Vitamin D metabolites and risk of first clinical diagnosis of central nervous system demyelination

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Abstract

Low 25-hydroxyvitamin D (25(OH)D) concentration is a recognised risk factor for multiple sclerosis (MS). Associations with vitamin D metabolites and vitamin D binding globulin (VDBG) have not been widely studied. We assessed the association between vitamin D metabolites ($25(OH)D_2$, $25(OH)D_3$, c3-epimer $25(OH)D_3$, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$)) measured by liquid chromatography-tandem mass spectrometry assays, VDBG measured using a polyclonal immunoassay, and calculated free and bioavailable 25(OH)D, free $1,25(OH)_2D_3$, and the $24,25(OH)_2D_3$: total 25(OH)D and total $1,25(OH)_2D$: total 25(OH)D ratios with risk of a first clinical diagnosis of CNS demyelination (FCD) in an Australian case-control study (n=196 cases, n=241 controls, matched on age, sex and study region). Higher 25(OH)D (adjusted odds ratio (AOR)=0.94 (95% confidence interval (CI) 0.85-1.03) per 10 nmol/L increment) and $24,25(OH)_2D_3$ (AOR=0.81 (95%CI 0.65-1.00) per 1 nmol/L increment) concentrations were associated with reduced FCD risk. Our results were compatible with no association for the other vitamin D metabolites, ratios, or VDBG with FCD risk. Thus, using standardised assays, and a comprehensive range of vitamin D metabolites, we confirmed the association of higher 25(OH)D and reduced FCD risk, and describe a similar effect for $24,25(OH)_2D_3$; free or bioavailable 25(OH)D were not associated with FCD risk.

Key words: multiple sclerosis; vitamin D, free vitamin D, vitamin D binding protein, first demyelinating event

Abbreviations: : AOR – adjusted odds ratio; CI – confidence interval; FCD – first clinical diagnosis of central nervous system demyelination; MS – multiple sclerosis; VDBG – vitamin D binding globulin

1. Introduction

Multiple sclerosis (MS) is an incurable chronic inflammatory and neurodegenerative autoimmune demyelinating disease of the central nervous system (CNS). Immune-mediated inflammation and neurodegeneration co-occur at all disease stages, but with weighting toward inflammation early in the disease (1, 2). Increasing incidence along a latitude gradient (3) and over recent time (at least in some locations) (4), as well as apparent changes in the sex ratio over time (5) have implicated a role for environmental risk factors. Indeed, we have shown that four key environmental risk factors for MS – smoking, past history of infectious mononucleosis, low vitamin D and low actinic damage (a measure of chronic past sun exposure) – together accounted for 53% of the risk of a first clinical diagnosis of CNS demyelination (FCD), a potential indicator of incipient MS (6).

In 2011 we published that low sun exposure and low serum 25-hydroxyvitamin D (25(OH)D) concentrations were independent risk factors for a first demyelinating event (FDE), using data from a multi-centre case-control study, the Ausimmune Study (7). The effect size for 25(OH)D level (per 50nmol/L increase, adjusted odds ratio (AOR)=0.69 (95% confidence interval (CI) 0.48-0.98)) was similar to that shown in a previous nested case-control study (AOR=0.59 (95%CI 0.36-0.97)) (8). Ensuing studies have confirmed a heightened risk of MS associated with low 25(OH)D concentrations, across a range of study types (reviewed in (9)), including Mendelian randomisation (10). A protective effect is thought to be through immune modulation (9, 11), although there is a lack of consistency in reports of cross-sectional correlations between 25(OH)D level and inflammatory markers in people with MS (9). Furthermore, the relevance of systemic markers of inflammation to the immune response in MS is not clear (12).

The metabolite 25(OH)D is an intermediary in the vitamin D pathway that has little biological activity. A further hydroxylation is required to make the bioactive metabolite, 1,25-dihyroxyvitamin D ($1,25(OH)_2D$), under the control of the 1α-hydroxylase enzyme (encoded by the gene *CYP27B1*) (13). Both 25(OH)D and $1,25(OH)_2D$ are catabolised by a 24-hydroxylase (encoded by *CYP24A1*), to form 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) and 1,24,25-trihydroxyvitamin D ($1,24,25(OH)_3D$), respectively (13). The concentrations of 25(OH)D and $24,25(OH)_2D$ are much greater than those of $1,25(OH)_2D$ and $1,24,25(OH)_3D$ (nmol/L compared to pmol/L). In recent years there has also been interest in the c-3 epimer of 25(OH)D (3-epi-25(OH)D) which has been shown to have biological activity (13). It is usually found only in high concentrations in infants, but can also occur at measurable concentrations in adults (14). All of the vitamin D metabolites circulate in blood tightly bound to a vitamin D binding globulin (VDBG), with small amounts free or loosely bound to albumin (15, 16). VDBG is polymorphic, with three main isoforms, characterised by different electrophoretic mobility and possibly affinity for 25(OH)D: Gc1F, Gc1S and Gc2 (16, 17). Gc1F is the predominant isoform in African Americans, with Gc1S or Gc2 more common in Caucasian populations (15). Polymorphisms in VDBG genes account for most of the genetic variation in 25(OH)D concentrations (18).

A 2013 study appeared to highlight the importance of free and loosely bound (bioavailable) 25(OH)D in relation to disease risk, rather than total 25(OH)D (19). In that study, total 25(OH)D concentrations were lower in black compared to white participants, but bone mineral density was higher in blacks than whites. However, the concentrations of bioavailable 25(OH)D, estimated using the serum VDBG concentrations and affinity constants specific for VDBG genotype, were similar between whites and blacks. The bioavailable 25(OH)D was thus thought likely to better reflect vitamin D bioavailability

compared to total 25(OH)D (19). However, the monoclonal antibody assay used to measure VDBG concentrations in that study is now recognised as not adequately detecting some VDBG phenotypes and the findings were likely spurious due to the measurement issues (15).

Nevertheless, the free hormone hypothesis predicts that the concentration of unbound hormone is important in mediating biological activity (15, 20, 21). Free hormone concentrations do appear to be particularly important for other hormones, e.g. thyroid hormone, with more limited support for vitamin D (for a comprehensive review, see (16)).

Several studies have examined concentrations of VDBG or vitamin D metabolites in people with MS, with conflicting findings (22, 23). Two recent studies have suggested an association of VDBG haplotypes (24), or VDBG serum concentrations (25) with MS onset, while one study showed lower 25(OH)D concentrations, but not bioavailable 25(OH)D, in people with clinically isolated syndrome compared to controls (26). Here we use data from the Ausimmune Study to examine the risk of FCD in relation to a comprehensive range of measured vitamin D metabolites and VDBG, including specifically testing the free hormone hypothesis which would suggest that FCD risk is more strongly associated with the concentrations of free and bioavailable 25(OH)D than total 25(OH)D.

2. Methods

The Ausimmune Study was a multi-centre case-control study investigating environmental risk factors for the onset of CNS demyelinating disease (27). The study methods have been detailed elsewhere (27). In brief, case participants were recruited between November 2003 and December 2006 in four Australian regions: Brisbane city (latitude 27°South), Newcastle city and surrounds (33°S), Geelong city and the Western Districts of Victoria (37°S) and the whole of Tasmania (43°S). Cases were aged between 18 and 59 years and had a FCD during

the study period. The diagnosis was confirmed by the study neurologist team, including confirming whether this was a classic first demyelinating event (FDE), FCD but with a history consistent with an unrecognised prior event, or a diagnosis of primary progressive MS. For each case, between 1 and 4 controls, matched by sex, age (within two years) and location, were randomly selected from the Australian Electoral Roll. The current study was based on a subset of all cases and controls due to funding constraints and availability of serum. We assayed as many case-control sets as was possible within those constraints, prioritising cases with a classic first demyelinating event and their matched controls.

Participants completed self-administered and nurse-administered questionnaires for demographic data, including ethnicity, the highest level of education completed, and past environmental exposures, including smoking, history of infectious mononucleosis, and level of physical activity (7). Height and weight were measured by a study nurse using standardised protocols.

Venous blood samples were collected at the study interview. Serum was separated and stored in 1ml aliquots at -80°C. In our previous study (7), serum 25(OH)D_2 and 25(OH)D_3 concentrations were measured using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay at the RMIT Drug Discovery Technologies (RDDT) laboratory, Melbourne. A recent study has shown that LC-MS/MS-measured 25(OH)D levels are stable in serum stored for 5 years at 80°C (28). Accurate and precise measurement of 25(OH)D concentration is now best ensured by using an assay standardised to a reference measurement procedure (RMP) under the International Vitamin D Standardisation Program (29). For the current study, we measured concentrations of vitamin D metabolites (25(OH)D_2, 25(OH)D_3, 3-epi-25(OH)D_3) on stored sera using a high performance LC-MS/MS

assay standardised to the RMP (CV% 6.5, 4.1, 3.6 for low, medium and high $25(OH)D_3$, respectively) and based at the University of Western Australia Metabolomics Australia node (UWAMA) (30).

We measured $1,25(OH)_2D_3$ (CV% 2.9) and $24,25(OH)_2D_3$ (CV% 5.0) in sera using Immunodiagnostick Solid Phase Extraction (KM1000) and liquid-liquid extraction, respectively, followed by 2D-LC-MS/MS (31).

Vitamin D binding globulin was measured using an automated polyclonal antibody method for Immunoturbimetric analysis (Dako, product code A0021) at UWAMA (CV% 5.5). A subset of samples (n=50) across the range of VDBG values was also measured using mass spectrometry at the University of Washington, Seattle (CV% 12.0) (32). Albumin was measured using a Bromocresol green colourimetric assay, Randox code AB 362 (CV% 3.3).

We used data on VDBG genotypes measured previously in the Ausimmune Study (33), focusing here on single nucleotide polymorphisms (SNPs) rs7041 and rs4588 to define the different haplotypes of VDBG (19).

2.1 Statistics Categorical data are described using number and percentage; continuous variables are described using mean and standard deviation (SD) or median and interquartile range (25^{th} – 75^{th} percentile) according to whether the data were normally or non-normally distributed. Body Mass Index (BMI) was calculated using the standard formula (weight (kg)/height $(m)^2$). We merged the categories for underweight and normal weight (BMI<25 kg/m^2), and for obese and obese II (>30 kg/m^2) (retaining overweight BMI, 25-30kg/m^2). For participants with smoking history, smoking was calculated as the total number of years smoking excluding periods of cessation, as per our previous work (7).

The limits of quantification (LOQ) for the UWAMA-assayed 25(OH)D_2 and 3-epi-25(OH)D_3 were 3 nmol/L and 2 nmol/L respectively. Where these metabolites were above the limit of detection (LOD) but below the LOQ, the concentration was replaced with the midpoint between zero and the LOQ. The sum of 25(OH)D_2 and 25(OH)D_3 is referred to as 'total 25(OH)D'.

We first tested agreement between the RDDT and UWAMA assays for 25(OH)D_3 concentration using an intraclass correlation (ICC) statistic with a two-way mixed effect layout and a Bland Altman plot (we excluded 25(OH)D_2 from this comparison due to different LOQs between the two assays and thus imputation of low values).

Agreement between the values from the two methods measuring VDBG concentration was assessed using an ICC and Bland Altman plots as above. We used a Deming regression model to derive an equation to allow imputation of VDBG according to the mass spectrometry values of the subsample (using MedCalc, https://www.medcalc.org/); these values were used in sensitivity analyses.

We calculated concentrations of free and bioavailable total 25(OH)D as previously described (19) and summarised in the Supplementary material. Bioavailable 25(OH)D is defined as the amount of 25(OH)D not bound to VDBG; that is, the sum of albumin-bound and free 25(OH)D. Affinity constants are defined for homozygous VDBG variants (19). We defined affinity constants for heterozygous variants as mid-way between the two relevant homozygous values (34). These affinity constants are outlined in Supplementary Table 1. We calculated the molar ratio of 1,25$(OH)_2$D:VDBG as an estimate of free 1,25$(OH)_2$D (16), and the ratios of 24,25$(OH)_2D_3$: total 25(OH)D and total 1,25$(OH)_2$D: total 25(OH)D as markers of vitamin D metabolism (35).

We tested the correlation between the different vitamin D metabolites and VDBG concentrations using Pearson correlation coefficients (r), separately for cases and controls. We tested for significant differences in correlation coefficients using Fisher's z transformation (36).

In regression analyses, for those vitamin D metabolites that showed significant seasonal variation (total 25(OH)D, 24,25$(OH)_2D_3$), we adjusted for season as in our previous work (7). In brief, seasonally-adjusted estimates were calculated by modelling the annual cycle in metabolite concentrations as single-harmonic sine and cosine waves with region-specific coefficients to account for the positive linear trend in amplitude with increasing latitude. Season-adjusted values of the metabolite were estimated by subtracting the fitted contribution of annual cycles from the observed measurements and adding this to the mean of all samples. We adjusted the control blood collection date to that of their matched case using the modelled sine and cosine curves (7). The distribution of 1,25$(OH)_2D_3$ was significantly skewed; values were (natural) log-transformed prior to use in regression analyses.

We tested the difference in VDBG haplotypes between cases and controls using univariate conditional logistic regression, with matching on age, sex, and study region. We used multivariable conditional logistic regression to test the associations between FCD risk and vitamin D metabolites and VDBG serum concentrations as both continuous and categorical (quintiles of the control distribution except for 3-epi-25$(OH)D_3$), the latter to examine the form of any dose-response relationship. For 3-epi-25$(OH)D_3$, we used three uneven categories (cuts at the median and 75th percentiles) due to a preponderance of

concentrations between the LOD and LOQ. We tested the trend across categories by modelling across the category rank scores of the exposure variable.

Participants with relevant missing data were excluded from those specific analyses. We tested the following potential confounders: total years smoking, physical activity, history of infectious mononucleosis, BMI, education, and ethnicity (analyses not shown). Final models are adjusted for variables meeting the criteria for confounding (independent risk factor for the outcome from literature and/or in the data ($p<0.1$); statistically associated with the exposure ($p<0.1$); not on the causal pathway between exposure and outcome): total years smoking, physical activity category, and history of infectious mononucleosis.

We undertook the following sensitivity analyses: 1) using deseasonalised total 25(OH)D in the estimation of free and bioavailable 25(OH)D; 2) using the imputed mass spectrometry VDBG values rather than the measured immunoassay values in the conditional logistic regression to assess association with case vs. control status; 3) using both deseasonalised total 25(OH)D and imputed VDBG to estimate free and bioavailable 25(OH)D; and, 4) using non-conditional multivariable logistic regression, adjusting for age, sex and study region in the assessment of the association between free and bioavailable total 25(OH)D and case vs. control status (to use all the data including unmatched cases and controls due to missing data on VDBG genotype).

All analyses were performed with Stata/SE for windows (version 14.0; StataCorp LP, College Station, TX). Adjusted odds ratios (AOR) and 95% confidence intervals (CI) are reported for associations, with interpretation of the results guided by these estimates rather than p-values (37). Statistical significance was set at $p<0.05$.

2.2 Study approval. The Ausimmune Study was approved by nine human research ethics committees, with the central approving committee the Human Research Ethics Committee of the Australian National University. All participants provided written informed consent prior to participation.

3. Results

Our study included 196 participants with a recent FCD and 241 control participants sourced from the population who did not have demyelinating disease and were matched on age (within 2 years), sex, and study region to case participants (with a variable matching ratio) (7). We selectively sampled the total Ausimmune Study participants to focus on cases with a classic FDE; 75% (n=147) of case participants in this study had had a classic FDE during the study period; 20% (n=40) had had a prior (unrecognised) demyelinating event; and 5% (n=9) had a first diagnosis of primary progressive MS. The characteristics of our study sample are provided in Table 1. The concentration of VDBG amongst controls varied according to the VDBG haplotype (*p=0.03*, see Supplementary material, Table 1), with isoforms carrying the 1s haplotype having higher VDBG concentrations. Of the participants with genotyping for VDBG, most were Caucasian (n=344, 94%); the most common VDBG isoform was Gc1S (70.3%), while for non-Caucasians, the Gc1F isoform predominated (47.8%), although the latter was based on small numbers. There was no significant difference in VDBG concentrations according to ethnicity (*p=0.76*). The 3-epi-25(OH)D_3 was detectable in all samples tested, but was below the LOQ (2 nmol/L) for nearly half of the samples (216/433). The highest value was 12.2 nmol/L.

We first considered agreement between different assays for 25(OH)D_3 (prior measurement (7) vs. current, see methods) and for VDBG. There was good agreement (38) between the

individual 25(OH)D_3 concentrations measured by the RMIT Drug Discovery Technologies (RDDT) and University of Western Australia Metabolomics Australia (UWAMA) assays based on the ICC (ICC=0.90 (95% CI 0.88, 0.91)). However, the RDDT measurements were on average 14.0 nmol/L higher than the UWAMA measurements (95% CI 12.9, 15.2) (Supplementary Fig 1), with greater difference and scatter as the average 25(OH)D_3 of the two measurements increased.

For the comparison of methods measuring VDBG, the individual ICC was poor (ICC=0.38 (95%C% 0.05-0.64)); the Bland Altman plot is shown in Supplementary Fig 2. The limits of agreement were 37.0 to 490.2 mg/L; mean difference 263.6 (95%CI 231.4, 295.8) mg/L. The correlation for ranking of values (Spearman r=0.53) was higher than for the actual values (Pearson correlation=0.40). Supplementary Figure 3 shows the results of the Deming regression analysis.

We next tested the correlation between the concentrations of vitamin D metabolites and VDBG measured for the current study (Pearson's r, (*p*)). Results for cases and controls were generally similar, with typically strong correlations between metabolites (see Table 2). For both cases and controls, concentrations of total 25(OH)D (25(OH)D_2 + 25(OH)D_3) were highly correlated with 24,25$(OH)_2D_3$, 3-epi-25(OH)D_3, and free and bioavailable total 25(OH)D. Weaker correlations were apparent for the concentration of 1,25$(OH)_2D_3$ with the concentrations for all other metabolites and VDBG, except for free 1,25$(OH)_2D_3$ (which is calculated from 1,25$(OH)_2D_3$ so that a high correlation is expected). Of interest, there were significant differences between cases and controls in the magnitude of the correlations for total 25(OH)D with 24,25$(OH)_2D_3$ (*p<0.001*), for free total 25(OH)D with free 1,25$(OH)_2D_3$ (*p=0.009*), and for bioavailable total 25(OH)D with free 1,25$(OH)_2D_3$ (*p=0.01*).

There was no marked seasonal variation for $1,25(OH)_2D_3$, 3-epi-$25(OH)D_3$, free total 25(OH)D or bioavailable total 25(OH)D and these were not deseasonalised. Concentrations of $24,25(OH)_2D_3$ displayed a significant seasonal trend and were deseasonalised as previously described (see Methods); the deseasonalised values were normally distributed.

Table 3 summarizes the analysis of the associations between the measured and calculated vitamin D metabolites and VDBG concentration with risk of FCD. We confirmed our previous finding of an association between higher total 25(OH)D concentration and reduced risk of FCD (for comparison to previous findings, for a 50nmol/L increment in 25(OH)D, the AOR was 0.71 (95%CI 0.45, 1.13), *p=0.15*). The analysis by quintiles for each of the 25(OH)D assays (Fig 1) shows a less convincing association for the UWAMA total 25(OH)D than for the RDDT total 25(OH)D, with the highest quintile cut-off considerably lower in the former than in the latter. We found a similar relationship for $24,25(OH)_2D_3$, with higher levels associated with reduced FCD risk and some evidence of a dose-response, shown in Fig. 2. The associations for the other vitamin D metabolites, ratios and VDBG were compatible with no association with FCD risk.

The results of the dose response and threshold analyses for the various metabolites and VDBG are shown graphically in Supplementary Figures 4-9. There was little evidence to support a dose response or threshold for the association with $1,25(OH)_2D_3$ ($p_{trend}=0.68$), free total 25(OH)D ($p_{trend}=0.78$), bioavailable total 25(OH)D ($p_{trend}=0.92$) or VDBG concentration ($p_{trend}=0.92$). Similarly, there was little evidence of an association across categories of 3-epi-$25(OH)D_3$ with FCD risk (Supplementary Fig. 9), including of a dose-response or threshold ($p_{trend}=0.40$)). We further found no evidence of an association between case vs. control status and VDBG haplotype (results not shown).

We undertook a number of sensitivity analyses. The results are summarized in Table 4; the findings are similar to those for the main analyses.

4. Discussion

This study presents a thorough examination of risk of FCD (as an indicator of MS) in relation to various facets of vitamin D metabolism. Our results confirm an association of higher concentrations of total 25(OH)D with reduced risk of FCD, and highlight the lower threshold concentration for an apparent protective effect when 25(OH)D measurements derive from a standardised assay, compared to previous studies (7, 8, 39). We found little evidence to support the free hormone hypothesis, with no convincing evidence of an association with free or bioavailable total 25(OH)D despite strong correlations of these metabolites with 25(OH)D concentration. In addition, there was no evidence of an association with concentrations of $1,25(OH)_2D_3$ (which are under tight physiological control) or the c3-epimer of $25(OH)D_3$.

A novel finding of the current analyses was the protective association with higher $24,25(OH)_2D_3$ concentration – a nearly 20% reduction in odds of being an FCD case per 1 nmol/L increase in concentration. We are not aware of any other studies reporting risk of MS onset in relation to $24,25(OH)_2D_3$ concentration. Our findings are however consistent with a study showing that disability levels in people with MS were inversely associated with serum $24,25(OH)_2D$ concentration (22). A recent study suggests that the concentration of $24,25(OH)_2D$ may represent a novel way to test activity of the vitamin D receptor, and found that $24,25(OH)_2D$ concentrations were more strongly associated with bone mineral density and incident hip fracture risk than were 25(OH)D concentrations (35). In accordance with

that study, we tested the ratio of 24,25(OH) $_2D_3$ to total 25(OH)D and also 1,25(OH)$_2D_3$ to total 25(OH)D in relation to FCD risk, but these analyses returned null results.

A further novel and intriguing finding was that the correlation between 25(OH)D and 24,25(OH)$_2D_3$ was significantly stronger in cases compared to controls, and that the correlation of free or bioavailable 25(OH)D with free 1,25(OH)$_2$D was significantly stronger in controls compared to cases. These findings may support a derangement of vitamin D metabolism in people with CNS demyelination or MS that requires further investigation.

Strengths of the Ausimmune Study are the inclusion of cases that have a FCD, rather than established MS, minimising disease-related alterations in behaviour and recall bias and effects of MS treatment post-diagnosis (at the time of the Ausimmune Study, treatment with disease modifying treatments was available only following a confirmed diagnosis of MS). An aspect of particular importance in this study is the use of standardised vitamin D assays so that the values reported are likely to be the true value of the metabolite. The design of the study allowed the comprehensive collection of data on exposures and potential confounders.

The study was however limited by missing genotype data for some participants, limiting the sample size for the calculation of free and bioavailable 25(OH)D. Further, measurement of VDBG using the (now standardised) mass spectrometry assay for VDBG (32) was limited to a subset of our samples. The latter may have affected the accuracy and precision of our calculations of free and bioavailable 25(OH)D, although our findings did not change when using the imputed VDBG values compared to the measured values to calculate these parameters. Nevertheless, the final sample size for these analyses was comparable to, or larger than, other recent studies examining similar questions (24, 25). We were unable to

directly measure free 25(OH)D due to the lack of available assays with adequate proven precision and accuracy (and our sample quantity was limited). This was mitigated by the incorporation of protein binding affinities specific to genotype, though for heterogeneous cases binding affinity was an estimated value. We did not have measurements for inflammatory markers to directly assess the correlation with 25(OH)D level.

The calculated serum concentration of bioavailable total 25(OH)D is largely determined (apart from total 25(OH)D concentration) by the VDBG concentration and binding affinity (16), with the latter governed by an individual's VDBG genotype (40, 41). Certain genotypes are more populous among different races: blacks predominately carry the VDBG phenotype Gc1F, while the Gc1S variant is more common among whites (19). Our sample was largely of European origin (94% noted by study nurses as 'Caucasian'). The limited heterogeneity in VDBG genotype among participants, in combination with the limited sample size, may have restricted the ability of this study to detect an association with concentrations of VDBG, or with free and bioavailable total 25(OH)D concentration.

We and others have previously demonstrated marked variability in 25(OH)D concentrations returned by various 25(OH)D assays, with LC-MS/MS assays commonly showing a positive bias, particularly at high 25(OH)D concentrations (42, 43). This is consistent with our current analyses. In our re-analysis of data from this Ausimmune Study subsample, the association with 25(OH)D concentration, while compatible with a reduction in MS risk with similar effect size to that previously shown, was not statistically significant as shown by the wider confidence interval (most likely a result of our smaller sample size in this study). It is important to consider the effects of both inaccuracy and imprecision of the 25(OH)D assays on the interpretation and use of findings from epidemiological studies (39). Imprecision may

result in wider confidence intervals. Clinical trials of vitamin D supplementation for MS that base power calculations on the effect sizes and confidence intervals reported in studies not using a standardised assay (hence, potentially imprecise) may be underpowered; this is consistent with the interpretation from several of these studies (44). Inaccuracy may lead to exaggeration of the absolute concentrations of 25(OH)D required to attain a protective effect compared to a standardised assay (7, 8). In the current study, and consistent with previous findings (7, 8), the 'protective' effect of 25(OH)D concentration appears to be for the highest compared to the lowest quintile/category, with little evidence of any association in intermediate categories. The cut-point for this highest category is thus of considerable importance to guide clinical targets for vitamin D supplementation to reduce disease risk.

Previous studies have returned misleading findings when a monoclonal antibody immunoassay was used to measure concentrations of VDBG (16, 19). Here we used a polyclonal immunoassay, but found that the agreement was low compared to concentrations measured using a mass spectrometry assay (with the former approximately two-fold higher than the latter, as previously observed) (45). Our sensitivity analysis using imputed values (based on Deming regression) showed results for associations that are similar to the main analysis, suggesting we would not change any conclusions from the current study.

Our results are compatible with no association between free or bioavailable total 25(OH)D concentration and FCD risk. This is consistent with another recent study which found that, while low serum 25(OH)D was a risk factor for clinically isolated syndrome, there was no association with bioavailable 25(OH)D (26). The VDBG has very high affinity for vitamin D metabolites and is present in human serum at higher molar concentrations than most

transport proteins. Thus, the free concentration of metabolites is very low, both absolutely and relative to other hormones such as cortisol, thyroxin or sex hormones. In addition, megalin and cubulin transport the VDBG-25(OH)D complex into cells, so that it is not necessary for vitamin D metabolites to be in their unbound form for this to occur (16).

An important finding from our analysis is the lack of association of $1,25(OH)_2D_3$ with FCD risk. This aligns with previous literature that concludes that this metabolite is so invariable as to offer little insight into how fluctuations in vitamin D status affect disease onset or outcomes (19, 46). It is a tightly regulated molecule with a very short half-life that exists in low concentrations so that at the time of measurement observations will be reflective of concentrations of transient $1,25(OH)_2D$ (46). Indeed, we founds a poor correlation between concentrations of 25(OH)D and of $1,25(OH)_2D_3$. Previous studies have also shown no association between $1,25(OH)_2D_3$ concentration MS risk (47) or disease activity (48), although for the latter the results are not completely consistent (49).

In conclusion, our study confirms our previously reported reduction in odds of FCD in association with higher 25(OH)D concentrations, now measured using a standardised assay for 25(OH)D. Higher $24,25(OH)_2D_3$ concentrations were also associated with reduced FCD risk, and we found no evidence of an association between FCD risk and concentrations of $1,25(OH)_2D$, 3-epi-25(OH)D, free or bioavailable 25(OH)D, or VDBG, and no association with VDBG haplotypes. Significantly different correlations between cases and controls for some vitamin D metabolites hint at derangements in vitamin D metabolism in the initial stages of clinical CNS demyelination and MS, further supporting evidence of a role of vitamin D in MS onset.

Author contributions

CT and RML undertook all analyses and drafted the manuscript. RML, ALP, BT, IvdM and the Ausimmune Investigator Group were responsible for collection of the original data and samples. MWC undertook the vitamin D assays and provided critical input to the measurement aspects of the manuscript. RML, LB, ALP, MWC and BT secured funding for the current study. All named authors reviewed the manuscript and gave critical feedback.

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Table 1. Descriptive statistics for Ausimmune Study participants included in this study.

		Controls (N=241)	Cases (N=196)
Age years, mean (SD)		39.5 (9.6)	38.9 (9.7)
Sex: Female, %(n)		78.0 (188)	77.6 (152)
Region (%(n))			
	Brisbane, 27°S	38.2 (94)	35.2 (69)
	Newcastle, 33°S	8.7 (21)	8.7 (17)
	Geelong, 37°S	26.1 (63)	24.5 (48)
	Tasmania, 43°S	27.0 (65)	31.6 (62)
Ethnicity[b] (%(n))			
	Caucasian	91.7 (220)	96.9 (189)
	Other	8.3 (20)	3.1 (6)
Physical activity[c] (%(n))			
	Low	21.7 (45)	16.6 (28)
	Medium	37.7 (78)	36.7 (62)
	High	40.6 (84)	46.8 (79)
Total years smoked (median (25th-75th percentile)), years[d]		0.0 (0.0-14.4)	5.4 (0.0-19.0)
History of infectious mononucleosis (%(n))			
	No	80.0 (192)	66.0 (128)
	Yes	14.2 (34)	27.3 (53)
	Don't know	5.8 (14)	6.7 (13)
RDDT 25(OH)D_3 (mean(SD)), nmol/L		76.6 (31.4)	72.0 (28.0)
UWAMA measures			
Mean (SD)			
	25(OH)D_3, nmol/L	61.4 (25.1)	59.3 (22.0)
	24,25$(OH)_2D_3$, nmol/L	1.9 (1.2)	1.7 (1.0)

	$24,25(OH)_2D_3$:total 25(OH)D ($*10^2$)	2.89 (1.81)	2.69 (0.99)
	$1,25(OH)_2D$:total 25(OH)D ratio ($*10^3$)	1.11 (0.60)(	1.15 (0.73)
Median (25^{th}-75^{th} percentile)			
	$1,25(OH)_2D_3$, pmol/L	57.8 (44.0-75.0)	54.8 (46.3-71.1)
	'Free' $1,25(OH)_2D_3$ (molar ratio $*10^6$)	0.11 (0.09-0.15)	0.11 (0.09-0.14)
	3-epi-$25(OH)D_3$, nmol/L	2.0 (1-3.1)	1.8 (1-3.0)
	Bioavailable 25(OH)D, nmol/L	6.7 (4.9 – 9.3)	6.6 (4.7 – 8.3)
	Free 25(OH)D, pmol/L	10.8 (8.0 – 15.5)	11.0 (7.8 – 14.3)
	Vitamin D binding globulin, mg/L	488 (436-560)	492 (440-576)
	Albumin, g/dL	5.2 (4.9-5.6)	5.2 (5.0-5.8)
Vitamin D binding globulin variants % (n)			
	Gc1F-1F	3.5 (7)	3.6 (6)
	Gc1S-1F	13.9 (28)	8.9 (15)
	Gc2-1F	31.8 (64)	37.5 (63)
	Gc1S-1S	33.3 (67)	36.3 (61)
	Gc1S-2	8.5 (17)	7.1 (12)
	Gc2-2	9.0 (18)	6.6 (11)

[a] Age at study interview; [b] Assessed by study nurse at interview; [c] Physical activity was scored and categorised according to the International Physical Activity Questionnaire; [d] Total years smoked; RDDT: RMIT Drug Discovery Technologies UWAMA: University of Western Australia Metabolomics Australia Numbers not adding to totals represent missing values. For free and bioavailable 25(OH)D, results are for the n=146 cases and n=181 controls for whom both genotype and serum measurements were available.

Table 2. Correlations (Pearson r, *p*) between different vitamin D metabolites and VDBG. Results above the diagonal are for cases only; below are for controls

	25(OH)D_3	**24,25$(OH)_2D_3$**	**3-epi-25(OH)D_3**	**1,25$(OH)_2D_3$**	**Free total 25(OH)D**	**Bioavailable total 25(OH)D**	**'Free' 1,25$(OH)_2$D**	**VDBG**
25(OH)D_3		0.85* *p<0.001*	0.67*, *p<0.001*	0.19*, *p=0.01*	0.70*, *p=0.001*	0.72*, *p<0.001*	0.05, *p=0.51*	0.22*, *p=0.003*
24,25$(OH)_2D_3$	0.73*, *p<0.001*		0.53*, *p<0.001*	0.09, *p=0.23*	0.59*, *p<0.001*	0.63*, *p<0.001*	-0.05, *p=0.47*	0.22*, *p=0.002*
3-epi-25(OH)D_3	0.71*, *p<0.001*	0.52*, *p<0.001*		0.08, *p=0.27*	0.46*, *p<0.001*	0.42*, *p<0.001*	0.01, *p=0.87*	0.11, *p=0.12*
1, 25$(OH)_2D_3$	0.29*, *p<0.001*	0.16*, *p=0.02*	0.23*, *p<0.001*		-0.09, *p=0.26*	-0.09, *p=0.28*	0.88*, *p<0.001*	0.15*, *p=0.04*
Free (total) 25(OH)D	0.72*, *p<0.001*	0.58*, *p<0.001*	0.50*, *p<0.001*	0.17*, *p=0.02*		0.95*, *<0.001*	0.01, *p=0.93*	-0.27*, *p<0.001*
Bioavailable total 25(OH)D	0.75*, *p<0.001*	0.61*, *p<0.001*	0.49*, *p<0.001*	0.16*, *p=0.03*	0.96*, *p<0.001*		-0.04, *p=0.64*	-0.17*, *p=0.03*

'Free' 1,25(OH)$_2$D	0.21*, *p=0.002*	0.10, *p=0.15*	0.18*, *p=0.007*	0.88*, *p<0.001*	0.28*, *p<0.001*	0.23*, *p=0.002*		-0.29*, p<0.001
VDBG	0.10, *p=0.12*	0.07, *p=0.26*	0.07, *p=0.28*	0.16*, *p=0.02*	-0.28*, *p<0.001*	-0.18*, *p=0.01*	-0.29*, *p<0.001*	

P values <0.05 are marked with an asterisk

Table 3. Summary of results of the association between risk of FCD and vitamin D metabolites

	AOR (95%CI)	*p*
RDDT 25(OH)D (10 nmol/ increment)	0.92 (0.85-1.00)	*0.05**
UWAMA 25(OH)D (10 nmol/L increment)	0.94 (0.85-1.03)	*0.15*
$24,25(OH)_2D_3$ (1 nmol/L increment)	0.81 (0.65-1.00)	*0.05*
$1,25(OH)_2D_3$ (log 1 pmol/L increment)	1.04 (0.58-1.89)	*0.89*
Free 25(OH)D (1 pmol/L increment)*	0.98 (0.94-1.04)	*0.54*
Bioavailable 25(OH)D (1 nmol/L increment)*	0.96 (0.87-1.07)	*0.48*
'Free' $1,25(OH)_2D_3$ (per molar ratio * 10^6 increment)	(0.99 (0.91-1.09)	*0.89*
$24,25(OH)_2D$:25(OH)D ratio (per SD increment)	0.84 (0.65-1.10)	*0.21*
$1,25(OH)_2D_3$:25(OH)D ratio (per SD increment)	1.16 (0.92-1.45)	*0.21*
Vitamin D binding globulin (per µg/L increment)	1.46 (0.18-11.89)	*0.72*

*Analyses for free and bioavailable 25(OH)D is based on n=146 cases and their matched n=181 controls with data on genotype, VDBG and albumin concentrations.

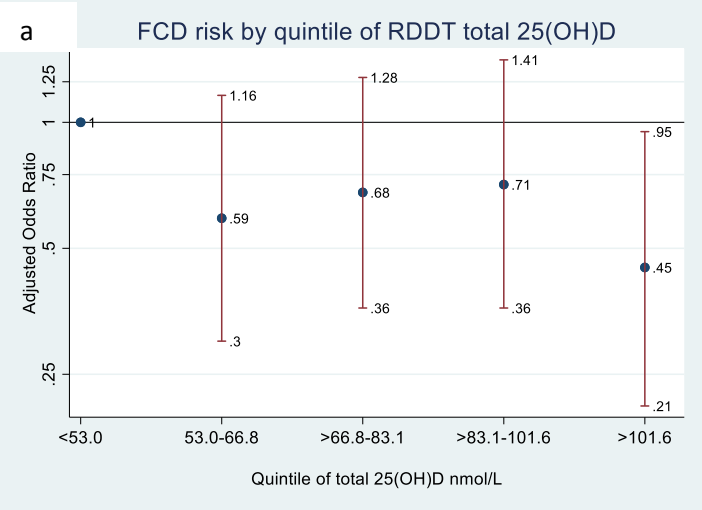
AORs are adjusted for total years smoking, physical activity category, and past history of infectious mononucleosis; P values <0.05 are marked with an asterisk

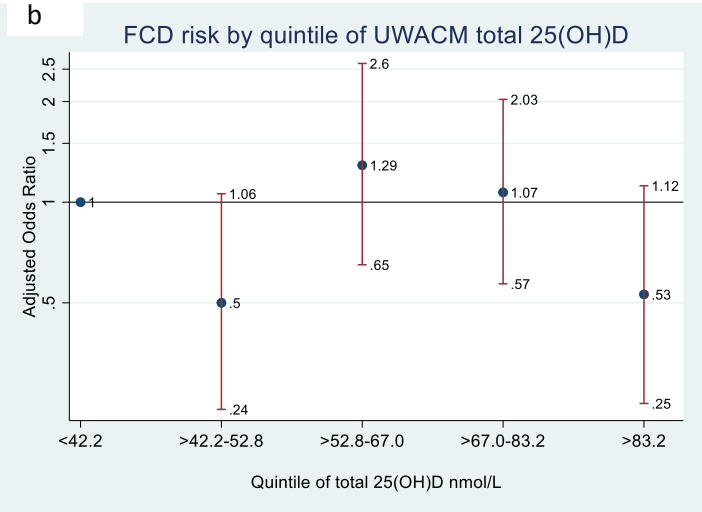
Table 4. Results of sensitivity analyses testing the association between vitamin D metabolites and VDBG with risk of FCD (see methods section for detail)

	Using:		AOR (95%CI)	*p*
1.	Deseasonalised total 25(OH)D	Free 25(OH)D	0.98 (0.94-1.04)	0.53
		Bioavailable 25(OH)D	0.96 (0.87-1.06)	0.48
2.	Imputed VDBG		1.00 (0.99-1.01)	0.72
3.	Imputed VDBG	Free 25(OH)D	0.99 (0.96-1.02)	0.57
		Bioavailable 25(OH)D	0.96 (0.87-1.07)	0.48
4.	Deseasonalised total 25(OH)D + imputed VDBG	Free 25(OH)D	0.99 (0.96-1.02)	0.57
		Bioavailable 25(OH)D	0.96 (0.87-1.07)	0.48
5.	Multivariable logistic regression	Free 25(OH)D	0.99 (0.97-1.02)	0.66
		Bioavailable 25(OH)D	0.97 (0.88-1.06)	0.49

P values <0.05 are marked with an asterisk

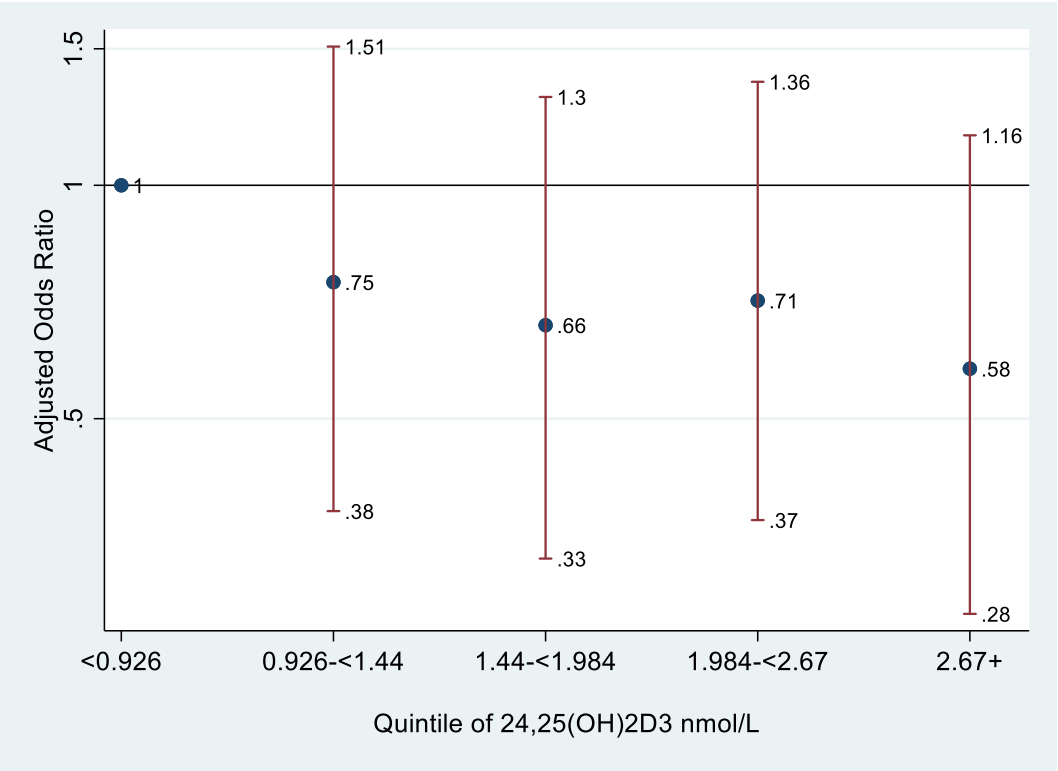
Fig 1. FCD risk by quintile of deseasonalised (total) 25(OH)D concentrations, nmol/L as measured by a). RDDT assay (p_{trend}=*0.10*) and b). UWAMA assay (p_{trend}=*0.60*)





Bars are Adjusted Odds ratios and 95% confidence intervals (adjusted for total years smoking, physical activity category, and past history of infectious mononucleosis)

Fig 2. FCD risk and quintile of serum 24,25(OH)$_2$D$_3$ concentrations, nmol/L (p_{trend}=*0.14*)



Bars are Adjusted Odds ratios and 95% confidence intervals (adjusted for total years smoking, physical activity category, and past history of infectious mononucleosis)

Supplementary material

The Ausimmune Investigator Group includes:

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Table 1 Genotype, affinity constants and mean serum concentration of vitamin D binding globulin for different haplotypes of VDBG, as defined by genotype based on rs7041 and rs4588 SNPs

	Genotype		Affinity Constant (M^{-1}), K	VDBG concentration (mean (SD))
Haplotype	rs4588	rs7041		
Gc1F-1F	T:T	C:C	1.12×10^9	449 (112)
Gc1S-1F	T:G	C:C	0.86×10^9	528 (100)
Gc2-1F	T:T	C:A	0.74×10^9	485 (76)
Gc1S-1S	G:G	C:C	0.60×10^9	518 (80)
Gc1S-2	T:G	C:A	0.48×10^9	514 (118)
Gc2-2	T:T	A:A	0.36×10^9	448 (78)

Calculation of free and bioavailable 25(OH)D

Calculations were based on Powe et al (1)

[Total D] = serum total 25(OH)D = ($25(OH)D_3$ + $25(OH)D_2$) concentration in mol/L

[Total VDBG] = serum VDBG concentration in mol/L

[Alb] = serum albumin concentration in mol/L

K_{alb} = the affinity constant for 25(OH)D for albumin = $6X10^5$ M^{-1}

K_{VDBG} = the affinity constant for 25(OH)D for VDBG = as defined according to genotype in Supplementary Table 1

$a = K_{VDBG} * K_{alb} * [Alb] + K_{VDBG}$

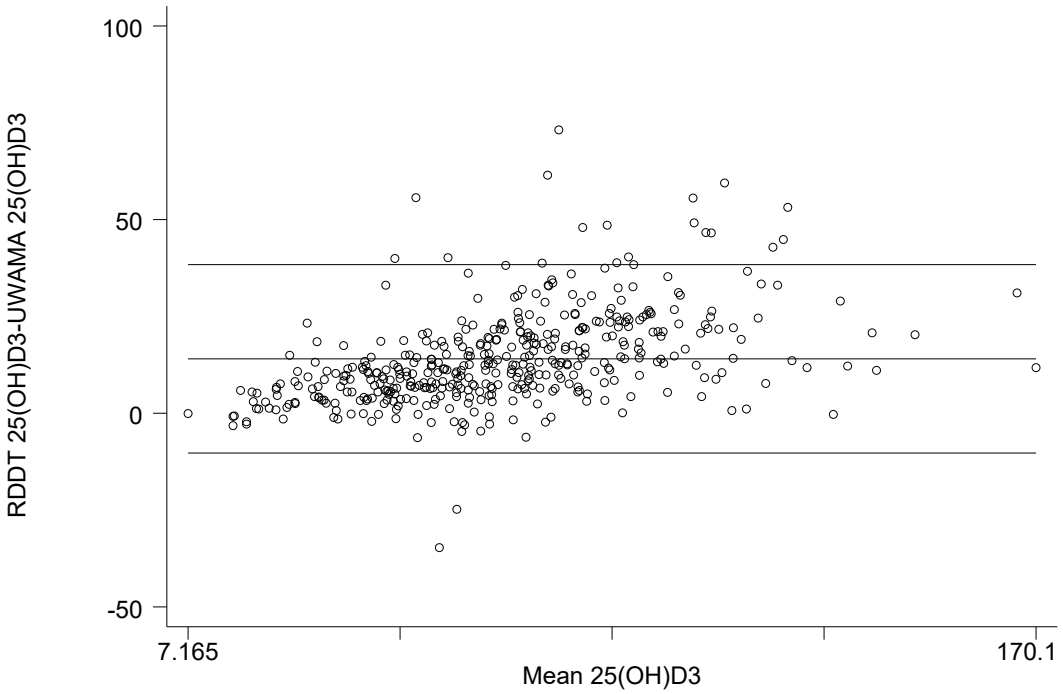
$b = K_{VDBG} * [\text{Total VDBG}] - K_{VDBG}*[\text{Total D}] + K_{alb}*[Alb] + 1$

$c = -[\text{Total D}]$

[Free 25(OH)D] = $\frac{-b + \sqrt{b^2 - 4ac}}{2a}$

Bioavailable 25(OH)D = (K_{alb} * [Alb] + 1)*[Free 25(OH)D])

Fig 1. Bland Altman plot comparing 25(OH)D3 measured at RMIT Drug Discovery Technologies laboratory (RDDT) vs. University of Western Australia Metabolomics node (UWAMA)



The solid lines indicate the mean bias, and the 95% limits of agreement (-10.3, 38.4 nmol/L).

Fig 2 Bland Altman plot comparing vitamin D binding globulin (VDBG) concentrations as assayed at UWAMA and the University of Washington (UoW)

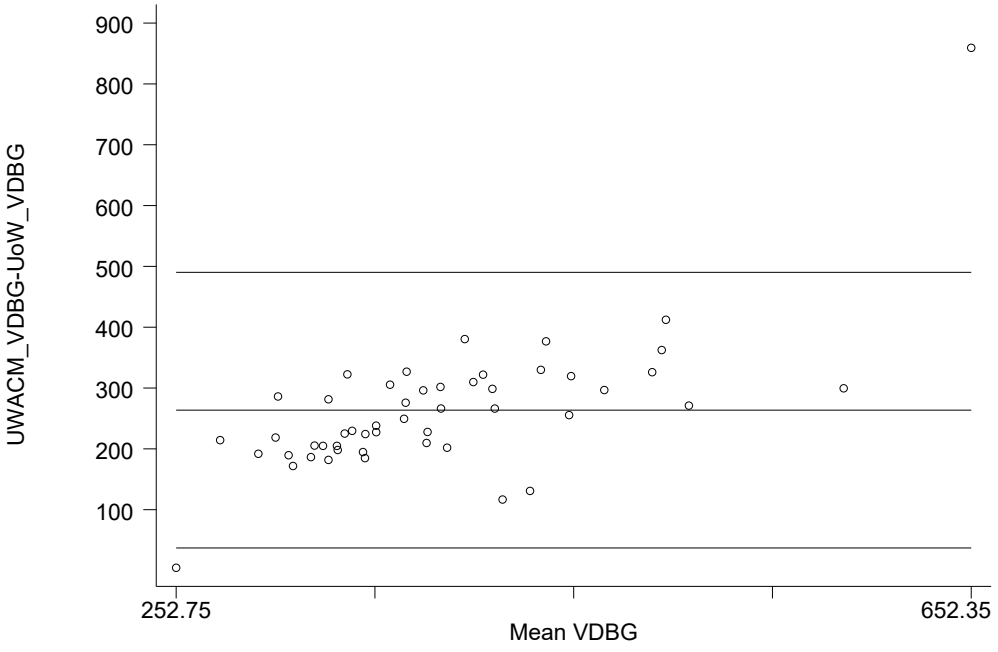
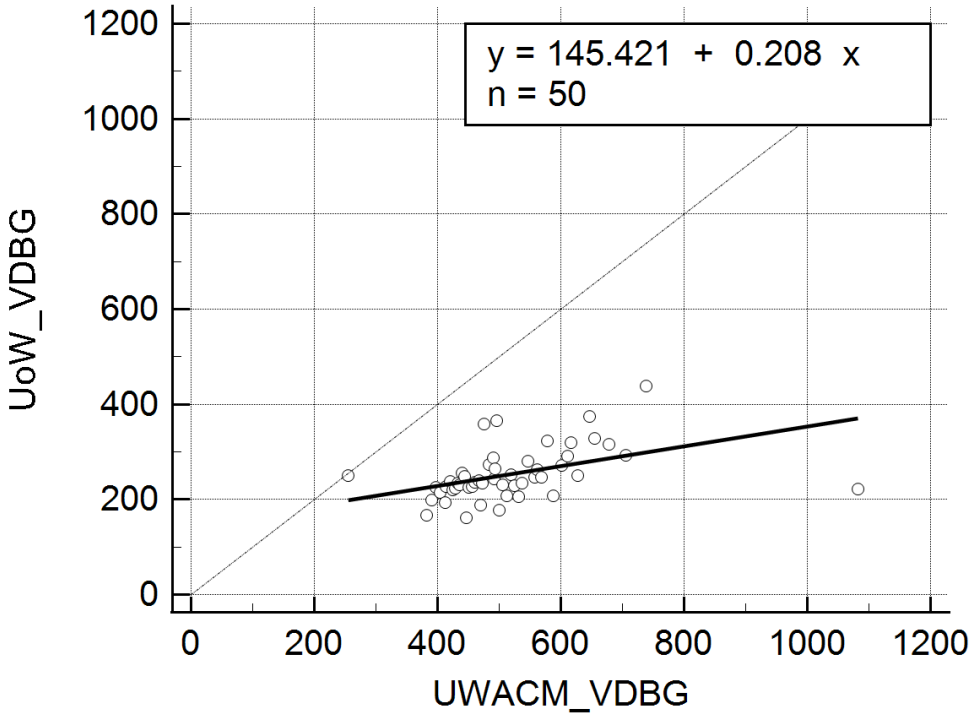


Fig 3. Agreement between assays measuring VDBG – results of the Deming regression



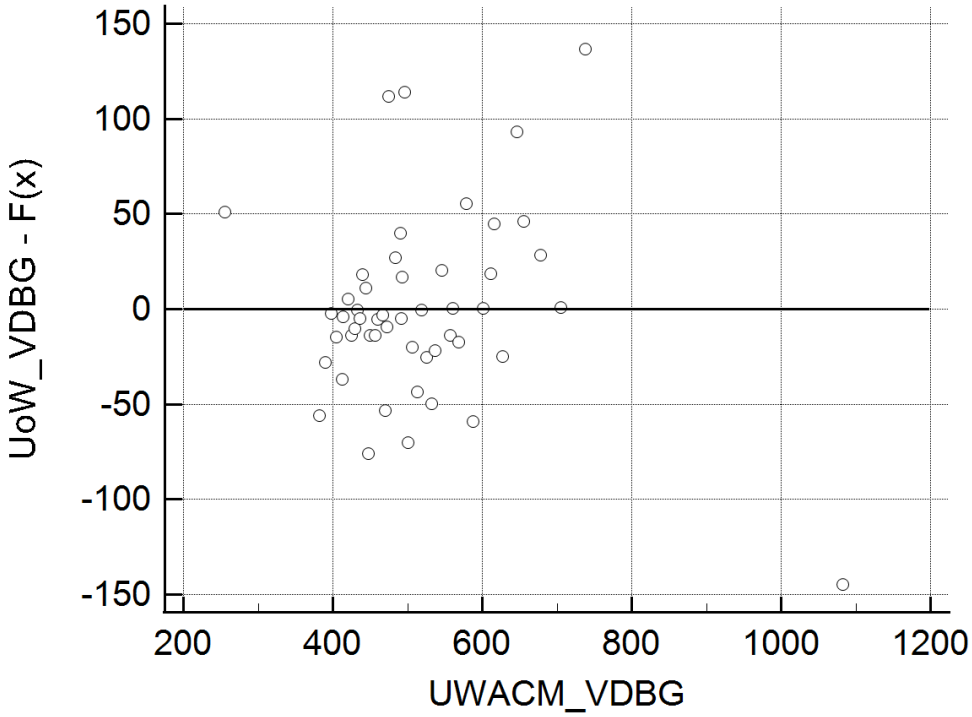
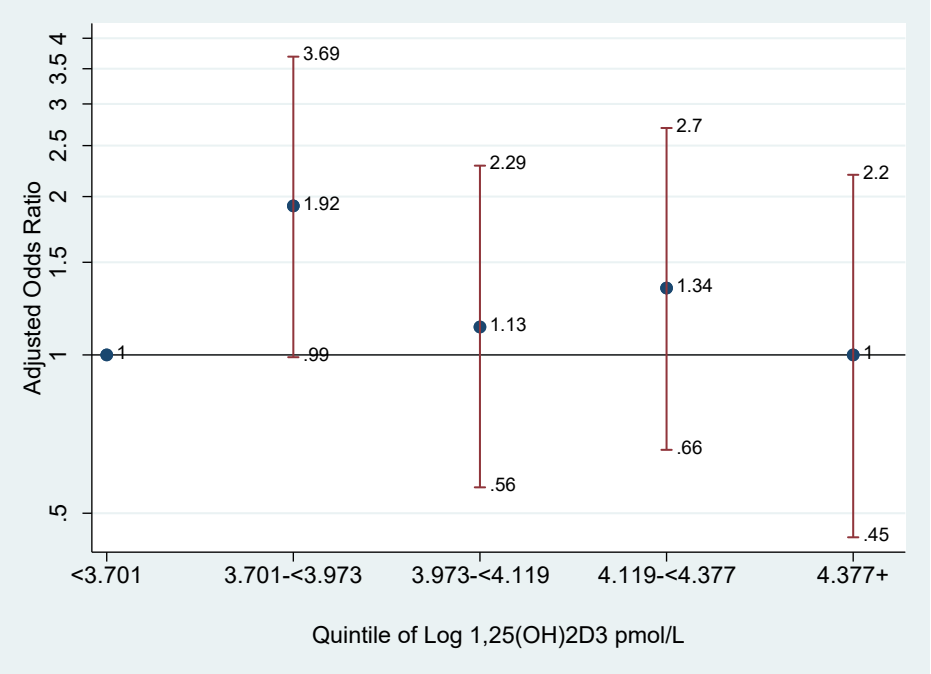
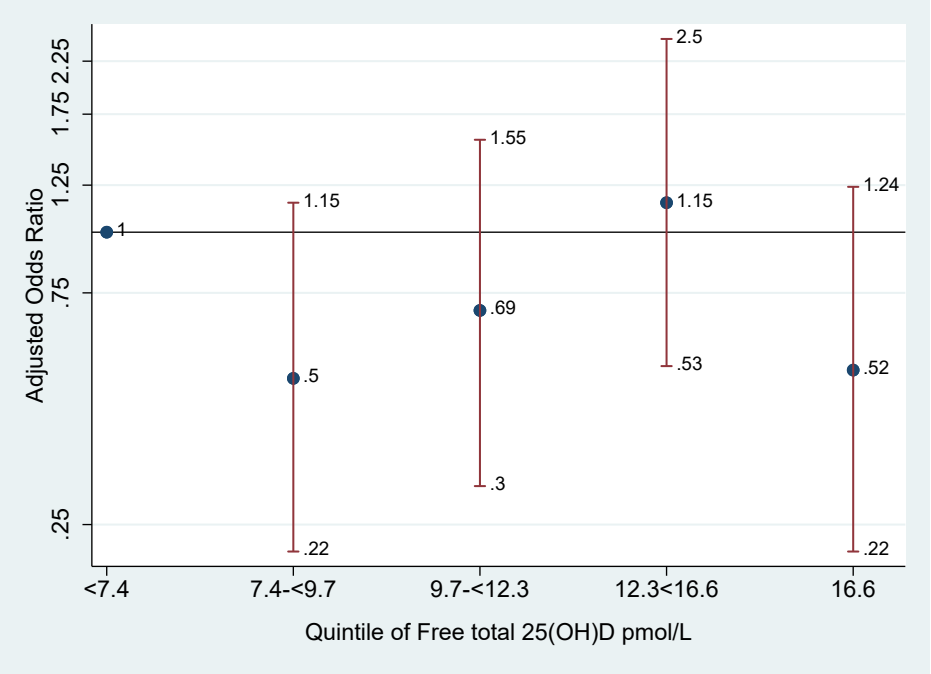


Fig 4. Risk of FCD according to quintile of $1,25(OH)_2D_3$ serum levels



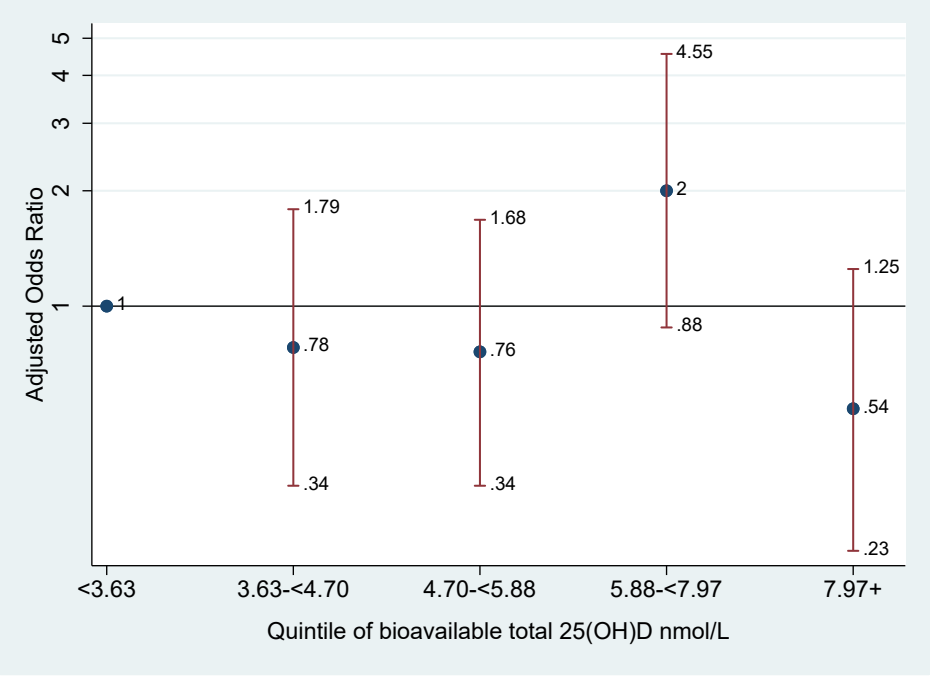
Bars are Adjusted Odds ratios and 95% confidence intervals (adjusted for total years smoking, physical activity category, and past history of infectious mononucleosis)

Fig 5. Risk of FCD according to quintiles of free total 25(OH)D serum levels



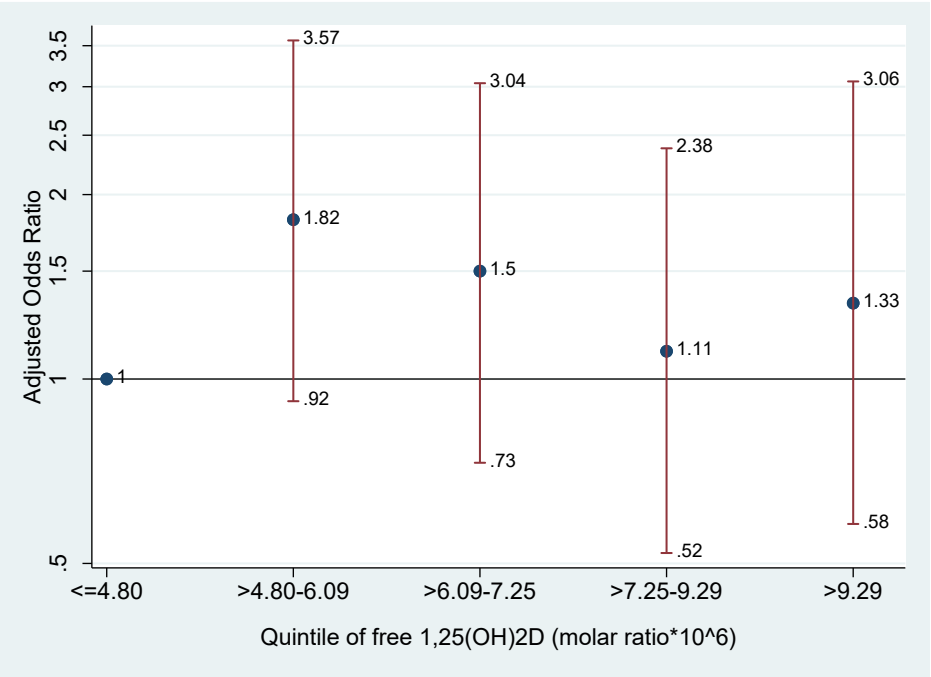
Bars are Adjusted Odds ratios and 95% confidence intervals (adjusted for total years smoking, physical activity category, and past history of infectious mononucleosis)

Fig 6. Risk of FCD according to quintiles of bioavailable total 25(OH)D serum levels



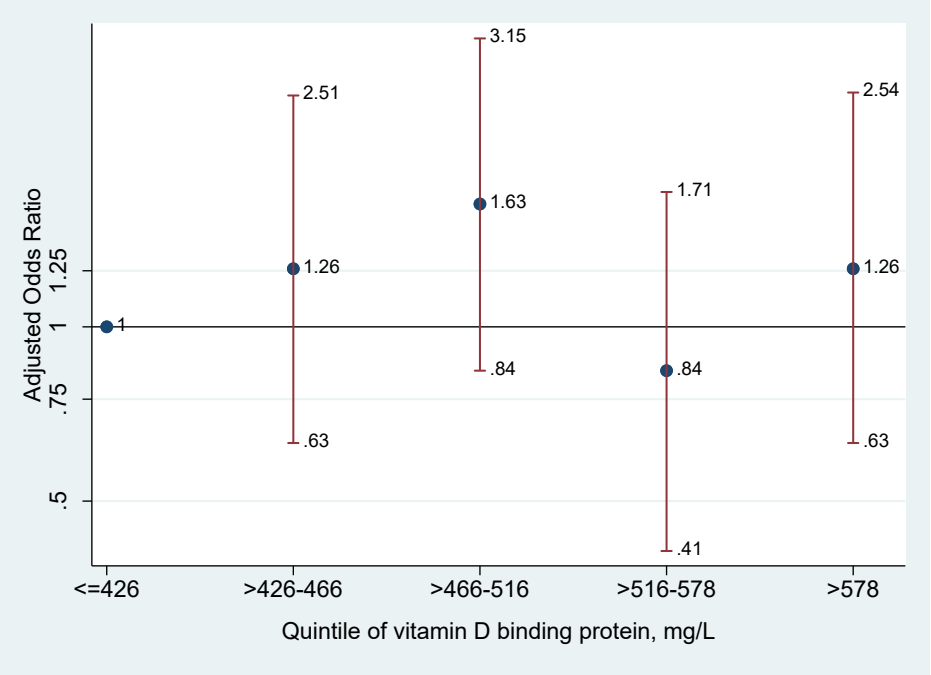
Bars are Adjusted Odds ratios and 95% confidence intervals (adjusted for total years smoking, physical activity category, and past history of infectious mononucleosis)

Fig 7. Risk of FCD according to quintiles of free 1,25(OH)$_2$D serum levels



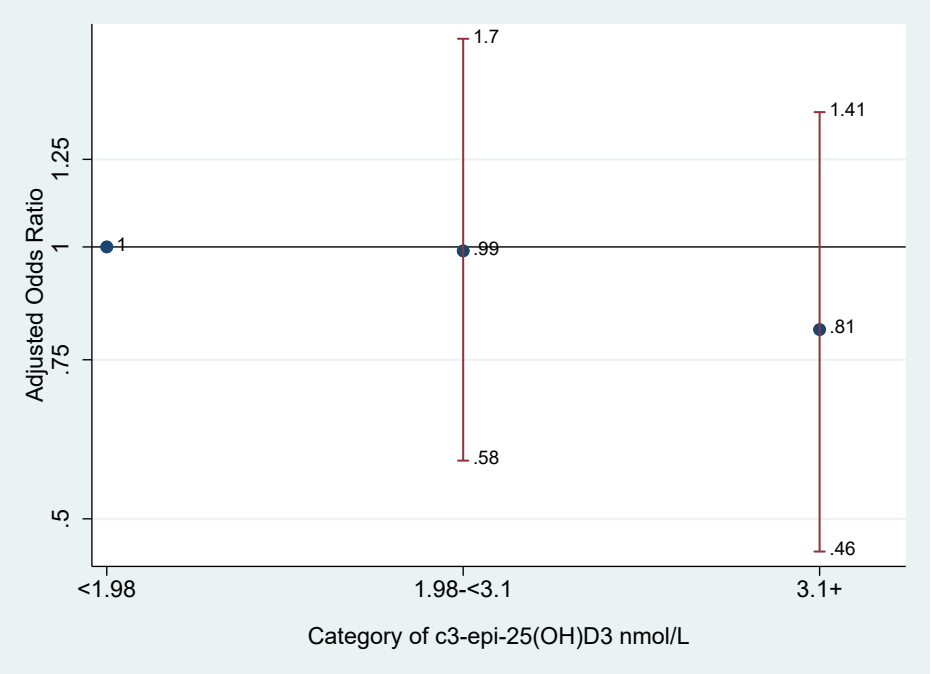
Bars are Adjusted Odds ratios and 95% confidence intervals (adjusted for total years smoking, physical activity category, and past history of infectious mononucleosis)

Fig 8 Risk of FCD according to quintiles of VDBG serum levels (Adjusted odds ratios and 95% confidence intervals)



Bars are Adjusted Odds ratios and 95% confidence intervals (adjusted for total years smoking, physical activity category, and past history of infectious mononucleosis)

Fig 9. Risk of FCD according to categories of c-3-epimer of 25(OH)D_3 concentration (Adjusted odds ratio and 95% confidence intervals)



Bars are Adjusted Odds ratios and 95% confidence intervals (adjusted for total years smoking, physical activity category, and past history of infectious mononucleosis)

References

1. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, et al. Vitamin D-binding protein and vitamin D status of black Americans and white Americans. *N Engl J Med.* 2013;369(21):1991-2000.