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ORIGINAL ARTICLE

Free vitamin D$_3$ index and vitamin D-binding protein in multiple sclerosis: A presymptomatic case–control study

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Abstract

Background and purpose: High levels of 25-hydroxyvitamin D$_3$ (25[OH]D$_3$) are associated with a lower risk for multiple sclerosis (MS). The bioavailability of 25(OH)D$_3$ is regulated by its main plasma carrier, vitamin D-binding protein (DBP). Free 25(OH)D$_3$ can be estimated by also measuring DBP concentration. In addition, DBP has immunomodulatory functions that may independently affect MS pathogenesis. No previous studies have assessed free 25(OH)D$_3$ or DBP in presymptomatically collected samples. This study was undertaken to assess free 25(OH)D$_3$ and DBP as risk factors for MS.

Methods: A nested case–control study was performed with presymptomatic serum samples identified through cross-linkage of MS registries and Swedish biobanks. Concentration of 25(OH)D$_3$ was measured with liquid chromatography and DBP levels with sandwich immunoassay. Free 25(OH)D$_3$ was approximated as free vitamin D$_3$ index: $(25[OH]D_3/DBP) \times 10^3$. MS risk was analyzed by conditional logistic regression, calculating odds ratios (ORs) with 95% confidence intervals (CIs).

Results: Serum samples from 660 pairs of matched cases and controls were included. At <20 years of age, high levels of free vitamin D$_3$ index were associated with a lower risk of MS (highest vs. lowest quintile: OR = 0.37, 95% CI = 0.15–0.91, p for trend across quintiles = 0.04). At age 30–39 years, high levels of DBP were associated with a lower MS risk (highest vs. lowest quintile: OR = 0.36, 95% CI = 0.15–0.85, p for trend = 0.02).

Conclusions: These findings support the hypothesis that high levels of free 25(OH)D$_3$ at a young age reduce the risk of MS later in life. They also implicate a role for DBP in MS etiology.

KEYWORDS

case–control studies, multiple sclerosis, vitamin D, vitamin D-binding protein

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease affecting the central nervous system. Observational MS studies indicate that higher levels of vitamin D are associated with a lower risk of developing MS and a decreased risk for inflammatory activity in patients with MS [1–6]. Two candidate genes coding for enzymes crucial for the vitamin D metabolism were identified in an MS genome-wide association study (GWAS) [7]. A decreased risk for MS has been observed among individuals with higher vitamin D intake [8], and results from Mendelian randomization studies suggest a protective role for vitamin D in MS etiology [9].

Vitamin D status is commonly assessed by measuring the concentration of 25-hydroxyvitamin D$_3$ (25[OH]D$_3$) in serum, as this is the main metabolite of vitamin D present in the blood. Commercially available assays routinely measure the sum of 25(OH)D$_3$ bound to proteins and the unbound fraction. The bioavailability of 25(OH)D$_3$ is largely regulated by its main plasma carrier, vitamin D-binding protein (DBP), which binds approximately 85% of the circulating 25(OH)D$_3$. Most of the remaining portion of 25(OH)D$_3$ is bound to albumin (~15% of total), and the free fraction is <0.1% [10]. According to the free hormone hypothesis, only the free fraction of the hormone is biologically active [10]. This hypothesis only partially applies to 25(OH)D$_3$, because some target organs, such as the kidney, express transmembrane receptors that mediate uptake of 25(OH)D$_3$ bound to DBP [11]. However, many cell types lack such abilities. This suggests that 25(OH)D$_3$ status should be assessed with regard to DBP concentration to determine the free fraction [11]. Only three studies have previously analyzed the free portion of 25(OH)D$_3$ as a risk marker for MS, and their results are diverging [12–14]. These studies included relatively few cases (n = 76, 91, and 77, respectively) and used samples drawn after diagnosis of MS or clinically isolated syndrome, which confers the risk of reverse causality. We are not aware of any studies of free 25(OH)D$_3$ in presymptomatically collected samples.

The molar concentration of DBP in serum is 50–100 times higher than that of the vitamin D metabolites, and only 5% of the circulating DBP is occupied by vitamin D ligands [15, 16]. The production of DBP is not regulated by vitamin D metabolites, but instead by estrogen, glucocorticoids, and inflammatory cytokines [10]. Apart from transporting vitamin D metabolites, DBP also has several other functions, including immunomodulation [15, 16]. DBP may thus influence MS pathophysiology both by itself and in conjunction with the vitamin D metabolites [17]. The role of DBP in MS etiopathogenesis has been assessed in several studies, also with inconclusive results [12–14, 18–23]. Again, these studies were small (n = 18–183) and performed on samples collected after disease onset. To our knowledge, no studies of DBP and MS have been performed on presymptomatically collected samples.

The primary aim of the present study was to determine whether free vitamin D$_3$ index, used as a proxy for free 25(OH)D$_3$, is inversely associated with MS risk, through a large study of presymptomatically collected serum samples. The secondary aim was to determine whether DBP levels are associated with the development of MS.

METHODS

Trial design and patients

Plasma or serum samples from individuals who later in life developed relapsing–remitting MS (RRMS) were identified and retrieved by cross-linkage of MS registries and microbiological biobanks. The Swedish MS registry and a local MS database in Umeå, Sweden were cross-linked with six Swedish biobanks located in Umeå, Skåne, Gothenburg, Linköping, Örebro, and the Public Health Agency of Sweden. These biobanks contain remainders of sera from clinical analyses. All included samples were donated before the age of 40 years and prior to MS symptom onset. For each case, one control was randomly selected, matched for biobank, sex, date of blood sampling, and date of birth (in order of priority). The process has previously been described in detail [6].

Laboratory procedures

The concentration of 25(OH)D$_3$ in plasma was analyzed by liquid chromatography with tandem mass spectrometry using an Agilent Technologies 1200/Sciex API 4000 and a Shimadzu Corporation Nexera/Sciex QTrap 5500 as described previously [24]. The analyses were performed at Clinical Chemistry, Laboratory Medicine, Umeå University Hospital, Sweden (Swedac accreditation no. 1397). External controls from Vitamin D External Quality Assessment Scheme (www.deqas.org) with values assigned by the National Institute of Standards and Technology Reference Measurement Procedure were used to ensure analytical quality. The concentration of DBP in plasma was analyzed by sandwich immunoassay using monoclonal antibodies, quantitating the different DBP alleles equally (Quantikine enzyme-linked immunosorbent assay [ELISA] kit, catalogue # DVDBP0B, R&D Systems). The samples from matched cases

and controls were analyzed consecutively but randomly with case–control status blinded for the technicians.

Measures

The commonly used methods for calculation of the free fraction of vitamin D require albumin concentration, which was not available for this study. Instead, we calculated free vitamin D_3 index as

$$\frac{25(OH)D_3 \ (nmol/L)}{DBP \ (nmol/L)} \times 10^3$$. This is a simple molar ratio, suggested to provide

a good indication for the real free $25(OH)D_3$ concentration [25]. A similar ratio has been used in a previous MS study [14].

Statistical analyses

Free vitamin D_3 index, $25(OH)D_3$ levels and DBP levels were divided into quintiles, as in all previous presymptomatic studies of vitamin D levels and MS [1, 4–6]. Due to significant differences in free vitamin D_3 index and DBP levels between men and women ($p < 0.001$) as well as between biobanks ($p < 0.001$), the quintiles were calculated separately for each biobank and sex (Tables S1 and S2), using cutoffs derived from the distribution among controls (Table S3). These assigned quintiles were then used in a pooled analysis, including the entire cohort. Similar pooling of biobank-specific quintiles has been used previously on this material, as well as in other biobank studies [6, 26].

Analyses were also stratified based on age at blood sampling: <20, 20–29, and 30–39 years of age. Matched pairs with participants on different sides of the age limits were both assigned to the group that contained fewer individuals. Sensitivity analyses were performed for individuals with samples drawn more than the median of 8 years before the onset of symptomatic MS.

Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated with conditional logistic regression, and in these analyses, quintiles of the free vitamin D_3 index or DBP were analyzed as categorical variables. For DBP, ORs were adjusted for vitamin D status by including $25(OH)D_3$ quintiles in the model. The trend across quintiles was analyzed by modeling the quintiles as continuous variables. Wilcoxon signed ranks test was used to compare differences between matched cases and controls, as variables were nonnormally distributed. Correlations between variables were analyzed by calculating Spearman rank correlation coefficient (ρ).

Statistical analyses were performed with IBM SPSS version 27.

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and approved by the Regional Ethical Review Board in Umeå (2011-198-31M with amendments 2013-226-32M, 2017-104-32, 2017-484-32, 2018-468-32M, 2019-03402, and 2020-00119). No written informed consent was required.

RESULTS

Serum or plasma was obtained from a total of 670 individuals who later developed RRMS and 670 matched controls. For 10 individuals, the sample volume was insufficient to analyze both DBP and $25(OH)D_3$ concentration. These individuals were excluded together with their matched case or control, leaving 660 complete sets of cases and controls for the final analysis. The mean absolute differences for sampling date and sampling age between cases and controls were 6 days and 149 days, respectively, and the median age at sampling was similar for cases and controls (Table 1). In the youngest group, <20 years of age, a weak correlation between DBP levels and age at sampling was present in the control group (Table S4). In the same group, a weak negative correlation between free vitamin D index and sampling age was observed. None of these correlations was observed in the remaining age strata or in the whole cohort.

Free vitamin D_3 index as a risk factor for MS

The free vitamin D_3 index was lower among cases than controls in the youngest group, but not in the remaining age groups or in the entire cohort (Table 1). Similarly, the logistic regression analysis for the youngest group showed that an elevated free vitamin D_3 index (quintiles three and five) was associated with a reduced risk of developing MS, compared to the reference (quintile one). The risk of developing MS showed a significant decreasing trend across quintiles of free vitamin D_3 index (Table 2). In the oldest group, an association with increased MS risk was observed for the middle quintile compared to the reference, although the CIs were wide. No significant associations were observed at age 20–29 years or in the total cohort.

The sensitivity analysis of individuals with 8 years or more from sampling to symptom onset resulted in similar effect sizes, although not statistically significant (data not shown).

DBP as a risk factor for MS

The median concentration of DBP did not differ significantly between cases and controls in the whole cohort or in any of the age groups (Table 1). A substantial proportion (15.7%) of the DBP measurements surpassed the highest standard for the assay (103/660 cases and 104/660 controls).

In the logistic regression analysis for the whole cohort, we observed that elevated DBP levels (fourth quintile compared to first quintile) were associated with a lower risk for MS and a significant trend across quintiles ($p < 0.0498$; Table 3). The risk reduction was most pronounced in the oldest group, where DBP levels in the top

TABLE 1 Baseline characteristics of cases and controls

Characteristic	Cases	Controls	p[a]
All sampling ages, *n* (male/female)	660 (107/553)	660 (107/553)	
Sampling age < 20 years	139 (32/107)	139 (32/107)	
Sampling age = 20–29 years	372 (53/319)	372 (53/319)	
Sampling age = 30–39 years	149 (22/127)	149 (22/127)	
Age at disease onset, years, median (IQR)	33.4 (27.8–39.7)	–	
Age at sampling, years, median (IQR)	25.0 (20.9–29.3)	25.0 (21.0–29.2)	
All sampling ages, median (IQR)			
Free vitamin D_3 index	8.5 (6.3–11.5)	8.8 (6.0–12.1)	0.49
DBP, nmol/L	6014 (4891–7626)	6187 (4906–7859)	0.20
Sampling age < 20 years, median (IQR)			
Free vitamin D_3 index	8.6 (6.4–11.6)	9.0 (7.0–12.8)	0.03[b]
DBP, nmol/L	5416 (4772–6814)	5701 (4436–7119)	0.30
Sampling age = 20–29 years, median (IQR)			
Free vitamin D_3 index	8.3 (6.1–10.8)	8.4 (5.7–11.1)	0.81
DBP, nmol/L	6347 (5105–7974)	6550 (5171–8124)	0.35
Sampling age 30–39 years, median (IQR)			
Free vitamin D_3 index	9.5 (7.2–13.6)	9.1 (6.0–13.3)	0.39
DBP, nmol/L	5509 (4355–6972)	5928 (4802–7756)	0.05
Umeå biobank, *n* (%)	99 (15.0%)	99 (15.0%)	
Free vitamin D_3 index, median (IQR)	9.5 (6.7–12.4)	10.2 (7.6–13.4)	0.23
DBP, nmol/L, median (IQR)	5134 (3956–6077)	5162 (4319–6225)	0.24
PHAS biobank, *n* (%)	137 (20.8%)	137 (20.8%)	
Free vitamin D_3 index, median (IQR)	10.0 (7.5–14.1)	10.0 (7.6–14.1)	0.75
DBP, nmol/L, median (IQR)	5595 (4490–7095)	5521 (4395–7353)	0.74
Örebro biobank, *n* (%)	29 (4.4%)	29 (4.4%)	
Free vitamin D_3 index, median (IQR)	8.4 (6.7–10.5)	8.4 (4.8–11.1)	0.44
DBP, nmol/L, median (IQR)	5984 (5022–6677)	6353 (5541–7195)	0.27
Gothenburg biobank, *n* (%)	47 (7.1%)	47 (7.1%)	
Free vitamin D_3 index, median (IQR)	10.4 (6.6–16.3)	8.0 (6.5–12.5)	0.03[b]
DBP, nmol/L, median (IQR)	5159 (4037–6535)	6107 (4649–7756)	0.002[b]
Skåne biobank, *n* (%)	309 (46.8%)	309 (46.8%)	
Free vitamin D_3 index, median (IQR)	8.0 (5.6–10.3)	8.1 (5.6–10.8)	0.21
DBP, nmol/L, median (IQR)	6694 (5288–8527)	6880 (5369–8474)	0.63
Linköping biobank, *n* (%)	39 (5.9%)	39 (5.9%)	
Free vitamin D_3 index, median (IQR)	7.8 (5.7–8.8)	6.6 (4.6–10.9)	0.69
DBP, nmol/L, median (IQR)	5759 (5137–7450)	6612 (5486–7599)	0.08

Note: Free vitamin D_3 index was calculated as $\frac{25(OH)D_3 \ (nmol/L)}{DBP \ (nmol/L)} \times 10^3$.

Abbreviations: 25(OH)D_3, 25-hydroxyvitamin D_3; DBP, vitamin D-binding protein; IQR, interquartile range; PHAS, Public Health Agency of Sweden.

[a]Calculated by Wilcoxon signed ranks test.

[b]Statistically significant results.

quintile compared to the first were associated with a reduced risk of developing MS. A trend across quintiles was also observed. These results remained statistically significant after adjustments for 25(OH)D_3 levels. The sensitivity analysis of individuals with samples drawn >8 years before the onset of symptomatic MS resulted in similar effect sizes, although not statistically significant (data not shown).

DISCUSSION

In this study on presymptomatically collected samples, we found that higher levels of free vitamin D_3 index among individuals <20 years of age were associated with a lower risk of developing MS later in life. In addition, higher DBP levels were associated with a lower MS risk.

TABLE 2 Risk of multiple sclerosis by quintiles of free vitamin D_3 index

	Quintiles of free vitamin D_3 index					
Sampling age	1 (ref)	2	3	4	5	p trend[a]
All ages	1.00	0.96 (0.67–1.36)	1.17 (0.82–1.67)	1.02 (0.70–1.50)	0.96 (0.65–1.43)	1.00
Age < 20 years	1.00	0.76 (0.34–1.71)	0.46 (0.22–0.98)[b]	0.75 (0.30–1.87)	0.37 (0.15–0.91)[b]	0.04[b]
Age = 20–29 years	1.00	1.07 (0.67–1.70)	1.39 (0.87–2.22)	1.04 (0.62–1.74)	1.13 (0.65–1.97)	0.69
Age = 30–39 years	1.00	0.84 (0.39–1.81)	2.68 (1.09–6.61)[b]	1.51 (0.69–3.27)	1.65 (0.75–3.63)	0.24

Note: Figures represent odds ratio (95% confidence interval).

[a]Calculated by analyzing quintiles as continuous variables in conditional logistic regression.

[b]Statistically significant results.

TABLE 3 Risk of multiple sclerosis by quintiles of vitamin D-binding protein

	Quintiles of vitamin D-binding protein					
Sampling age	1 [ref]	2	3	4	5	p trend[a]
All ages	1.00	0.77 (0.55–1.08)	0.83 (0.58–1.18)	0.64 (0.43–0.95)[b]	0.69 (0.47–1.02)	0.05[b]
All ages, adjusted[c]	1.00	0.78 (0.55–1.10)	0.85 (0.59–1.22)	0.66 (0.44–0.98)[b]	0.73 (0.49–1.10)	0.07
Age < 20 years	1.00	0.86 (0.39–1.88)	1.48 (0.68–3.21)	0.82 (0.32–2.12)	1.57 (0.59–4.15)	0.35
Age < 20 years, adjusted[c]	1.00	0.87 (0.40–1.93)	1.80 (0.80–4.08)	0.99 (0.37–2.67)	2.10 (0.74–5.98)	0.24
Age = 20–29 years	1.00	0.83 (0.52–1.34)	0.72 (0.45–1.16)	0.69 (0.42–1.13)	0.68 (0.41–1.13)	0.11
Age = 20–29 years, adjusted[c]	1.00	0.83 (0.51–1.34)	0.74 (0.45–1.19)	0.70 (0.42–1.17)	0.72 (0.42–1.21)	0.12
Age = 30–39 years	1.00	0.56 (0.29–1.10)	0.63 (0.28–1.43)	0.43 (0.17–1.07)	0.36 (0.15–0.85)[b]	0.02[b]
Age = 30–39 years, adjusted[c]	1.00	0.60 (0.30–1.21)	0.66 (0.28–1.54)	0.42 (0.16–1.08)	0.37 (0.15–0.92)[b]	0.03[b]

Note: Figures represent odds ratio (95% confidence interval).

[a]Calculated by analyzing quintiles as continuous variables in conditional logistic regression.

[b]Statistically significant results.

[c]Adjusted for quintiles of 25-hydroxyvitamin D_3.

This risk reduction was most profound among individuals at age 30–39 years. Adjustments for 25(OH)D_3 status did not alter the results, arguing against confounding.

The major strength of this study is the large number of samples collected before onset of symptomatic MS, as well as the inclusion of samples collected from individuals of young age. The latter is essential, considering that an individual's risk of developing MS seems to be influenced by environmental factors early in life, thus making it preferable to study such factors when their effect appears to be most profound. To our knowledge, no previous studies have assessed the free proportion of vitamin D_3 or DBP as risk factors for MS in presymptomatically collected samples.

Although the exact role of vitamin D in MS etiology remains uncertain, and other effects from ultraviolet B exposure may be involved [2, 27], it is widely recognized that vitamin D has immunomodulatory effects with potential relevance for MS etiopathogenesis. In vitro studies have demonstrated that the active form of vitamin D_3, 1,25(OH)$_2$$D_3$, modulates the differentiation of CD4$^+$ T cells, promoting Th2 and Treg cells and inhibiting Th1 cells [28]. The augmentation of Treg cells is suggested to further shift the balance between Th1 and Th2 cells, resulting in an anti-inflammatory immune response [28]. However, the effective concentration of 1,25(OH)$_2$$D_3$ in most in vitro studies far surpasses the physiological levels [29]. The mechanisms in vivo remain uncertain, but it has been suggested that 25(OH)D_3 is locally converted to 1,25(OH)$_2$$D_3$, which is then used as a paracrine/autocrine agent through the vitamin D receptor (VDR) [29]. This mechanism has been demonstrated in vitro in activated antigen-presenting cells and in activated T cells [29, 30]. Both cell types express VDR as well as the enzyme 25(OH)D-1α-hydroxylase, converting 25(OH)D_3 to 1,25(OH)$_2$$D_3$ in levels sufficient to affect gene expression [30]. Interestingly, a variant in the gene for this enzyme has been associated with increased MS risk in a recent GWAS [7]. Furthermore, the process of paracrine/autocrine activation is inhibited in vitro in the presence of DBP [30]. This indicates that the free hormone hypothesis is applicable to the immunomodulatory effects of vitamin D, which are thus regulated by DBP concentration. The DBP concentration in lymph nodes, where immunological responses are generally initiated, is unknown but appears to be lower than in serum [30]. Hypothetically, the shift to an anti-inflammatory immune response could thus be determined by the balance of 25(OH)D_3 and DBP levels.

Consistent with that, we observed that a high free vitamin D_3 index was associated with a lower MS risk in the youngest age group. It is also worth noting that our previous study on the same samples found no significant association between $25(OH)D_3$ levels and MS risk in that age category (top vs. lowest quintile, OR = 0.62, 95% CI = 0.27–1.42, p for trend = 0.34) [6]. A similar observation has been reported for the association of vitamin D and bone mass, where free $25(OH)D_3$, but not total $25(OH)D_3$, correlated with bone mineral density [31]. We hypothesize that free vitamin D provides a better marker for MS risk than $25(OH)D_3$ status.

Previous studies of free $25(OH)D_3$ as a risk factor for MS have shown different results [12–14]. As all previous studies were performed on subjects with confirmed disease, reverse causation is a possibility. However, our results are consistent with a previous study of total $25(OH)D$ and MS risk that was performed on presymptomatically collected samples and reported that the effect of vitamin D on MS risk was most profound at a younger age [1].

In addition to binding and transporting vitamin D metabolites, DBP also has immunomodulatory functions, such as influencing T-cell activation through a complex interplay that also involves $25(OH)D_3$ [15, 16]. Furthermore, DBP regulates neutrophile chemotaxis, which is best described for C5a-induced chemotaxis [16] but may also apply to CXCL1 [32]. DBP is suggested to act either as a direct positive regulator of C5a-induced chemotaxis or by scavenging inhibitory factors such as oleic acid [16]. Interestingly, the enhancement of C5a-induced chemotaxis appears to be inhibited by physiological concentrations of $1,25(OH)_2D_3$, but not by $25(OH)D_3$ [33]. This further underlines the intricate interplay of these factors regarding their effect on the immune system.

In the present study, higher DBP levels were associated with a lower MS risk. However, the enhancement of neutrophile chemotaxis indicates that DBP has proinflammatory functions, which would seem to contradict our results. Previous studies of DBP as a risk factor for MS have shown diverging results [12–14, 18–23]. These studies were also performed on samples drawn after MS diagnosis, which could affect the results by reverse causation. Some studies have found lower levels of DBP in cerebrospinal fluid among MS patients compared to controls [34, 35]. These latter findings are consistent with our results and argue against an exclusively proinflammatory effect of DBP. In accordance with our results, some studies indicate that DBP has neuroprotective functions. This is best described in relation to Alzheimer disease, where DBP appears to scavenge amyloid β peptide and thereby reduce its pathological effects [36, 37]. Similarly, the actin scavenging function of DBP has been suggested to be involved in MS pathogenesis [17].

However, reverse causation also needs to be considered a possible explanation for the observed association between DBP and reduced MS risk in the present study, because the association was mainly driven by the oldest group of subjects, and some of these may have developed a subclinical MS prodrome. Median follow-up time from sampling to MS symptom onset was 8 years. However, evidence of a long prodromal phase of MS is emerging, suggesting that even 8 years might be insufficient to ensure that samples are collected before the onset of disease [38]. Possibly, actin released from injured neurons could leak into the blood circulation and bind to DBP, which in turn would then be cleared from the blood stream, reducing DBP levels [17]. Further studies are needed to explore the role of DBP in MS etiology.

Confounding and reverse causation could also affect the results for free vitamin D index. Reduced sun exposure may increase MS risk not only via vitamin D, but through additional mechanisms [2]. The MS prodrome may lead to consumption of vitamin D from an activated immune system. However, we found a similar effect size in the subgroup of individuals with 8 years or more from sampling to symptom onset, arguing against reverse causation.

The present study also has additional limitations that merit discussion. For example, the samples used in the study originate from six geographically separated biobanks, with a maximum distance of >900 km in latitude, causing considerable differences in sun exposure. The biobanks also used different procedures for analysis and storage and did not have the same inclusion criteria. These factors are likely explanations for the observed differences of DBP and $25(OH)D_3$ levels between the biobanks. To reduce the effect of these differences, each case was matched with a control from the same biobank. This matching was kept intact in the analyses by using conditional logistic regression. Furthermore, the regression analyses were modeled on quintiles, which were defined separately for each biobank. Similar pooling of biobank-specific quintiles has been used in previous studies [6, 26].

Quintiles were used to allow for comparison with previous prospective studies of $25(OH)D_3$ and MS, which all used quintiles [1, 4–6, 8]. However, this generated a few very small groups, which could affect the results. We thus performed post hoc analyses of quintiles defined from all controls, regardless of biobank or sex. The results remained statistically significant; high free vitamin D_3 index among individuals younger than 20 years was associated with a lower MS risk (highest vs. lowest quintile, OR = 0.27, 95% CI = 0.09–0.78). Similarly, DBP at age 30–39 years was inversely associated with MS risk (highest vs. lowest quintile, OR = 0.34, 95% CI = 0.12–0.92).

Another limitation is that some measured DBP values were above the highest standard for the assay, resulting in a reduced precision compared to samples with concentrations within the defined range. However, all DBP values were within the range that has previously been reported [25]. The effect of the increased uncertainty regarding these high values was reduced by modeling the DBP levels as quintiles and analyzing them categorically, rather than using a linear method. The same approach was used for free vitamin D_3 index, which was calculated from the $25(OH)D_3$ and DBP levels.

Finally, it should also be noted that free vitamin D_3 index is used as a proxy for the unbound portion of $25(OH)D_3$. The index is a molar ratio and not equal to the actual free $25(OH)D_3$. Several different methods have been suggested to determine the free $25(OH)D_3$, such as centrifugal ultrafiltration, direct measurement by two-step ELISA, or calculation based on concentration and affinity binding constants for albumin and DBP [10]. Each of these methods also has problems, such as the disparity between assays

and a variety in affinity between different DBP alleles or under different clinical conditions [10]. Nonetheless, all these methods are more precise than the approximative free vitamin D_3 index, but none of them was applicable because of the sparse amounts of serum available for this study. Neither could we assess the different DBP genotypes, which to a degree affect the free fraction of vitamin D by differing affinity to $25(OH)D_3$. Although the free vitamin D_3 index appears to be a good indicator of the actual unbound portion of $25(OH)D_3$ [25, 39], and previous studies have used similar ratios [14], further studies using more precise methods are needed to confirm our findings.

In conclusion, this study supports the hypothesis that high levels of free $25(OH)D_3$ at young age are associated with a reduced risk of developing MS later in life. In addition, an association between high DBP levels and a lower risk of developing MS is reported. This may indicate an independent role of DBP in MS etiology.

AUTHOR CONTRIBUTIONS

Viktor Grut: Data curation (supporting); formal analysis (lead); funding acquisition (supporting); investigation (supporting); project administration (equal); writing – original draft (lead); writing – review and editing (lead). **Martin Biström:** Conceptualization (supporting); data curation (lead); formal analysis (supporting); investigation (supporting); methodology (supporting); project administration (supporting); supervision (supporting); validation (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Jonatan Salzer:** Conceptualization (supporting); formal analysis (supporting); methodology (supporting); supervision (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Pernilla Strid:** Formal analysis (supporting); investigation (supporting); supervision (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Anna Lindam:** Formal analysis (supporting); methodology (supporting); software (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Lucia Alonso-Magdalena:** Investigation (supporting); project administration (supporting); validation (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Oluf Andersen:** Formal analysis (supporting); methodology (supporting); validation (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Daniel Jons:** Project administration (supporting); validation (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Martin Gunnarsson:** Project administration (supporting); validation (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Magnus Vrethem:** Formal analysis (supporting); project administration (supporting); validation (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Johan Hultdin:** Conceptualization (lead); formal analysis (supporting); investigation (supporting); methodology (supporting); resources (lead); supervision (lead); writing – original draft (supporting); writing – review and editing (supporting). **Peter Sundström:** Conceptualization (lead); formal analysis (supporting); funding acquisition (lead); investigation (supporting); methodology (supporting); project administration (supporting); supervision (lead); writing – original draft (supporting); writing – review and editing (supporting).

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CONFLICT OF INTEREST

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Table S1-S4