

Vitamin D and brain health: an observational and Mendelian randomization study

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Abbreviations: **25(OH)D:** 25-hydroxyvitamin D, an indicator of vitamin D status; **β :** beta; **BMI:** body mass index; **CHIAG:** community health index advisory group; **CI:** confidence interval; **EHR:** electronic health record; **GRS:** genetic risk score; **GS:** genetic score; **GWAS:** genome wide association study; **HR:** hazards ratio; **HREC:** human research ethics committee; **IVW:** inverse variance weighted; **LACE:** localized average causal effect; **MR:** mendelian randomization; **MREC:** multi-research ethics committee; **MRI:** magnetic resonance imaging; **MR-PRESSO:** mendelian randomization pleiotropy residual sum and outlier; **NLMR:** non-linear mendelian randomization; **OR:** odds ratio; **PIF:** potential impact fraction; **RCTs:** randomized control trials; **RF:** radiofrequency; **SD:** standard deviation; **SNP:** single nucleotide polymorphism; **SUNLIGHT:** study of underlying genetic determinants of vitamin D and highly related traits; **T2-FLAIR:** T2- fluid attenuated inversion recovery; **TE:** time of echo; **TR:** time of repetition; **UK BiLEVE:** UK biobank lung exome evaluation;

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Abstract

Background: Higher vitamin D status has been suggested to have beneficial effects on the brain.

Objective: To investigate the association between 25-hydroxyvitamin D [25(OH)D], neuroimaging features and the risk of dementia and stroke.

Design: We used prospective data from the UK Biobank (37-73 years at baseline) to examine the association between 25(OH)D concentrations with neuroimaging outcomes (N=33,523) and the risk of dementia and stroke (N=427,690; 3,414 and 5,339 incident cases respectively). Observational analyses were adjusted for age, sex, ethnicity, month, center, socioeconomic, lifestyle, sun behavior and illness-related factors. Non-linear Mendelian randomization (MR) analyses were used to test for underlying causality for neuroimaging outcomes (N=23,901) and dementia and stroke (N=294,514; 2,399 and 3,760 cases, respectively).

Results: Associations between 25(OH)D and total, grey matter, white matter and hippocampal volumes were non-linear, with lower volumes both for low and high concentrations (adjusted $P_{\text{non-linear}} \leq 0.04$). 25(OH)D had an inverse association with white matter hyperintensity volume (per 10nmol/L 25(OH)D, adjusted β : -6.1, 95%CI -11.5, -7.0). Vitamin D deficiency was associated with an increased risk of dementia and stroke, with the strongest associations for those with 25(OH)D <25nmol/L (vs. 50-75.9 nmol/L, adjusted HR: 1.79, 95%CI 1.57, 2.04, and HR: 1.40, 95%CI 1.26, 1.56, respectively). Non-linear MR analyses confirmed the threshold effect of 25(OH)D on dementia, with the risk predicted to be 54% (95%CI 1.21, 1.96) higher for participants at 25nmol/l compared to 50nmol/l. 25(OH)D was not associated with neuroimaging outcomes or the risk of stroke in MR analyses. Potential impact fraction suggests 17% (95%CI 7.22, 30.58) of dementia could be prevented by increasing 25(OH)D to 50nmol/L.

Conclusions: Low vitamin D status was associated with neuroimaging outcomes and the risks of dementia and stroke even after extensive covariate adjustment. MR analyses support a causal effect of vitamin D deficiency on dementia but not on stroke risk.

Keywords: 25-hydroxyvitamin D, Vitamin D, Mendelian randomization, dementia, stroke, Magnetic Resonance Imaging, UK Biobank, prospective cohort study, brain volume

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Introduction

Low 25-hydroxyvitamin D (25(OH)D, an indicator of vitamin D status) concentrations are common and the prevalence of severe vitamin D deficiency (<25 nmol/L) ranges from 5% to 50%, depending on location and population characteristics (1). Vitamin D is a hormone precursor which is increasingly recognized for widespread effects, including on brain health (2, 3). There are various mechanisms by which active vitamin D may affect the brain, including the regulation of neurotrophic growth factors, influences on inflammation and thrombosis (4, 5). With a growing interest in identifying modifiable risk factors for dementia and stroke, vitamin D has become an attractive candidate, as supplementation, diet, and sunlight exposure can maintain adequate serum concentrations (6).

Several aspects of brain morphometry can reflect cognitive decline and neurocognitive disease (see Supplementary Table 1 for a brief review). Association between 25(OH)D and brain morphometry has been mostly investigated in cross-sectional studies but with promising findings (7). A systematic review conducted in 2014 concluded that vitamin D depletion is associated with lower total brain volumes, while associations with sub-volumes were more mixed (7). Several studies have been conducted since this review, some of which suggest associations that are the strongest for individuals with vitamin D deficiency, such as those who have been institutionalized or hospitalized (8-11). Interestingly, higher 25(OH)D concentrations have been associated with brain markers reflecting cerebrovascular disease, including larger hippocampal volumes (11, 12) and a lower prevalence of white matter hyperintensities (8-10). However, most of the studies on brain morphometry have been relatively small, and causality is yet to be established as 25(OH)D concentrations have been measured around the time of brain morphology assessment and these associations could reflect confounding or disease-associated differences ('reverse causation'). Many prospective studies are looking into the associations of 25(OH)D concentrations with dementia and stroke, some of which suggest a threshold effect, where the associations are the strongest or restricted to those with the lowest concentrations (13, 14). However, the

causality of the association between vitamin D and dementia has not been confirmed, and the few randomized controlled trials (RCTs) have not provided convincing evidence for the role of vitamin D on dementia or other related outcomes (2).

Mendelian randomization (MR) is a genetic approach, which allows testing for underlying causality when RCTs are deemed infeasible or unethical (15). In an MR study, genetic variants, typically single nucleotide polymorphisms (SNPs), are used as proxy indicators for the exposure (15). Given that SNPs are randomly assigned and fixed at conception, they do not change in response to our behavior, or disease experiences, thereby reducing methodological problems related to confounding and reverse causality (Figure 1) (15). This approach has been used in at least three earlier studies investigating the association between 25(OH)D and the risk of Alzheimer's disease, which provided some support for an association (16-18). However, causal evidence for an association between 25(OH)D and stroke risk is inconclusive (19, 20). Additionally, all MR studies in this area, so far, have assumed linear associations. This type of approach is likely to miss an association in the context of a strong threshold effect, where the association is restricted to the correction of nutritional deficiency.

In this large-scale prospective study, we use information from up to 33,523 participants from the UK Biobank to examine the association between 25(OH)D concentrations with a range of brain neuroimaging features. Extending the analyses to 427,690 participants, we also examine the associations with the risks of dementia and stroke, for the first time, using the MR approach to test for the causal effects of increasing 25(OH)D concentrations in the context of severe vitamin D deficiency.

Methods:

Study population: UK Biobank is an ongoing prospective cohort study of 502,504 participants aged 37-73 years (99.5% 40-69 years) at recruitment (21). Participants were recruited across 22 centers in England, Scotland, and Wales from March 13, 2006, to October 1, 2010 (21). During baseline data collection, detailed information about the participants' socioeconomic status, lifestyle and health was obtained from self-reported touch-screen questionnaires, computer-assisted interviews, physical measurements and collection of blood samples (21). In 2014, the UK Biobank incorporated an imaging sub-study aiming to conduct magnetic resonance imaging (MRI) of the brain, heart and body on approximately 100,000 participants (22). Data collection is still ongoing and at the time of the data analyses, information for over 39,000 participants had been released (23).

In this study, we excluded participants who had more than two members per family, missing or low quality 25(OH)D data, or who had a history of dementia or stroke (Supplementary Figure 1). For the imaging sub-sample, we made further exclusions of participants with missing brain volume data and outliers, which were defined as any brain volume data \pm 3 standard deviations (SD) from the mean brain volume. Our final analysis samples included 427,690 participants for disease outcomes (3,414 incident dementia and 5,339 incident stroke cases), with brain neuroimaging sub-sample including up to 33,523 participants (Supplementary Figure 1). For our MR analyses, we further excluded participants without genetic data, who had a mismatch between genetic and self-reported sex, non-white British participants, and participants with more than one member from each family. The final sample size for the MR study was 294,514 for disease outcomes (including 2,399 incident dementia and 3,760 incident stroke cases) and up to 23,901 participants for imaging outcomes (Supplementary Figure 1, Supplementary Text S1).

Ethics: All participants provided informed consent before data collection by the UK Biobank. All data collection protocols for the UK Biobank have been approved by external ethics committees including North West Multi-center Research Ethics Committee (MREC),

the National Information Governance Board for Health & Social Care in England and Community Health Index Advisory Group (CHIAG) in Scotland (24). Ethics approval for our analysis was obtained from the UK Biobank Ethics and Governance Council and Human Research Ethics Committee (HREC) of the University of South Australia and all data has been de-identified for analysis.

Vitamin D: Serum 25(OH)D concentration was determined from samples collected at baseline using direct competitive chemiluminescent immunoassay (DiaSorin Liaison XL), with the assay having a measuring range of 10-375nmol/L, and coefficient of variation 5.04% to 6.14% (Supplementary Text S2). 25(OH)D values below (n=2,654) or above (n=2) the reportable limit were replaced with missing (25). Furthermore, given sample dilution issues, we excluded participants (n=10,002) with 25(OH)D data from aliquot 3 (Supplementary Figure 1). For categorical analysis, 25(OH)D was grouped as <24.9nmol/L, 25-49.9nmol/L, 50-74.9 nmol/L, 75-99.9 nmol/L, 100-124.9 nmol/L and 125-240nmol/L based on literature and Institute of Medicine and Endocrine Society Clinical Practice guidelines (6).

MRI brain volumes: The UK Biobank conducted MRI across sites at Reading, Newcastle and Cheadle Manchester (22). Scanning was performed using a Siemens Skyra 3T scanner running on VD13A SP4 software with a Siemens 32-channel Radiofrequency (RF) receive head coil (22) (Supplementary Text S3). Scanning was conducted from the top of the head to the neck using a 256cm superior-inferior field of view (22). The protocol consisted of sagittal T1-weighted images (1x1x1 mm resolution, TR=2000ms, TE=2ms) and T2-fluid attenuated inversion recovery (FLAIR) images with fat saturation (1.05x1x1 mm resolution, TR 5000ms, TE 395ms) (26). All acquired images were pre-processed and then checked for quality (Supplementary Text S3) (22). While total brain, grey matter, white matter and hippocampal volumes were extracted from processed T1 images only, white matter hyperintensities were identified from both processed T2-FLAIR images and T1-weighted images (22). All brain volumes were calculated using FreeSurfer software and

normalized for the head size using a T1-based head sizing scaling factor (scaled brain volume = brain volume * head size scaling factor) (22).

Dementia and stroke incidence: Data on incident dementia or stroke cases were obtained by the UK biobank from linkages to available national datasets including primary care data, hospital admissions electronic health records (EHR), self-reported health information from touch-screen questionnaires and the national death registry (27). Incidence of stroke or dementia was defined as any stroke or all-cause dementia cases after the baseline assessment and before the end of follow-up on February 1, 2020.

Covariates: Information on all covariates were obtained during the baseline assessment, with full information provided in Supplementary Table 2. Covariates were decided based on theory and literature. Basic characteristics included the month and participants age at baseline data collection, sex, self-reported ethnic group, and assessment center location. Sociodemographic factors included Townsend deprivation index (23, 28), education and employment status, and lifestyle covariates included the type of physical activity, diet quality (29), use of any dietary supplements (yes/no) and body mass index (BMI in kg/m^2), calculated based on measured weights and heights and categorized according to criteria by World Health Organisation as underweight ($<18.5\text{kg}/\text{m}^2$), normal ($18.5 - 24.9\text{kg}/\text{m}^2$), overweight ($25 - 29.9\text{kg}/\text{m}^2$) and obese ($\geq 30\text{kg}/\text{m}^2$) (23, 30). To minimize the possible influences by reverse causality, we further adjusted for sun exposure behaviors including time spent outdoors in summer and winter, frequency of sun protection and disease-related indicators including long-standing illness/disability/infirmary and depression.

Genetic Instruments: The most recent genome-wide association study (GWAS) identified 143 genetic variants associated with 25(OH)D using information from 417,580 European ancestry individuals from UK Biobank (31). Of these, 122 were autosomal SNPs of which we selected 35 common SNPs (minor allele frequency $>5\%$) that had at least nominally significant and directionally consistent association with 25(OH)D in an earlier independent GWAS by the SUNLIGHT Consortium, which did not include UK Biobank (32)

(Supplementary Table 3). We extracted these variants from the third release of imputed genetic data by the UK Biobank (33). For genotyping, two arrays (UK Biobank Lung Exome Evaluation (UK BiLEVE) for around 50,000 and UK Biobank axion array for around 450,000 participants) with 95% marker similarities were used, and here, we adjusted for the genotyping array. Haplotype reference consortium, UK10K and 1000 genome reference panel were used for imputation. Genotyping, imputation, and related quality control were carried out by UK Biobank central team, with full details reported elsewhere (33). We calculated a weighted genetic risk scores (GRS) using the 35 variants with weights taken from SUNLIGHT consortium (32) (Supplementary Text S4).

Statistical analysis:

Linear regression was used to investigate the association between 25(OH)D and brain neuroimaging outcomes. White matter hyperintensity was log-transformed due to skewness, while other brain volume indicators were normally distributed. Cox proportional hazards model was used to investigate the association between 25(OH)D and risk of dementia and stroke. The proportional hazards assumption was examined using Schoenfeld residuals and were satisfied in all models for dementia and stroke.

Adjustments were performed progressively, adjusting for additional groups of confounders with each new model. The basic model was adjusted for age, sex, assessment center, ethnicity, and month. Further adjustments were done for socioeconomic factors (education, Townsend deprivation index and employment status), lifestyles (BMI, type of physical activity, diet quality and any use of dietary supplements), sun behaviors (time spent outdoors in summer, time spent outdoors in winter and sun protection) and illnesses (long-standing illnesses/disability/infirmity and depression). Models were weighted by (1-kinship coefficient) to account for relatedness (34). For analyses involving brain volumes, we performed sensitivity analyses additionally adjusting for the duration between the baseline and imaging study visits, and after excluding incident cases of stroke or dementia occurring

before the imaging visit. We tested for two-way and three-way interactions of age, sex, ethnicity, and BMI in each of the associations, and conducted further stratified analyses in the presence of effect modification. In these models, interactions were based on likelihood ratio tests where age and BMI were included as continuous indicators, while sex and ethnicity were categorical. We tested for non-linearity using the quadratic form of 25(OH)D and interpreted the associations based on stratified associations in presence of curvature.

We used linear and non-linear MR analyses to explore the causal effects of 25(OH)D on brain volumes and the risk of dementia and stroke (Supplementary Text S4). For the linear MR analyses, we used two-sample random-effects inverse variance weight (IVW) MR as the primary approach, with this method providing valid causal estimates if there is no unbalanced directional horizontal pleiotropy (35). For additional sensitivity analyses, we included weighted median (36), weighted mode (37), Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) (38) and MR-Egger (39) analyses that all rely on different assumptions relating to underlying pleiotropy (Supplementary Text S5) (15). The two-sample MR analyses relied on variant-25(OH)D estimates taken from SUNLIGHT consortium (32), and variant-outcome estimates from UK biobank from a model adjusted for age, sex, assessment center, 40 principal components, genotyping array, and birth location. In contrast, the non-linear MR approach uses GRS in a one-sample setting that involves the following steps: first, we generated 40 strata using “instrument-free” 25(OH)D information, reflecting 25(OH)D concentrations from which the genetic effects had been removed, obtained by taking the residual from the regression of the 25(OH)D concentration on 25(OH)D GRS (40). We then generated localized average causal effect (LACE) estimates (which is the ratio of the GRS-outcome association estimates to GRS-25(OH)D association estimates) within each stratum and conducted a meta-regression of the LACE estimates against the mean of 25(OH)D in each stratum to test whether the fractional polynomial model fits better than the linear model (40, 41). To test for the model assumptions of a uniform GRS-25(OH)D association across all 40 strata, we examined the stratum specific

associations. We found that the 1st-2nd and 37th-40th strata were outliers (Supplementary Figure 2), and hence, we use non-linear MR analysis removing these strata for our primary results. Furthermore, we repeated the linear MR analyses including the pleiotropy robust methods in models stratifying according to baseline concentrations genetic instrument-free 25(OH)D (<24.9nmol/L, 25-49.9nmol/L, 50-74.9nmol/L and ≥75nmol/L) (15, 42). We further examined possible pleiotropy using leave-block-out analysis (Supplementary Text S6) which involves repeating the non-linear MR analysis using GRS excluding SNPs in the block (functional blocks and variants included in the block found in Supplementary Table 4). Potential impact fraction (PIF) (43) was calculated to estimate dementia incidence that may be preventable by correction of low vitamin D status in this population using estimates obtained from the non-linear MR analyses.

All observational analyses were conducted using the STATA SE version 14.1 software, and the MR analyses were conducted in R version 3.6.1 using TwoSampleMR (44), MR-PRESSO (38), and NLMR packages (40).

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Results

The median follow-up for both dementia and stroke incidence were 10.9 years. The total person-years at risk is 5,467,740.9 years for dementia and 5,371,196.5 years for stroke. Table 1 represents the distribution of the study population by baseline characteristics. Most of the participants were from British, Irish, or white backgrounds, females and aged 60-73 years at baseline without long-standing illnesses or depression. 25(OH)D concentrations varied by social and lifestyle covariates, and the type of physical activity, time spent outdoors, sun protection use, oily fish consumption, and dietary restrictions were also associated with brain volumes, and the incidence of dementia and stroke (Supplementary Table 5). Across the covariates, categories associated with lower total brain volume tended to reflect a higher rate of dementia and stroke (Supplementary Table 5).

Observational analysis

Association between 25(OH)D and all neuroimaging features strengthened after adjustment for socioeconomic factors (Table 2). Accounting for lifestyle, sun behavior and illness-related covariates led to some attenuation, however, all associations persisted even after full adjustment (Table 2). Sensitivity analyses including further adjustment for the duration between the baseline and imaging visits, or where participants with incident stroke or dementia were excluded also supported an association between 25(OH)D and all neuroimaging features (Supplementary Figure 3).

The association between 25(OH)D and total brain, grey matter, white matter, and hippocampal volume was non-linear ($P_{\text{non-linear}} < 0.04$ for all) (Table 2). For total brain, grey matter and white matter volume, the association with 25(OH)D concentrations were U-shaped with both low and high 25(OH)D concentrations associated with lower brain volumes, while for hippocampal volume, the association was apparent only in the upper extreme (Figure 2, Supplementary Figure 4). There was also some evidence for an association between lower 25(OH)D concentrations and white matter hyperintensity volume (25-49.9nmol/L vs. 50-74.9nmol, adjusted $\beta = 0.03$, 95%CI: 0.01, 0.05) (Figure 2A). There

was some heterogeneity in the strength of the association between 25(OH)D and total brain, grey matter and white matter volume between males and females ($P_{\text{interaction}} < 0.01$ for all), and while the coefficients were generally in the same direction, associations appeared stronger for males than for females (Supplementary Table 6). No evidence for interaction was observed by age, ethnicity, or BMI in the association of 25(OH)D concentrations with any of the neuroimaging features.

There was an association between 25(OH)D and dementia and stroke risk which persisted after full adjustment (Table 2). These associations were non-linear (adjusted $P_{\text{non-linear}} = 2.1 \times 10^{-9}$ and 0.01, respectively), but we did not observe any evidence for effect modification by age, sex, ethnicity, or BMI ($P_{\text{interaction}} > 0.05$ for all comparisons). The highest risks of both dementia and stroke were seen for participants with the lowest concentrations (<50nmol/L) of 25(OH)D, with no differences in risk by increasing concentrations (Figure 2B, Supplementary Figure 5).

Mendelian randomization:

25(OH)D concentrations were instrumented by GRS including 35 variants, which jointly explained 2.8% of the variation in the UKB (F-statistic=8672, $p < 1.0 \times 10^{-300}$). 25(OH)D GRS was not associated with confounders (uncorrected $P > 0.09$ for all, Supplementary Table 7).

In linear MR analyses, 25(OH)D was not associated with any of the neuroimaging outcomes or the risk of dementia or stroke (Supplementary Table 8, $P > 0.10$ for all linear associations). There was no evidence for pleiotropy (MR-Egger $P_{\text{intercept}} > 0.90$ for all associations), and MR-PRESSO outlier test did not detect evidence for pleiotropic variants. However, we found evidence for a non-linear inverse association between genetically determined 25(OH)D and dementia risk, with the odds of dementia decreasing with higher 25(OH)D concentrations until about 50nmol/l (Figure 3, Supplementary Figure 6). Based on the non-linear MR, individuals with serum 25(OH)D at 25 nmol/L had 54% (95%CI 1.21,

1.96) higher odds of dementia compared to those with 50 nmol/L. The shape of the polynomial model was affected by inclusion of variants in the “blood traits functional block”, with further investigation using leave-one-out analysis showing that the shape of the fractional polynomial was sensitive to the inclusion of GC variant to the GRS (Supplementary Table 9). In stratified MR analyses, higher 25(OH)D was associated with lower odds of dementia among those with the lowest concentrations (<25nmol/L, inverse variance weighted MR OR= 0.35, 95%CI 0.19-0.63 per 10 nmol/L higher), with no evidence for pleiotropy and consistent findings across all MR approaches (Supplementary Figure 7). There was no consistent evidence for lower dementia risk by higher 25(OH)D for individuals with concentrations >25nmol/l (Supplementary Figure 7). According to PIF calculation, up to 17% (95%CI 7.22, 30.58) of dementia could be prevented in this population by increasing the serum 25(OH)D to 50nmol/L for those with serum value below this threshold (Supplementary Figure 8).

Discussion

Our analyses using a large cohort of UK participants provides some evidence for a beneficial role for adequate 25(OH)D concentrations on brain health. In the observational analyses, low 25(OH)D concentration was associated with differences in several aspects of brain morphometry, in addition to increased risks of dementia and stroke. While we were not able to confirm causality concerning the effects on brain volumes or the risk of stroke, we did observe evidence for a non-linear causal association between 25(OH)D and dementia risk in the MR analyses. This suggests that the benefit of improving 25(OH)D concentrations is likely to be strongest and may be restricted to the context of alleviating vitamin D deficiency, and that attempts to increase concentrations beyond 50 nmol/L may provide limited further benefit. These findings highlight the importance of treating and preventing vitamin D deficiency, appreciating the challenges to obtain evidence from RCTs, which may be

deemed unethical or infeasible to confirm the causal effects of supplementation in those with very low 25(OH)D concentrations (15).

Findings on the association between 25(OH)D and brain volume have been largely based on cross-sectional studies, which cannot explore temporal associations. Consistent with a meta-analysis conducted in 2014, we found that lower 25(OH)D is associated with lower total brain volume (7). Furthermore, in line with a recent cross-sectional study (45), our study supports a threshold effect, where both lower and higher 25(OH)D concentrations are associated with lower total brain, grey matter and white matter volumes, all of which are markers reflecting brain atrophy and an elevated risk of cognitive decline and dementia. However, in our study, vitamin D deficiency was not associated with lower hippocampal volume, which is a key prognostic marker for dementia risk, and we only observed an association with high concentrations ($>125\text{nmol/L}$). This contrasts with at least two cross-sectional (11, 12) and one prospective study (47), which have all reported an association between vitamin D deficiency and lower hippocampal volumes. We did observe an association between low 25(OH)D and greater white matter hyperintensity volume, which is interesting as a greater prevalence of these lesions in the brain could suggest increases in dementia risk (46). Indeed, an association between low 25(OH)D and white matter hyperintensity volume has been consistently supported by earlier cross-sectional studies in institutionalized and hospitalized elderly participants, in whom also vitamin D deficiency is relatively common (8-10). However, to our knowledge there is only one earlier prospective study (N=1,658) which was conducted on a general population sample, and which did not find evidence for an association (47). While studies on the association between 25(OH)D concentrations and neuroimaging features appear to suggest a potential role in brain morphology, further well-designed prospective studies are required to clarify these associations and possible threshold effects.

The finding that the strongest effects on dementia risk are seen for those with the lowest 25(OH)D concentrations in our study was consistent both in observational and MR

analyses. Evidence for a threshold effect has also been observed in other studies, including a meta-analysis of five prospective studies which found a pooled 33% and 14% higher risk of dementia in vitamin D deficient (<30nmol/L) and insufficient (30-50nmol/L) participants respectively, when compared to those with sufficient concentrations (>50nmol/L) (13). Evidence from RCTs using vitamin D supplementation to prevent dementia or stroke is limited, and these studies have typically been small, of short duration and may not have included participants with overt deficiency (2). While other prospective studies have reported a similar U-shaped association with the risk of stroke as seen in our study (14), to our knowledge evidence for a causal association between 25(OH)D and stroke risk has not been reported by any of the MR studies conducted to date (19, 20). In contrast, there are three linear MR studies which support a causal association between 25(OH)D and Alzheimer's disease (16-18). Our non-linear MR analyses suggested a threshold effect, where increases in 25(OH)D would mainly benefit individuals who have an overt vitamin D deficiency. Interestingly, in our study and in the previous linear MR studies (16-18), the association between 25(OH)D and dementia appears to be sensitive to the removal of GC variants. GC encodes the vitamin D binding protein, and it is the strongest individual variant affecting 25(OH)D concentrations. As in our study, the association between 25(OH)D and dementia within the deficiency threshold was consistent across all MR methods, and no influential variants were observed, the apparent sensitivity to GC may reflect lack of power in the analyses excluding this variant.

A protective effect of higher 25(OH)D on brain health is biologically plausible and could be explained by at least three potential mechanisms. Firstly, the presence of vitamin D receptors in the hypothalamus has suggested a neurosteroid function for active vitamin D, promoting the growth and maturation of neurons (48, 49). Secondly, there may be vascular mechanisms as active vitamin D has been associated with reduced thrombosis and regulation of the renin-angiotensin system (5). Thirdly, replete levels of active vitamin D may act as a neuroprotectant through the suppression of excess inflammatory neurovascular

damage caused by pro-inflammatory cytokines and attenuation of amyloid proteins, commonly observed in Alzheimer's disease (3, 4).

There are several strengths to our study. Firstly, as one of the largest population-based prospective and MR studies investigating the association between 25(OH)D and brain health, our study had more statistical power to detect associations than past smaller studies. Secondly, the extensive data available allowed for comprehensive adjustments of confounders and enabled the investigation of effects at very low concentrations of 25(OH)D. Thirdly, due to the nature of MR studies, the findings from our MR study are less influenced by reverse causality and confounding (15), with our results being robust across several sensitivity analyses including pleiotropy robust methods. Finally, this is the first study to conduct non-linear MR analyses and to provide causal evidence for a role of 25(OH)D for which dementia risk appears to operate only below the deficiency threshold.

There were also several limitations to our study. Although we used extensive adjustment strategies, we cannot rule out influences by residual confounding in our observational analyses. Additionally, although the UK Biobank cohort is diverse, it is vulnerable to healthy volunteer bias as the baseline population were mostly less deprived, had intermediate to high education, and had a normal to overweight BMI (50). As the MR analyses were restricted to participants from white British ancestry, these findings may not be generalizable to other populations. While the genetic instruments included in our analyses all had a replicated associations with 25(OH)D concentrations, we cannot fully exclude the possibility of horizontal pleiotropy in which the genetic variant of interest affects the outcome through another phenotype or pathway (15). In particular, the possible independent role of vitamin D binding protein (GC) requires further scrutiny, even if extensive sensitivity analyses using multiple MR approaches did not find evidence for pleiotropy. Additionally, the sub-sample available for brain morphometry analyses may have been too small to detect a difference in the MR analyses, despite us using the largest study available. For instance, in contrast to the 23,901 participants with MR information, the

sample would need to be increased ten-fold ($N \sim 279,893$) to provide 80% power for detecting the type of difference in total brain volumes seen in our study at 5% level. Furthermore, given differences in 25(OH)D concentrations measured by different assays, and the small number of individuals with 25(OH)D >100 nmol/L in our study, it is not possible to infer potential effects of high concentrations. It might also be argued that as residual concentrations (where the effect of genetic variation was removed) were used to establish thresholds for 25(OH)D in the non-linear MR analyses, it is unclear how those relate to measured levels. However, given the correlation between residual and measured 25(OH)D concentrations is very strong ($r=0.986$), any difference is likely to be trivial.

In conclusion, our study supports a role of vitamin D deficiency on brain health, notably for the risk of dementia. Larger MR studies are needed to confirm causality for the proposed associations between 25(OH)D concentrations and brain morphometry. Our MR results suggest no clear association with stroke, while a causal relationship with dementia risk provides an important opportunity for prevention.

Author contributions:

SN wrote the first draft, conducted literature reviews, and analyzed the data. AM and AZ advised on analyses, analyzed the data, and drafted the paper; EH designed research, advised on analyses, provided essential material, supervised the study, and wrote the paper. DJL advised on literature and the data. All authors revised the paper, interpreted results, read, and approved the final manuscript.

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Table 1: Summary of baseline characteristics of the UK Biobank cohort

Characteristic		N (%)
Sex	Male	199,275 (46.6)
	Female	228,415 (53.4)
Age	40-49 years	102,588 (24.0)
	50-59 years	142,304 (33.3)
	60-73 years	182,798 (42.7)
Ethnic background	British, Irish, or White	403,475 (94.3)
	Indian, Pakistani, Bangladeshi, or Asian	7,709 (1.8)
	African, Caribbean, or Black	6,793 (1.6)
	Chinese	1,362 (0.3)
	Mixed or other ethnic groups	6,366 (1.5)
	Missing	1,985 (0.5)
BMI	Underweight <18.5 kg/m ²	2,196 (0.5)
	Normal [18.5, 25) kg/m ²	140,027 (32.7)
	Overweight [25,30) kg/m ²	180,706 (42.3)
	Obese ≥30kg/ m ²	103,161 (24.1)
	Missing	1,600 (0.4)
Education	None	71,245 (16.7)
	Intermediate (NVQ/CSE/A-levels)	149,049 (34.8)
	High (degree/professional)	202,414 (47.3)
	Missing	4,982 (1.2)
Townsend deprivation index	Less deprived	215,258 (50.3)
	Highly deprived	211,912 (49.6)
	Missing	520 (0.1)
Type of physical activity	None	26,736 (6.2)
	Light/moderate	354,236 (82.8)
	Strenuous sport	44,464 (10.4)
	Missing	2,254 (0.5)
Time spent outdoors in summer	None	871 (0.2)
	<2 hours	142,051 (33.2)
	3-5 hours	177,648 (41.5)
	>6 hours	82,238 (19.2)
	Missing	24,882 (5.8)
Frequency of sun protection use	Never goes out in the sunshine	2,533 (0.6)
	Never/rarely	42,940 (10.0)
	Sometimes	142,328 (33.3)
	Most of the time	150,926 (35.3)
	Always	87,612 (20.5)
	Missing	1,351 (0.3)
Depression	yes	44,394 (10.4)
	no	383,296 (89.6)
Longstanding illness/infirmity/disability	Yes	133,428 (31.2)
	No	283,384 (66.3)
	Missing	10,878 (2.5)

Table 2: The association between 25(OH)D (per 10 nmol/L) with brain volumes and the risk of dementia and stroke with progressive covariate adjustment.¹

Outcome	Model	β (LCI, UCI)	P_{trend}	$P_{\text{non-linear}}^3$
Total (N=31,025)	Basic	586.9 (230.9, 943.0)	1.2×10^{-3}	2.8×10^{-4}
	Socioeconomic	742.7 (382.9, 1102.5)	5.2×10^{-5}	1.2×10^{-4}
	Lifestyle	636.6 (261.4, 1011.8)	8.8×10^{-4}	3.3×10^{-5}
	Sun behaviors	561.8 (183.5, 940.1)	3.6×10^{-3}	5.4×10^{-5}
	Illness	548.0 (170.0, 925.9)	4.5×10^{-3}	9.2×10^{-5}
Grey matter (N=31,037)	Basic	452.5 (234.6, 670.4)	4.7×10^{-5}	5.0×10^{-4}
	Socioeconomic	563.3 (343.4, 783.2)	5.2×10^{-7}	2.0×10^{-4}
	Lifestyle	403.8 (175.5, 632.1)	5.3×10^{-4}	9.3×10^{-5}
	Sun behaviors	389.0 (158.8, 619.2)	9.3×10^{-4}	1.2×10^{-4}
	Illness	374.5 (144.5, 604.4)	1.4×10^{-3}	2.1×10^{-4}
White matter (N=31,037)	Basic	134.4 (-87.0, 355.8)	0.23	0.02
	Socioeconomic	179.4 (-44.5, 403.2)	0.12	0.01
	Lifestyle	232.8 (-1.5, 467.1)	0.05	6.6×10^{-3}
	Sun behaviors	172.8 (-63.6, 409.2)	0.15	9.5×10^{-3}
	Illness	173.5 (-62.8, 409.8)	0.15	0.01
Hippocampal (N=31,025)	Basic	3.1 (-2.6, 8.8)	0.29	0.04
	Socioeconomic	4.7 (-1.0, 10.5)	0.11	0.04
	Lifestyle	2.8 (-3.2, 8.8)	0.36	0.02
	Sun behaviors	1.6 (-4.5, 7.7)	0.61	0.03
	Illness	1.4 (-4.7, 7.5)	0.65	0.04
White matter hyperintensities² (N=29,989)	Basic	-7.7 (-12.9, -2.6)	3.0×10^{-3}	0.34
	Socioeconomic	-11.9 (-17.1, -6.7)	6.4×10^{-6}	0.27
	Lifestyle	-5.2 (-10.6, 2.0)	0.06	0.40
	Sun behaviors	-6.5 (-11.9, -1.1)	0.02	0.37
	Illness	-6.1 (-11.5, -7.0)	0.03	0.55
		HR (LCI, UCI)	P_{trend}	$P_{\text{non-linear}}^3$
Dementia (N=372,232)	Basic	0.86 (0.83, 0.88)	2.7×10^{-23}	5.6×10^{-22}
	Socioeconomic	0.84 (0.82, 0.87)	3.4×10^{-27}	5.7×10^{-19}
	Lifestyle	0.85 (0.82, 0.88)	6.0×10^{-23}	1.4×10^{-15}
	Sun behaviors	0.86 (0.83, 0.88)	4.2×10^{-21}	8.5×10^{-14}
	Illnesses	0.88 (0.85, 0.9)	3.4×10^{-16}	2.1×10^{-9}
Stroke (N=372,232)	Basic	0.93 (0.90, 0.95)	3.4×10^{-11}	9.7×10^{-7}
	Socioeconomic	0.92 (0.90, 0.95)	1.6×10^{-11}	2.9×10^{-5}
	Lifestyle	0.94 (0.92, 0.97)	1.9×10^{-6}	8.7×10^{-4}
	Sun behaviors	0.94 (0.92, 0.97)	1.4×10^{-6}	1.3×10^{-3}
	Illnesses	0.95 (0.93, 0.97)	3.8×10^{-5}	0.01

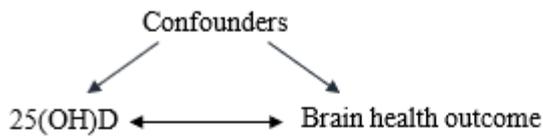
¹ Hazard Ratio (HR), lower confidence interval (LCI), upper confidence interval (UCI). Estimates for brain volumes from linear regression, risks of dementia and stroke assessed using Cox proportional hazards models. Adjustments were as follows: **Basic covariates (model 1)**: age, sex, assessment center, ethnicity, month; **Socioeconomic (model 2)**: model 1+ education, Townsend deprivation index, employment status; **Lifestyle factors (model 3)**: model 2+ BMI (categorical), type of physical activity, diet quality, any use of dietary supplements; **Sun behaviors (model 4)**: model 3+ time spent outdoors in summer, time spent outdoors in

winter, sun protection; **Illnesses (All covariates):** model 4+ long-standing illnesses/disability/infirmity +depression

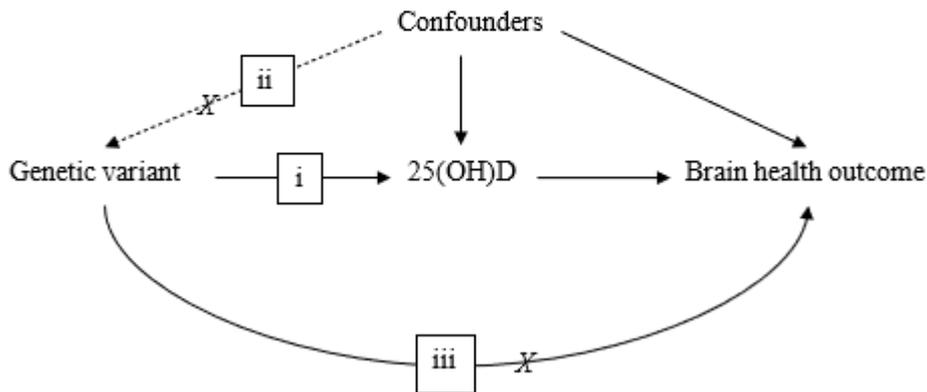
² White matter hyperintensity volume log transformed in analyses due to skewness.

³ P_{non-linear} from a model using a quadratic term of 25(OH)D in nmol/L

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A: Observational studies



B: MR studies

Figure 1: Characteristics of observational and Mendelian randomization (MR) studies. Observational studies (panel A) may be affected by confounders and reverse causality. MR studies (panel B) analyse the association between genetic variants, which are proxy indicators for the exposure (here, 25(OH)D), and the outcome to provide proof of principle for underlying causality (15). The diagram illustrates the core assumptions in a MR study, where genetic variants (i) should be associated only with the exposure, (ii) should not be affected by the confounders, and (iii) should not associate with the outcome through pathways or phenotypes other than the exposure.

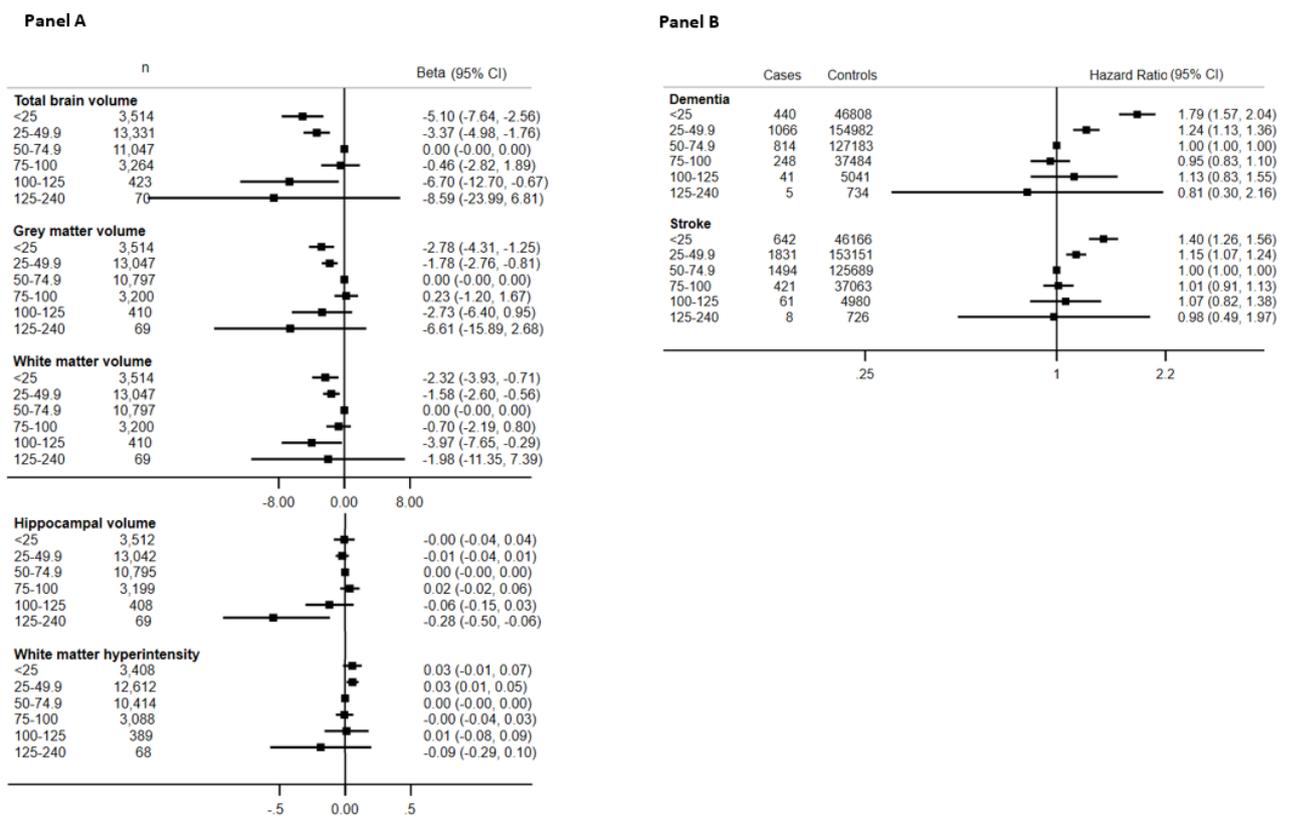


Figure 2: The adjusted observational associations between categories of 25(OH)D with neuroimaging outcomes (panel A) and disease outcomes (panel B). Confidence interval (CI). Participants with 25(OH)D 50-74.9nmol/L are used as reference. Estimates obtained from linear regression for neuroimaging outcomes, and from Cox proportional hazards model for disease outcomes. All models are adjusted for basic covariates (age, sex, assessment center, ethnicity, month), socioeconomic factors (education, Townsend deprivation index, employment status), lifestyle factors (BMI (categorical), type of physical activity, diet quality, any use of dietary supplements), sun behaviors (time spent outdoors in summer, time spent outdoors in winter, sun protection) and disease outcomes (long-standing illnesses/disability/infirmary, depression).

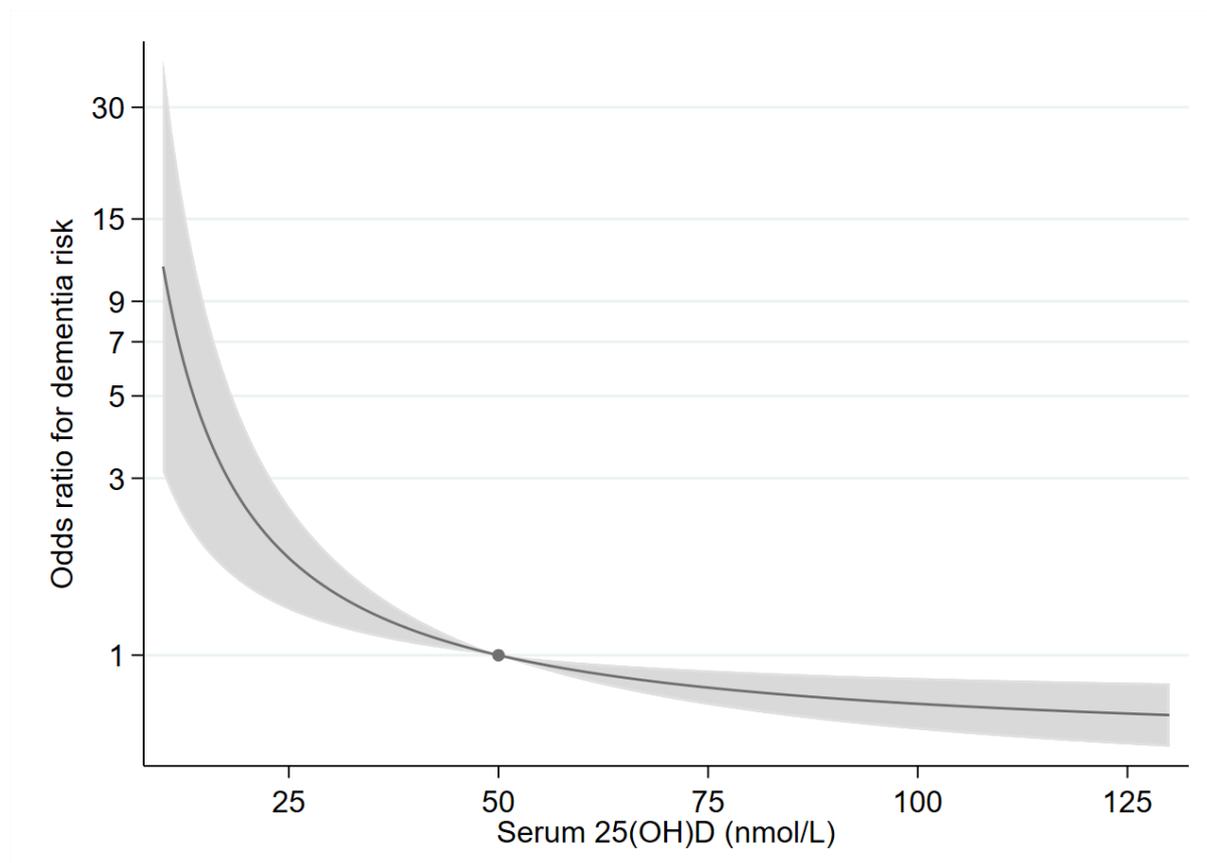


Figure 3: The association between genetically determined 25(OH)D and odds of dementia using a fractional polynomial model with the outlying strata removed ($P_{\text{non-linearity}}=5.9 \times 10^{-3}$ for this association). The dot indicates the reference point at 50nmol/L and the shaded areas represent the 95% confidence intervals.

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