptions required

by the residual stratification method, non-linear Mendelian randomisation analyses using the doubly ranked method require additional assumptions beyond standard population-based Mendelian randomisation analyses; namely the "rank preserving assumption" that the ranking of participants by their exposure values would be the same for all values of the genetic instrument. This assumption is generally plausible, but cannot be empirically tested. Third, to reduce the scope for confounding by ethnicity (population stratification), our analyses were limited to middle-aged participants of European ancestries. This limitation means that our findings might not be applicable to other populations. In particular, further analyses are needed to assess the potential effect of vitamin D supplementation in individuals with dark skin, as this correlates with lower 25(OH)D concentrations. Fourth, UK Biobank and EPIC-CVD are not fully representative samples of the UK and European populations, further limiting the applicability of findings. Selection into these studies is dependent on age, sex, and other covariates, which can lead to bias in Mendelian randomisation estimates.[31] While the effect of moderate selection bias on Mendelian randomisation estimates is often slight, one specific concern in non-linear Mendelian randomisation is that selection bias might affect stratum-specific estimates non-differentially; that is, bias may be greater in some strata than in others. Fifth, fewer genetic variants were available in the Copenhagen studies, limiting comparability between datasets; however, although this will reduce power for analysis, it should not lead to bias. Sixth, we do not have information from all studies on the accuracy of 25(OH)D measurements from external quality control programmes; however, there was no indication that mean 25(OH)D estimates varied by assay type, and as any such variation is probably non-differential to morbidity and mortality outcomes it would bias the results toward the null. Seventh, our primary genetic analyses for cardiovascular disease considered both prevalent and incident events. Stratification into categories according to residual 25(OH)D concentration might therefore be affected by reverse causation. However, genetic associations with disease outcomes within each of the strata will not be affected by reverse causation, as genotype is fixed from conception. Finally, while our Mendelian randomisation analyses provided confidence intervals that overlapped the null in all strata, it is not possible to prove a null finding; we may not have sufficient power to detect a small causal effect, particularly in the stratified analyses, as the sample size is naturally reduced for these analyses.

In conclusion, while observational analyses found a threshold association between 25(OH)D and cardiovascular and mortality outcomes, this was not confirmed in genetic analyses. This suggests the absence of a causal effect of 25(OH)D on cardiovascular and mortality outcomes, even at low 25(OH)D concentrations.

Contributors
ES, SKK, EA, MGA, AMM, RC, LAMP, LS, PW, JD, AMW, EDA, ASB, and SB conceived and designed the study. ES, SKK, SA, TJ, TRB, and SB did the analyses. ES, SKK, DG, AMW, EDA, ASB, JD, and SB drafted the manuscript. ES, SKK, SA, and SB verified the underlying data. SB is responsible for the decision to submit the manuscript. SB has seen and verified all the data. SKK has seen and verified the observational analysis. ES has seen and verified the genetic analyses of UK Biobank and EPIC-CVD. SA has seen and verified the genetic analyses of the Copenhagen studies. All authors acquired and interpreted the data, critically revised the paper, and had final responsibility for the decision to submit for publication.

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