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DISSERTATION

**The impact of hypovitaminosis D and vitamin D
supplementation on disease activity and immune profile in
patients with multiple sclerosis**

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1. Summary

1.1. Abstract [English]

Deficient 25-hydroxyvitamin D [25(OH)D] (hypovitaminosis D) levels are associated with increased risk, disease activity and severity, and multiple sclerosis (MS) progression. It remains unclear whether high-dose vitamin D intake ameliorates disease activity and whether it exerts immunologic effects in MS.

This PhD project aimed to investigate the: 1) association of hypovitaminosis D with markers of disease severity in MS, 2) effects of high-dose versus low-dose cholecalciferol (vitamin D3) supplementation, on disease activity and brain magnetic resonance imaging (MRI) markers of MS and clinically isolated syndrome (CIS), 3) effects of vitamin D3 on immune cell landscape and N-glycan branching on immune cells.

These objectives were addressed in the context of the EVIDIMS study (Efficacy of Vitamin D Supplementation in Multiple Sclerosis), ClinicalTrials.gov identifier, NCT01440062. EVIDIMS was a multicenter, randomized, actively-controlled explorative phase 2a pilot trial with a double-blind vitamin D3 intervention (20,400 international units (IU) vs. 400 IU). Vitamin D3 was administered for 18 months, as an add-on to interferon β 1b treatment in 53 MS/CIS patients. We studied the association of clinical and brain MRI features with hypovitaminosis D (Project 1) or with vitamin D3 supplementation (Project 2). In Project 3, the immunologic effects of vitamin D3 supplementation on T, B and natural killer (NK) cell subpopulations were investigated using flow cytometry.

Hypovitaminosis D was associated with increased MRI T2-weighted brain lesion counts and clinical disability scores. Vitamin D supplementation did not affect clinical and MRI measures of disease activity longitudinally. Moreover, high-dose supplementation did not affect the frequencies of broad, regulatory/effector immune cell subpopulations, but did reduce N-glycan branching in broad T cells and NK cell subtypes.

These projects suggest – in line with the literature - that hypovitaminosis D in MS patients is associated with increased disease activity, while vitamin D intake did not affect clinical outcomes of MS or the immune profile in the blood. It shows, however, that vitamin D regulates N-glycan branching on immune cells.

1.2. Abstract [German]

Ein 25-Hydroxyvitamin D Mangel (Hypovitaminose D) ist mit einem erhöhten Erkrankungsrisiko und erhöhter Krankheitsaktivität einer Multiplen Sklerose (MS) verbunden. Es ist unklar, ob eine Vitamin-D Nahrungsergänzung die MS-Krankheitsaktivität reduziert.

Das Promotionsprojekt hatte folgende Ziele: Untersuchen der 1) Assoziation von Hypovitaminose D mit MS Erkrankungsschwere, 2) Auswirkungen einer hochdosierten im Vergleich zu einer niedrigdosierten Vitamin D3 Nahrungsergänzung auf die Krankheitsaktivität bei MS und klinisch isoliertem Syndrom (CIS), 3) Auswirkungen von Vitamin D3 auf die Immzelllandschaft und N-Glycan-Verzweigung auf Immunzellen.

Die Untersuchungen wurden im Rahmen der EVIDIMS-Studie (Wirksamkeit der Vitamin-D-Supplementierung bei Multipler Sklerose) durchgeführt. EVIDIMS war eine multizentrische, randomisierte, aktiv kontrollierte explorative Phase-2a-Pilotstudie mit einer doppelblinden Vitamin-D3 Intervention (20.400 internationale Einheiten (IE) gegenüber 400 IE). Vitamin D3 wurde 53 MS/CIS-Patienten 18 Monate lang als Ergänzung zur Interferon- β 1b-Behandlung verabreicht.

Wir untersuchten den Zusammenhang von klinischen und magnetresonanztomographischen (MRT)-Merkmalen der MS mit Hypovitaminose D (Projekt 1) und mit Vitamin D3-Nahrungsergänzung (Projekt 2). Im Projekt 3 wurde die Wirkung der Vitamin D3-Nahrungsergänzung auf T-, B- und Natural Killer (NK) -Zell-Subpopulationen mittels Durchflusszytometrie untersucht.

Eine Hypovitaminose D war mit einer erhöhten T2-gewichteten Läsionslast im MRT und der klinischen Behinderung assoziiert. Eine Vitamin-D- Nahrungsergänzung hatte keinen Einfluss auf die klinischen und MRT-Messungen der Krankheitsaktivität. Eine hochdosierte Nahrungsergänzung hatte keinen Einfluss auf die Häufigkeit von Immunzellpopulationen, reduzierte jedoch die N-Glycan-Verzweigung in T-Zellen und NK-Zell-Subtypen. Die Arbeit zeigt, dass Hypovitaminose D bei MS-Patienten mit einer erhöhten Krankheitsaktivität verbunden ist, während die hochdosierte Einnahme von Vitamin D die klinischen Ergebnisse von MS oder das Immunprofil im Blut nicht beeinflusst. Die Regulierung der Verzweigung von N-Glycan auf Immunzellen stellt einen möglichen Mechanismus dar, wie Vitamin D die Aktivität von Immunzellen beeinflusst.

1.3. Introduction

MS is a chronic inflammatory and neurodegenerative autoimmune disease of the central nervous system (CNS) with unknown cause and global prevalence around 2.5 million[1]. It is predominant in females and a leading cause of disability and early retirement in young adults[2,3]. MS is clinically characterized by repetitive subacute bouts of CNS dysfunction, such as reduced motor function, poor coordination and balance, fatigue, sensory disturbances, visual impairment and cognitive dysfunction[4,5]. Clinical isolated syndrome (CIS) on the other hand, refers to the first clinical incidence such as evidence of one brain MRI lesion suggestive of MS onset[6].

Epidemiological evidence link MS onset with an array of environmental factors; increased serum antibody titers of the Epstein-Barr virus (EBV), deficient vitamin D levels and smoking[7]. Of these, vitamin D is a well-established environmental factor associated with MS for several decades. Low levels of vitamin D are associated with higher MS risk, younger age of onset, increased disease severity, worse progression and worse outcome. In this line, a plethora of studies have reported deficient vitamin D levels (hypovitaminosis D) in MS patients[8–11].

Vitamin D is a hormone metabolized in the skin via ultraviolet B (UVB) radiations, which converts 7-dehydrocholesterol to cholecalciferol (vitamin D₃). In the blood, vitamin D levels are easily quantified by measuring (25(OH)D) levels produced in the liver[12]. The active form with shorter life span is 1,25-dihydroxycholecalciferol (1,25(OH)₂D), produced in the kidneys. Increasing evidence from MS studies shows an association between sunlight exposure and MS prevalence. UVB radiations account for 90% of vitamin D while the remaining 10% is attainable from dietary sources[13].

Immune cells express the vitamin D receptor (VDR) and the vitamin D enzyme, 1 α -hydroxylase (CyP27B1, CyP2R1, CyP27A1) of the cytochrome P family[14] and the vitamin D catalyst enzyme 24-hydroxylase (CyP24A1); hence they can regulate the availability and effectiveness of vitamin D on-site [14–16].

Hypovitaminosis D is defined by serum or plasma 25(OH)D levels of < 20 to 30 ng/ml (<50 to 75 nM)[17–19]. MS patients with 25(OH)D levels > 30 ng/ml (75nM) have a more favorable disease course in terms of relapse rates, disease progression, T2-weighted and contrast-enhancing brain lesions and brain atrophy[20–22]. As a result, patients with deficient vitamin D levels are often supplemented to boost the levels.

Several epidemiological, population-based, cross-sectional, preclinical and clinical studies investigating the influence of vitamin D in MS have produced conflicting results[23]. The few interventional trials conducted so far are also inconclusive regarding clinical benefits perhaps due

to potential interactions with disease-modifying treatments (DMTs), varying vitamin D doses and sample sizes. Regardless of these disparities, vitamin D intake is considered safe, as reported by supplementation studies with varying doses[24–26].

The exact mechanism by which vitamin D exerts its therapeutic benefits in MS remains unclear, although preclinical *in vitro* and *in vivo* studies suggest that it may skew T-cell responses from pathogenic T helper 1 (Th1) and T helper 17 (Th17) to anti-inflammatory T helper 2 (Th2) phenotype, and/or favor regulatory T cells (Treg) proportions and/or function[27,28]. Additionally, Vitamin D is suggested to modulate cytokine production[25,29], immune cell landscape[25] and cell-surface proteins expressed on immune cells[30,31].

MS is not only T cell-mediated but also B cells and NK cells are implicated in its pathogenesis[32,33]. Consequently, disruption or reduced function of these immune cells characterize the disease[16,34]. Vitamin D exerts its effects on immune cells via its receptors and the enzyme 1 α -hydroxylase. VDR expression is a prerequisite for being amenable to vitamin D signaling[35]. Nevertheless, VDR expression by immune cells is partly dependent on the state of the immune cells. For example, resting T and B cells do express little to no VDR compared to activated T or B cells[36].

While the relationship between vitamin D and immune cells proportions remains unclear[24,25,29], its impact on cell-surface proteins has not been extensively studied *in vivo*. *In vitro* studies suggest an immune regulatory role of vitamin D via cell-surface protein glycosylation[30]. Cell-surface protein glycosylation is one of the least studied yet very fundamental functional tool of all cells including immune cells[37].

Glycosylation is the addition of sugar molecules (e.g N-acetyl-D-glucosamine (GlcNAc)) to proteins by acetylglucosaminyltransferases (Mgat) in the Golgi apparatus[38,39]. The addition of N- GlcNAc to the protein asparagine (N) via glycosidic bonds generates N-glycans via four Mgat enzymes (Mgat 1, 2, 4 and 5)[40] involved in the branching process. These enzymes act sequentially with declining efficiency to generate mono, bi, tri and tetra-antennary GlcNAc-branched N-glycans respectively[40,41]. Depending on the activity of the enzymes and substrate availability, they can either reduce or upregulate branching on cell-surface proteins[31]. N-glycans expressed on immune cell surfaces influence their activity and functional properties[42]. In this line, alterations in immune cell glycomes (the complement of sugars expressed on immune cells) is associated with autoimmunity[42].

In vitro and *in vivo* studies suggest vitamin D as an important mediator in regulating N-glycans. The ability of vitamin D to upregulate or downregulate N-glycan branching on immune cells

depends on the cell's activation state and substrate availability[30,31]. As such, resting and activated immune cells behave differently regarding N-glycan branching. For example, activated T cells express higher N-glycan branching compared to resting T cells[36]. Evidence from the experimental autoimmune encephalitis, EAE model of MS, showed that increase in N-glycan branching in T cells attenuated autoimmune responses while a reduction in branching induced spontaneous autoimmunity[43]. Against this background we hypothesized that in MS, upregulation of N-glycan branching by vitamin D may suppress activated or partially activated T cells, thus reducing autoimmunity. What remains unclear is whether vitamin D's impact on N-glycans is of clinical relevance in MS. This question is difficult to answer, as no human interventional studies have investigated this so far.

Based on the above-elaborated impact of vitamin D in MS, the 'Efficacy of Vitamin D supplementation In Multiple Sclerosis' (EVIDIMS) trial was conducted involving Caucasian RRMS/CIS patients from north-eastern Germany[44].

1.4 Objectives

The PhD project aimed to evaluate and confirm hypovitaminosis D in our cohort and to assess and confirm the safety and impact of vitamin D3 intake on clinical and brain MRI markers of disease activity. We then investigated the impact of vitamin D3 intake on the frequencies of circulating immune phenotypes and, for the first time in a human interventional study, we investigated the effect of vitamin D3 on N-glycan branching. The PhD project, therefore, addressed three hypotheses using these specific objectives related to vitamin D in MS:

VD_cross-sectional (Project 1) [11]

Hypothesis 1: Hypovitaminosis D is associated with disease activity in MS.

1. To confirm if the EVIDIMS cohort is a representative MS cohort with hypovitaminosis D at baseline before vitamin D3 supplementation.
2. To investigate the associations between hypovitaminosis D and clinical and brain MRI markers of disease activity.

VD_interventional (Project 2) [45]

Hypothesis 2: High-dose vitamin D supplementation is safe and provides more clinical benefits compared to low-dose supplementation.

1. To investigate the safety of supplementing patients with 20,400 IU or 400 IU cholecalciferol.
2. To compare the effects of high (20,400 IU) vs low-dose (400 IU) cholecalciferol intake on clinical and brain MRI markers of disease activity.

VD_immunological (Project 3)[46]

Hypothesis 3: Vitamin D is an important player in immune regulation and modulates N-glycan branching.

1. To investigate the impact of vitamin D intake on the frequencies of immune cells using flow cytometry.
2. To investigate the influence of vitamin D supplementation on N-glycan branching on immune cells using flow cytometry and mean fluorescence intensity of L-PHA (*phaseolus vulgaris leukoagglutinin*) stain.

1.5. Methods

This section describes the study design and main techniques used in the PhD thesis.

Details of methods used for each study, the elaborated patient inclusion and exclusion criteria, study design, outcome measures and statistical analysis are described in the respective publications[11,45,46].

1.5.1 EVIDIMS trial

1.5.2 Participants

Patients were recruited from seven centers in north-eastern Germany (Berlin (4 centers), Potsdam, Halle and Teupitz) by experienced MS neurologists and were stratified according to gender and serum 25(OH)D level (< or ≥ 20 ng/ml or 50 nmol/l)[11,45]. Briefly, patients eligible for inclusion were: aged 18- 65 years (at randomization), diagnosed with MS according to revised McDonald criteria (2005)[47] or CIS, and treated with interferon-β1b for at least 3 months (at randomization). The exclusion criteria were any other disease courses than RRMS/CIS and vitamin D supplementation >500 IU/day within 6 months before recruitment and randomization. At the end of the study, 51 RRMS and 2 CIS patients were included. Depending on the research questions and objectives of each project, a sub-cohort or the whole cohort of 53 patients were included in the analysis (as described in Projects 1 and 2)[11,45].

1.5.3 Study protocol

Screening and regular study visits took place at the different study centers. Patients were monitored at baseline, 6, 12 and 18 months. At each visit, blood samples were collected for serum, peripheral blood mononuclear cells (PBMCs) isolation and pharmacovigilance assays[11]. At the end of the study, patients who violated the study protocol were excluded from the analysis. The per-protocol (PP) definition was both, regular study termination and protocol-conform study drug intake. Protocol violations applied when a patient was lost to follow-up, changed MS

medication, personal reasons, new MRI contraindications and psychological problems. All randomized patients were included in the intention-to-treat (ITT) analysis regardless of whether they completed the study or not.

1.5.6 Ethics

The EVIDIMS trial was registered at the European Clinical Trials Database (EudraCT 2011-002785-20) and ClinicalTrials.gov (NCT01440062)[44] and was approved by the responsible national competent authority (BfArM, 4037578) and local ethic committees (11/0386-ZS EK 13) of Germany. The study was conducted adhering to the study protocol and applicable national laws, the Harmonized Tripartite Guideline for Good Clinical Practice (ICH GCP) and the principles of the Declaration of Helsinki in its applicable version. All participants gave written informed consent[45].

1.5.7 Project-specific methods

1.5.8 Safety, Clinical examination and Serum 25(OH)D measurement

Safety parameters (urine calcium and creatinine levels) were measured four weeks after starting vitamin D intake and thereafter every three months.

The clinical examination included the measuring of blood pressure and Electrocardiogram (ECG), serum vitamin D levels, body weight and height. Patients were also monitored for relapses defined as the periods where the disease worsens and radiological activities progress[48]. Disease progression and disability were assessed using expanded disability status scores (EDSS)[49], multiple sclerosis functional composite (MSFC)[50] scores, brain MRI and visual function tests using optical coherence tomography. EDSS evaluates the ability to walk freely or with walking aid[49] (ambulation) while the MSFC assesses both ambulatory, hand and cognitive functions using the 25-foot walk, the 9-hole peg test and the paced auditory serial addition test (PASAT)[50]. EDSS scores were performed at screening and thereafter, every six months by experienced neurologists trained in the Neurostatus score. Serum 25(OH)D levels were measured by Bioscientia Institute for Medical Diagnostics, GmbH Berlin. PBMCs were isolated from 10 ml blood samples the same day after collection and frozen in liquid nitrogen with cryoprotection until flow cytometric analysis was performed.

1.5.9 Magnetic Resonance Imaging (MRI)

Standardized MRI scans were performed at screening, 12 and 18 months using the same Magnetom TIM TRIO 3 Tesla MRI (Siemens, Healthineers, Erlangen, Germany) and protocol at the Berlin Center for Advanced Neuroimaging (BCAN) at the Charité study center, Campus Mitte Berlin. For details of the MRI scan protocols please refer to Projects 1 and 2[11,45].

1.6 STUDY DESIGN

1.6.1 VD_Cross-sectional (Project 1)[11]

Details of the study design are described in Project 1[11]. Briefly, the 53 recruited patients were subgrouped based on their serum 25(OH)D levels at baseline before randomization and supplementation. Based on the suggested cut-offs for deficient 25(OH)D levels, two groups were created: 1) vitamin D deficiency (< 30 ng/ml) and 2) vitamin D sufficiency (\geq 30 ng/ml). These two groups were compared regarding i) T2c, T2 lesion volume (T2v), white matter volume (WM), gray matter volume (GM), total brain volume and ii) disease progression as measured by the EDSS score (Project 1)[11].

1.6.2 VD_Interventional (Project 2)[45]

Patients were randomized in a 1:1 ratio to either high dose or low dose treatment arms as an add-on to continuous treatment with IFN β -1b. In the high-dose arm, participants received 20,400 IU (1 ml cholecalciferol oil (20.000 IU) + 1 tablet (400 IU)) every other day for 18 months, while the low dose arm received 400 IU (1 ml placebo oil (0 IU) + 1 tablet (400 IU))[45]. Patients were monitored for safety parameters, disease course, brain MRI and blood immunophenotypes.

1.6.3 VD_ immunological (Project 3)[46]

38 patients (n = 21 in the high-dose arm and n = 17 in the low-dose arm) who fulfilled the per-protocol (PP) analysis criteria were included. Two independent sub-analyses were conducted: a) Analysis 1, the effect of vitamin D supplementation on the frequencies of T cells, B cells and NK cells was investigated in 29 RRMS patients at baseline and after 12 months. Only 29 patients out of 38 were included due to lack of source material at the time of experiments. b) In Analysis 2, the effect of vitamin D intake on the intensity of β 1,6 N-glycan expression on immune cells was investigated in 38 RRMS patients at baseline, and after 6, 12 and 18 months[46].

1.7 FLOW CYTOMETRIC CHARACTERIZATION OF EFFECTOR AND REGULATORY IMMUNE CELLS (ANALYSIS 1)

Immunophenotyping provides information on the composition and function of immune cells. Accurate measurement of immune profiles is obtained by following standard protocols during the cell staining and acquisition processes. To minimize errors due to frequent pipetting of the many antibodies, ready-to-use multicolor cocktails tube were obtained. Effector and regulatory T, B and NK cells subpopulations were characterized using custom-made single-batch LyotubesTM (BD, Cat. #625148, BD, USA) containing antibody cocktails. This enabled us to investigate broader

immune subpopulations with higher accuracy. The detailed protocols for the isolation of PBMCs, staining and acquisition are elaborated in the methods section under Project 3[46].

Briefly, thawed PBMCs were stained with the required antibody cocktails at 4 °C for 30 min. Dead cells were identified by staining using fixable viable stain, FVS 520 (BD, Cat. #564407) for 10 min at 4 °C and washed. The acquisition was done immediately without fixation using FACSCanto II, Diva 6.1.3 8 (BD Pharmingen, Franklin Lakes, NJ, USA).

1.7.2 FLOW CYTOMETRIC ANALYSIS FOR β 1,6 N-GLYCAN EXPRESSION ON BROAD T, B AND NK CELLS (ANALYSIS 2)

The expression of sugars, β 1,6-N-acetylglucosamine (N-GlcNAc or N-glycan) on the surfaces of immune cells can be quantified using L-PHA conjugated to FITC (L-PHA-FITC), a fluorochrome that binds to the β 1,6-GlcNAc. Through the binding of L-PHA-FITC to β 1,6-GlcNAc, we can estimate N-glycan branching based on the mean fluorescence intensity (MFI) of L-PHA. The amount of N-GlcNAc present in N-glycans increases with branching complexity and is, therefore, an accurate marker for N-glycan branching[31].

PBMCs were stained using a cocktail of antibodies together with L-PHA-FITC. For details of the staining protocol and dilution factor of antibodies, please refer to Project 3[46]. A summary of the immune cells investigated are listed below, and the antibody panels are shown in Tables 1 (*Adapted from Project 3, Bäcker-Koduah et al., ACTN, 2020*)[46].

1.7.3. BLINDING

The trial was blinded such that clinicians, patients and persons who conducted part of the study were not aware of the group allocations or treatments received by patients. All immunological assays were performed blinded until statistical analysis.

1.7.4 Statistical Analysis

Details of all statistical analyses used in this PhD thesis are indicated in the statistics section of Projects 1,2 and 3[11,45,46]. Briefly, all variables are given as mean \pm standard deviation (SD) if not indicated otherwise. Due to the small sample size and nonparametric distribution of the data, the exact Mann-Whitney U test was used for comparing independent groups. The following statistical methods were applied for the analyses: simple linear regression analyses with adjustment for age, disease duration and sex in multivariate linear regressions. Nonparametric analysis of covariance (Project 1)[11] and multivariate nonparametric analysis for longitudinal data (MANOVA). The immunology data were analyzed using nonparametric analysis of covariance (ANCOVA) for longitudinal data with baseline as a covariate, nonparametric exact

Mann-Whitney test and Spearman`s Rho correlational analyses (Project 3)[46]. In the ITT population, missing values were replaced by multiple imputations[51] using the stochastic regression imputation method. In this method, missing values are predicted from regression and a random residual value (in this case the existing baseline value)[51]. The following missing parameters were imputed for 8 patients at 18 months for the EDSS score, MSFC score, T2c, T2v, gadolinium-enhancing lesion count (gdc), thalamic volume, brain parenchymal fraction and normalized brain volume (nbv) (Project 2)[45]. Statistical tests were performed using R 3.6.0 (2019-04-26) with packages ggplot2, psych, geepack, lme4, lmerTest, MuMIn, Rmisc. To complement the analysis in R IBM® SPSS® Statistics, 24, © Copyright 1989, 2016 SPSS Inc., an IBM Company and SAS 9.4 [TS1M3] Copyright © 2002 by SAS Institute Inc., Cary, NC, USA were used.

Immune cell subpopulations investigated in Project

3 Analysis 1.

B/NK tube: total CD19+, CD19+CD24highCD38low (B-memory), CD19+CD24lowCD38low (B-mature), CD19+CD24highCD38high (B-reg), CD19+CD24-CD38high (B-plasma), CD19+CD24highCD38- (B-memory-atypical), CD16+CD56low (CD56 dim), CD16+CD56high (CD56 bright)

Treg tube: CD3+, CD3+CD4+, CD3+CD8+, CD45RA+CD25low (naïve Treg), CD4+CD25+CD127- (CD4+ Treg), CD3+CD8+CD28-CD127- (CD8+ Treg)

Teff tube: CD3+CD4+CCR6-CD161-CXCR3+(Th1), CD3+CD4+CCR6-CD161+CXCR3+ (Th1 non-classic), CD3+CD4+CCR6+CD161+CCR4+ (Th17).

Analysis 2. CD3+, CD4+, CD8+; B cells, CD19+CD16+ CD56 low (CD56dim), CD16+ CD56 high (CD56bright).

Table 1. FACS staining panel

Analysis 1									
Fluorochrome									
		BV421	V500-C	FITC	PE	PerCp-Cy5.5	PE-Cy7	APC	APC-H7
B/NK tube	Specificity	CD45	CD3	FVS520	CD24	CD16	CD19	CD38	CD14
	Clone	NCAM16.2	SK7		ML5	3G8	SJ25C1	HB7	MφP9
	Cat #	562751	652896	LIQUID DROP IN	555428	338440	341113	345807	560180
Treg tube	Specificity	CD127	CD3	FVS520	CD28	CD8	CD25	CD45RA	CD4
	Clone	HIL-7RM21	SK7		L293	SK1	2A3/MA251	HI100	SK3
	Cat #	562436	652896	LIQUID DROP IN	348047	341050	335824	550855	641398
Teff tube	Specificity	CD161	CD3	FVS520	CXCR3		CCR4	CCR6	CD4
	Clone	DX12	SK7		1C6/CXCR3		1G1	11A9	SK3
	Cat #	562615	652896	LIQUID DROP IN	550633		557864	560619	641398
Analysis 2									
Fluorochrome									
		FITC	PerCP	APC	APC-Cy7	PB	BV510	APC-Cy7	PB
T/B/NK cells	Specificity	L-PHA	CD45	CD3	CD4	CD8a	CD19	CD56	CD16
	Clone								
	Cat #	FL-1111	368506	317318	317418	301033	302242	302018	362520
Per 100 µl		2µg/ml	1:100	0.5:100	0.5:100	0.5:100	1:100	0.5:100	0.5:100

1.8. Results

This section summarizes the key findings from the three publications chronologically. All other results are elaborated in the results section of the respective publications.

1.8.1 COHORT DESCRIPTION:

The mean age of the 53 patients was 43 ± 10 years with 37 (69.8 %) females and a mean disease duration of 9 ± 9 years. The detailed baseline characteristics of patients included for the cross-sectional, interventional and immunological studies are presented under Projects 1, 2 and 3[11,45,46] respectively. The consort diagram is illustrated in Project 2 (Dörr & Bäcker-Koduah *et al.*, MSJ-ETC, 2020, Project 2)[45]. At baseline, 91% (48 out of 53) of patients had deficient

25(OH)D levels of 18 (12; 24) ng/ml [median (25; 75% Q)]. Specifically, the <30 ng/ml 25(OH)D group had 15 (10,21) ng/ml [median (25; 75% Q)] compared to 33 (33; 35) ng/ml ($p < 0.001$) in the group with 25(OH)D ≥ 30 ng/ml (*Adapted from Project 1, Bäcker-Koduah et al., Front. Neurol, 2020*) [11].

1.8.2 VD_Cross-sectional (Project 1)[11]

Deficient serum VD levels were associated with increased disease activity.

At baseline, deficient (<30 ng/ml) 25(OH)D levels were associated with higher EDSS scores of 2.5 (1.5; 3) [median (25; 75% Q)] compared to 1.0 (1; 2) in the sufficient (≥ 30 ng/ml) 25(OH)D group ($p = 0.04$). Overall, EDSS scores correlated negatively with serum 25(OH)D levels after adjustment for age, sex and disease duration ($R^2 = 0.34$, $p < 0.001$). Additionally, the number of T2c recorded in the <30 ng/ml 25(OH)D group was higher, 60 (36; 84) [median (25; 75% Q)] compared to the 25 (24; 33) in the ≥ 30 ng/ml 25(OH)D ($p = 0.03$) (*Adapted from Project 1, Bäcker-Koduah et al., Front. Neurol, 2020*) [11].

1.8.3 VD_Interventional (Project 2)[45]

Safety parameters, Serum vitamin D levels and Primary outcome

Safety markers such as urine creatinine, calcium or their ratios were not different between arms (Figure 1A, B). Similar types and numbers of adverse events were recorded in both arms; the most common were musculoskeletal complaints, respiratory infections, gastrointestinal symptoms, skin lesion and headache (*for details, see the supplementary material in Dörr & Bäcker-Koduah et al., MSJ-ETC, 2020, Project 2*)[45].

Supplementation with cholecalciferol increased the serum 25(OH)D levels in both high- and low-dose arms (Figure 2). In the high-dose arm, serum 25(OH)D levels increased from 18.8 ± 1.9 ng/ml [Mean \pm SE] to $65.0 (5.5)$ ng/ml compared to $17.8 (1.7)$ to $23 (1.4)$ ng/ml in the low-dose arm at 18 months ($p < 0.001$). Supplementation with 20400 IU led to significantly higher serum 25(OH)D levels at all time points compared with 400 IU ($p < 0.001$) in the ITT population ($n = 53$). The primary efficacy outcome - number of new T2w hyperintense lesions on brain MRI at month 18 compared with the baseline value was not different between arms, both in the ITT and PP populations. The cumulative new T2w lesion counts recorded in the high-dose arm was 2.6 ± 1.4 [Mean \pm SE] compared to 7.3 ± 5.4 in the low-dose arm ($p = 0.747$) (ITT=53). No significant differences were found in the change in T2w lesion count ($p = 0.15$) and T2v ($p = 0.98$) (Figure 2 A, B). The change in thalamic volume ($p = 0.95$) and percentage brain volume change ($p = 0.84$) between arms in the ITT nor PP population was not different (*Dörr & Bäcker-Koduah et al., MSJ-ETC, 2020, Project 2*)[45].

1.8.4 VD_immunological (Project 3)[46]

Vitamin D on Immunophenotypes and N-glycan branching

Vitamin D supplementation did not affect the fractions of broad CD3+, CD4+, CD8+ T cells nor that of B/NK regulatory or effector cells or T effector cells when comparing the change from baseline to 12 months (Project 3, Figures 2, 3 and 4)[46]. A reduction in the proportions of CD8+CD28-CD127- (CD8+ Treg) cells was observed in the low-dose compared to the high-dose arm ($p = 0.030$). However, this did not correlate with the proportions of effector T cells (*for details of data and gating strategies see in Figures 2 to 4 and Fig. S2 in Bäcker-Koduah et al., ACTN, 2020, Project 3*)[46].

1.8.5 High-dose vitamin D supplementation reduced N-glycan branching in T and NK cells.

Next, the effect of vitamin D3 supplementation on the β 1,6-GlcNAc (N-glycan) branching on broad T cells, B cells and NK cells was investigated. N-glycan branching was quantified by the MFI of L-PHA. High-dose supplementation led to a decrease in the MFI of L-PHA, hence N-glycan branching at 6 months in broad CD3+ ($p = 0.007$), CD4+ ($p = 0.005$), CD8+ ($p = 0.026$), CD56dim ($p = 0.028$) and CD56bright ($p = 0.020$) NK cells. CD19+ B cells showed a trend towards a decrease in N-glycan branching (Figure 3A-F). This decrease in N-glycan branching in the high-dose arm remained until 18 months (*for details, refer to Figure 5A-F in Bäcker-Koduah et al., ACTN 2020, Project 3*)[46].

Figure 1

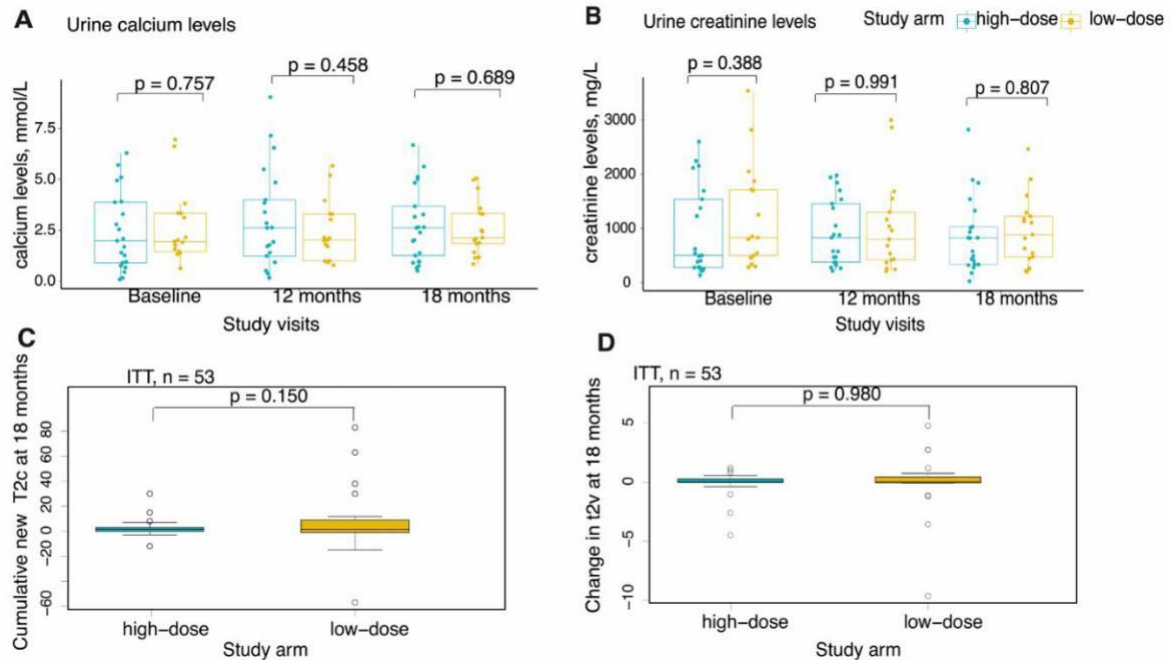


Figure 1. Safety parameters, primary outcome and T2 lesion volume in both arms. Boxplot of the safety parameters (A) urine calcium and (B) urine creatinine levels at baseline, 12 and 18 months. The cumulative new T2c (C) and the change T2v (D) at 18 months compared to baseline, (n = 53 (ITT)). High-dose arm (Green), low-dose arm (Gold). (Adapted and modified from Project 2, Dörr & Bäcker-Koduah et al., 2020 MSJ-ETC, 2020)[45].

Figure 2

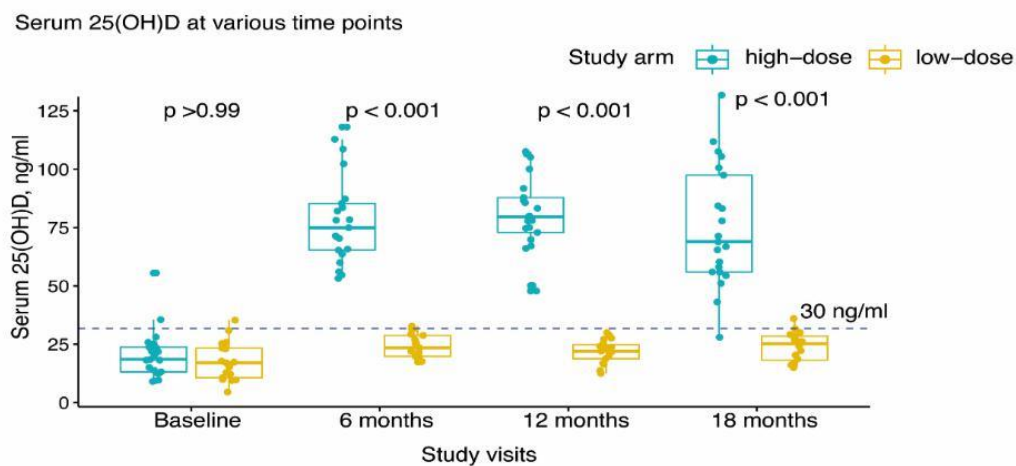


Figure 2. Serum 25hydroxyvitamin D, 25(OH)D levels in the treatment arms with time. Boxplot of the serum 25(OH)D levels measured at baseline, 6, 12 and 18 months. The dashed line marks deficient 25(OH)D levels (<30 ng/ml). Data from the ITT population, n at baseline = 53 (Adapted and modified from Project 3, Bäcker-Koduah et al., ACTN, 2020)[46].

Figure 3

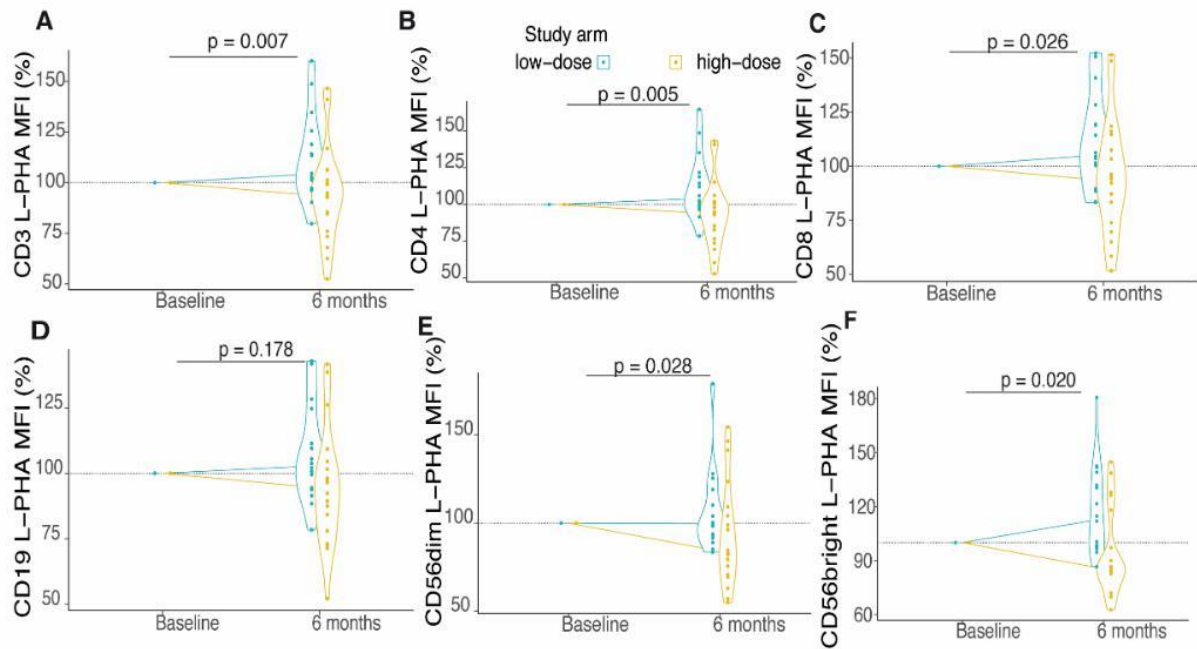


Fig 3. Immune cells L-PHA MFI over different time points.

The L-PHA MFI on immune cells was expressed as percentage fold change in T, B and NK cells. At 6 months a decrease in MFI of L-PHA was observed in the high-dose arm (green) compared to the low-dose arm (gold) in (A) CD3+ ($p=0.007$), (B) CD4+ ($p=0.005$), (C) CD8+ ($p=0.026$) T cells, (D) CD19+ B cells ($p=0.178$), (E) CD56dim ($p=0.028$) and (F) CD56bright, ($p=0.020$) NK cells (*adapted and modified from Bäcker-Koduah et al., ACTN 2020, Project 3*)[46]. ANCOVA with baseline as covariate ($n=38$) (for details, refer to Figure 5A-F in Bäcker-Koduah et al., ACTN 2020, Project 3)[46].

1.9. Discussion

The key findings of the three projects used in this PhD thesis are as follows: we confirmed that MS patients indeed have deficient vitamin D levels which are associated with disease severity. High-dose vitamin D3 supplementation is not superior to low-dose regarding clinical benefits. To our knowledge, our study is the first to investigate the effect of vitamin D3 supplementation on N-glycan branching in MS in the context of a clinical trial. We did not observe any significant impact of vitamin D3 on the proportions of MS relevant immune cells, however, we observed an effect of vitamin D3 on N-glycan branching.

VD_CROSS-SECTIONAL (PROJECT 1)[11]

In Project 1, our hypothesis that hypovitaminosis D is associated with disease activity in MS was confirmed. Hypovitaminosis D was observed in RRMS/CIS patients living in the north-eastern parts of Germany and this is representative of a typical MS cohort published by other cross-sectional studies[52–55]. Higher 25(OH)D levels (≥ 30 ng/ml) was associated with reduced EDSS scores and fewer MRI T2w lesion counts[11]. Previous cross-sectional and population-based studies also demonstrated a high prevalence of vitamin D deficiency in MS patients[53,54]. These studies also confirmed an association of hypovitaminosis D with disability score[55,56] and MRI parameters such as T2c[57,58]. Besides these associations of hypovitaminosis D with disease severity in MS, it remains unclear whether hypovitaminosis D is a risk factor for MS or rather a consequence of the disease (reverse causality) for example by reduced sunlight exposure due to reduced mobility[59]. However, it is evident from a previous study of CIS and early MS patients that hypovitaminosis D exists already in the early phase of the disease where patients are fully ambulatory[52]. Additionally, the patients used in our study were relatively young with shorter disease duration which argues against reverse causality.

VD_INTERVENTIONAL (PROJECT 2)[45]

Here, we hypothesized that high-dose vitamin D3 supplementation is safe and provides more clinical benefits compared to low-dose supplementation. However, supplementation with 20,400 IU cholecalciferol was not superior to 400 IU when comparing the cumulative number of new T2c, T2v, total brain volume, gray matter or white matter volumes and EDSS scores. To date, the benefit of vitamin D supplementation in MS is inconclusive regarding clinical benefits as different clinical trials have produced varying results. A randomized controlled trial that supplemented vitamin D3 as an add-on to IFN- β -1b reported reduced T2c and gadolinium-enhancing lesions[60]. Contrarily, no benefits of vitamin D3 were observed on the relapse rate or EDSS scores in other randomized control trials[57,61]. The largest randomized placebo-controlled trial that investigated the effect of high-dose vitamin D3 supplementation in MS patients on IFN- β -1b

found no statistically significant difference between the two arms in terms of the evidence of disease activity (NEDA-3). However, exploratory secondary outcome analyses revealed a significant reduction in total T2v in the high-dose arm at 48 weeks[62,63].

Several reasons could explain the controversy of vitamin D benefit in MS. Different doses ranging from 200 IU up to 280,000 IU have been used to investigate the impact of vitamin D in MS. Additionally, varying supplementation protocols, either daily, every other day, weekly and the varying study endpoints (a few months to years)[25,45,60,61,63,64] may contribute to the inconclusive results. Most importantly, the EVIDIMS trial had some limitations such as small sample size caused by the increasing trend to self-supplement vitamin D due to its availability on the market, the mild disease activity perhaps due to the pretreatment with interferon β and the restriction of analyses to certain time points due to limited source material[45].

VD_IMMUNOLOGICAL (PROJECT 3)[46]

In Project 3, we hypothesized that vitamin D is an important player in immune regulation and modulates N-glycan branching. However, supplementation with vitamin D3 neither affected the proportions T, B and NK cells subpopulations. Previous studies which investigated the effect of vitamin D on peripheral immune cells could also not detect any effect on the proportions of Th17, Th1, Treg, naïve T cells, or Bregs in MS patients[24,27,29,65]. The effect of vitamin D on NK cells is inadequately investigated although, NK cells play an important role in the regulation of adaptive immunity[66].

On the other hand, high-dose vitamin D3 supplementation reduced N-glycan branching on T cells and NK cells independent of their cell frequencies. Previous evidence from mouse and human studies showed that cell pre-activation increases N-glycan due to increased VDR expression[36,67]. Evidence from animal studies suggests an increase in branching within days to weeks after exposure to vitamin D which we did not observe at 6 months. Depending on the level of 25(OH)D, a high activation state of immune cells could be induced leading to increase in branching shortly after vitamin D intake. Over time, lower branching occurs when the activated cells revert to the resting state as observed in the high-dose arm[46]. Nevertheless, animal and *in vitro* studies[30,68] cannot be directly compared with human studies due to the following: i) existing comorbidities, ii) cross-talk of vitamin D with MS therapies and other vitamin D sensitive cells, iii) the activation state of immune cells, iv) and different analysis times. Additionally, genetic and molecular analysis revealed different responders to vitamin D; low, mid and high responders[69]. Thus, different individuals may need varying vitamin D doses to evoke a clinically relevant effect.

The Projects 1-3 of this PhD thesis are, however, limited by the small sample size and low power, early termination of the study since most MS patients' self-supplement vitamin D and the lack of functional assays due to limited source material. The PhD thesis, however, addresses key points in vitamin D supplementation which might be relevant for consideration in future clinical trials. Future trials, (i) should aim at increasing the sample size, (ii) could focus on the advanced stage of MS to ascertain if vitamin D intake is more clinically beneficial at this stage, (iii) could vary supplementation doses and prolong supplementation periods to ascertain the time point at which vitamin D intake may be clinically relevant for the patients. Additionally, the effect of vitamin D intake on N-glycan branching at the early stage of supplementation should be investigated to confirm the evidence from animal studies. More especially, differential glycosylation effects of vitamin D on effector/regulatory immune cells should be further investigated.

In conclusion, we confirmed that hypovitaminosis D in MS patients is associated with increased disease severity and higher T2-weighted lesion counts. Supplementation with 20,400 IU vitamin D3 is safe and tolerable in MS patients. Vitamin D3 supplementation does not have a substantial effect on the proportions of circulating immune cells but seems to modulate N-glycan branching. Future trials should consider the vitamin D response index by categorizing patients based on their serum 25(OH)D levels before study entry and supplement patients to reach sufficient levels to properly elucidate vitamin D effect in MS.

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3.0. Affidavit

I, Priscilla Bäcker-Koduah, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic, **“The impact of hypovitaminosis D and vitamin D supplementation on disease activity and immune profile in patients with multiple sclerosis”**. I wrote this thesis independently and without assistance from third parties and used no other aids other than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see “uniform requirements for manuscripts (URM)” the ICMJE www.icmje.org) indicated. The sections on methodology and results correspond to the URM and are answered by me. My contributions in the selected publications for this thesis correspond to those that are specified in the following joint declaration with the responsible person and supervisors. All publications resulting from this thesis and which I am an author of, correspond to the URM and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156, 161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date

Priscilla Bäcker-Koduah

4.0 Declaration of contribution to the publications

I, Priscilla Bäcker-Koduah, contributed to these publications as follows:

Project 1: **Bäcker-Koduah P**, Bellmann-Strobl J, Scheel M, Wuerfel J, Wernecke K-D, Dörr J, Brandt AU and Paul F. **Vitamin D and Disease Severity in Multiple Sclerosis-Baseline Data From the Randomized Controlled Trial (EVIDIMS)**. *Front Neurol.* 2020;11:129. Published 2020 Feb 25. doi:10.3389/fneur.2020.00129

Impact factor = 3.552

Contribution: Priscilla Bäcker-Koduah collected patients medical records and created instrument for data digitalization into a Research Electronic Data Capture (Redcap) system, monitored the data, performed double entry which was also double-checked by an independent person to ensure correct data entry, processed, cleaned, and prepared the data for statistical analysis, performed the statistical analysis under supervision of K-D.W and A.U.B, created Tables 1, 2 and Figure 1, interpreted the data together with A.U.B and F.P, wrote the manuscript and revisions under supervision and in agreement with all other co-authors.

The contributions of all other co-authors are explained under the author contribution section of the publication.

Project 2: Dörr J*, **Bäcker-Koduah P***, Wernecke KD, Becker E, Hoffmann F, Faiss J, Brockmeier B, Hoffmann O, Anvari K, Wuerfel J, Piper SK, Bellmann-Strobl J, Brandt AU and Paul F. **High-dose vitamin D supplementation in multiple sclerosis - results from the randomized EVIDIMS (efficacy of vitamin D supplementation in multiple sclerosis) trial.**

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Contribution: Priscilla Bäcker-Koduah performed data entry from patient medical records into Redcap, monitored the database, collected data and processed data, performed statistical analyses under supervision of K-D.W and A.U.B, created Tables 1, 2 and supplementary table 1 together with J.D based on the analysis, designed and created the Consort diagram, interpreted the data together with J.D, A.U.B and F.P and wrote parts of the manuscript and revisions together with Jan Dörr and in agreement with all other co-authors.

The contributions of all other co-authors are explained under the author contribution sections of the publications.

Project 3: **Bäcker-Koduah P**, Infante-Duarte C, Ivaldi F, Uccelli A, Bellmann-Strobl J, Wernecke K-D, Sy M, Demetriou M, Dörr J, Paul F, Brandt AU. **Effect of vitamin D supplementation on N-glycan branching and cellular immunophenotypes in MS**. ACTN. 2020.

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Contribution: Priscilla Bäcker-Koduah contributed to the study design, performed all immunological assays, acquired cells at the flow cytometry, gated the immune cells and quantified immune cell subpopulations in Flowjo under supervision of C.I-D and F.I, exported the cell frequencies and mean fluorescence intensities from Flowjo and performed statistical analyses under supervision of A.U.B and K-D.W, created Table 1 and Figures 1 to 7, created the supplementary Figures S1 and S2, interpreted the data together with A.U.B, M.S, M.D and F.P, wrote the manuscript and responded to reviewer questions during revisions in agreement with all other co-authors. All other co-authors also contributed to the design, data interpretation and manuscript revision.

Signature, Date and Stamp of the supervisor

Signature of the doctoral candidate

5.0 Print versions of the selected publications

5.1 Publication 1- VD_Cross-sectional (Project 1)

Bäcker-Koduah P, Bellmann-Strobl J, Scheel M, Wuerfel J, Wernecke K-D, Dörr J, Brandt AU and Paul F. Vitamin D and Disease Severity in Multiple Sclerosis-Baseline Data From the Randomized Controlled Trial (EVIDIMS). *Front. Neurol.* 2020;11:129. Published 2020 Feb 25. doi:10.3389/Front. Neurol .2020.00129

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Vitamin D and Disease Severity in Multiple Sclerosis—Baseline Data From the Randomized Controlled Trial (EVIDIMS)

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Objective: To investigate the associations between hypovitaminosis D and disease activity in a cohort of relapsing remitting multiple sclerosis (RRMS) and clinically isolated syndrome (CIS) patients.

Methods: In 51 RRMS and 2 CIS patients on stable interferon- β -1b (IFN- β -1b) treatment recruited to the EVIDIMS study (Efficacy of Vitamin D Supplementation in Multiple Sclerosis (NCT01440062) baseline serum vitamin D levels were evaluated. Patients were dichotomized based on the definition of vitamin D deficiency which is reflected by a < 30 vs. ≥ 30 ng/ml level of 25-hydroxyvitamin D (25(OH)D). Possible associations between vitamin D deficiency and both clinical and MRI features of the disease were analyzed.

Results: Median (25, 75% quartiles, Q) 25(OH)D level was 18 ng/ml (12, 24). Forty eight out of 53 (91%) patients had 25(OH)D levels < 30 ng/ml ($p < 0.001$). Patients with 25(OH)D ≥ 30 ng/ml had lower median (25, 75% Q) T2-weighted lesion counts [25 (24, 33)] compared to patients with 25(OH)D < 30 ng/ml [60 (36, 84), $p = 0.03$; adjusted for age, gender and disease duration: $p < 0.001$]. Expanded disability status scale (EDSS) score was negatively associated with serum 25(OH)D levels in a multiple linear regression, including age, sex, and disease duration (adjusted: $p < 0.001$).

Interpretation: Most patients recruited in the EVIDIMS study were vitamin D deficient. Higher 25(OH)D levels were associated with reduced T2 weighted lesion count and lower EDSS scores.

Keywords: multiple sclerosis, vitamin D, vitamin D deficiency, T2w lesion count, EDSS score, disease severity, EVIDIMS

INTRODUCTION

The exact cause of multiple sclerosis (MS), a chronic inflammatory and neurodegenerative autoimmune disease of the central nervous system (1) is unknown. However, several environmental and genetic factors have been associated with MS pathogenesis, among them are vitamin D (VD) serum levels (2–5), vitamin D receptor polymorphisms (VDP) (6–10), and sunlight exposure (11, 12). In fact, increasing evidence suggests that VD deficiency may affect disease progression and outcome in MS.

VD is considered a potent immunomodulator which may reduce MS risk based on epidemiological and experimental evidence (13–15). High serum VD levels have been associated with lower MS risk, reduced relapse rates (16) and better disease outcome (2, 13, 17). Circulating VD levels are lower during relapses compared to phases of disease stability (18). *In vitro* studies also showed a reduced proliferation of CD4⁺ T cells and myelin basic protein (MBP)-specific T cells in the presence of 1,25(OH)D₃, the active metabolite of vitamin D (18). The immunomodulatory effect of vitamin D in upregulating anti-inflammatory cytokines points to an important role in the homeostasis of T cells (18) hence its relevance in autoimmune diseases.

The high prevalence of hypovitaminosis D worldwide (19, 20) and the continuously increasing incidence of MS incidence (21) highlights the need to investigate possible associations.

The American Institute of Medicine (IOM) (22), the Japan Endocrine Society (23), the Osteoporosis Council of Canada (24), and the International Osteoporosis Foundation (25) have suggested divergent cut-offs for vitamin D deficiency ranging from < 20 to < 30 ng/ml. These definitions are mainly based on the prevention of osteoporosis (22, 23, 26–28). In healthy populations, serum vitamin D levels \geq 30 ng/ml are considered sufficient (29–31). In the past decade, several clinical interventional trials have investigated the effect of vitamin D supplementation on the pathogenesis of MS, among them the EVIDIMS (NCT01440062) (32), the SOLAR (33), the VIDAMS (34), and the CHOLINE trials (35). Moreover, data from observational studies suggest that there may be a beneficial interaction of vitamin D and interferon- β regarding their immunomodulatory effect (36, 37).

In this paper, we dichotomized patients enrolled in the EVIDIMS study at baseline prior to randomization and vitamin D supplementation according to their serum 25(OH)D levels and based on VD deficiency. We then investigated possible associations of the VD status with clinical and MRI parameters. The EVIDIMS study investigated the effect of oral VD supplementation on clinical and MRI parameters in MS patients on stable immunomodulatory interferon- β treatment. The primary outcomes of the EVIDIMS trial are presented elsewhere (38). The goal of this analysis is to investigate if the EVIDIMS cohort is representative of a VD deficient RRMS cohort by (a) investigating VD serum levels and (b) investigating correlations with disease severity.

METHODS

Patients

The cohort in this study represents the baseline cohort of phase II interventional EVIDIMS trial (NCT01440062) (32) and comprised 51 patients with RRMS and 2 CIS patients, all Caucasians, according to the 2005 McDonald criteria (39). The detailed in- and exclusion criteria for the EVIDIMS trials have been published earlier (32, 38). Briefly, inclusion criteria were age of 18–65 years, Expanded Disability Status Score (EDSS) below 6.5, stable interferon- β 1b treatment for a minimum of 3 months, freedom of relapses for at least 30 days prior to study entry, and a relapsing remitting disease course or CIS. The exclusion criteria were other immunomodulatory therapies than interferon- β 1b, VD intake within 6 months before study entry, pregnancy or lactation and kidney disease, bone marrow dysfunction or hypercalcemia. To account for sunlight intensity as a possible confounder, patients were recruited from a single geographic region in the north-eastern part of Germany.

Disability was evaluated using EDSS scores. All clinical and MRI assessments were performed blinded to serum VD status.

Measurement of Serum 25(OH)D Levels

Serum 25(OH)D levels were measured by the Bioscientia Institute for Medical Diagnostics GmbH (Berlin, Germany) using the LIAISON[®] chemiluminescence analyzer, DiaSorin (Dietzenbach, Germany).

Definitions of Groups

Subgroup analyses based on serum 25(OH)D levels were performed based on the suggested cut-offs for VD levels for bone health as proposed by the American Institute of Medicine, the Japan Endocrine Society and the global definition for vitamin D deficiency and sufficiency. Hence, dichotomous analyses comparing serum 25(OH)D levels of (< 30 vs. \geq 30 ng/ml) were performed. These groups were compared with respect to the following (i) MRI imaging parameters: T2 weighted lesion count (T2C), T2 lesion volume (T2V), white matter volume (WM), gray matter volume (GM), total brain volume and (ii) clinical parameters such as EDSS scores.

MRI Protocol

The same MRI machine and protocol were used in all patients using a Magnetom TIM TRIO 3 Tesla MRI (Siemens, Healthineers, Erlangen, Germany). High resolution images were acquired using a sagittal three-dimensional (3D) T2-weighted (T2w) SPACE sequence (repetition time (TR) ms/echo time (TE) ms/inversion time (TI) ms (5,000/502/900; flip angle 9°), isotropic resolution 1 mm³, Generalized Autocalibrating Partially Parallel Acquisition (GRAPPA 2), a 3D SPACE-FLAIR (TR ms/TE ms/ TI ms, 6,000/388/2,100) with similar spatial parameters and a three-dimension (3D) T1w, magnetization-prepared rapid gradient-echo (MP-RAGE) sequence (TR ms/echo time ms/ TI ms, 1,900/3.03/900; flip angle 9°, isotropic resolution 1 mm³, GRAPPA 2). Five minutes after injection of 0.1 mmol/kg gadolinium-labeled diethylenetriaminepentaacetic acid (Gd-DTPA, Magnevist, Bayer-Schering, Berlin, Germany), a 3D T1w gradient recall echo volumetric interpolated breath-hold

examination (VIBE) sequence (TR ms/TE ms, 4.8/2.2; flip angle 9°, isotropic resolution 1 mm³, GRAPPA 2) was applied. The quality of acquired images was reviewed, and raw data were transferred to a Linux workstation and processed semi-automatically using an image coregistration (FMRIB's Linear Image Registration Tool, FMRIB Analysis Group, University of Oxford, Oxford, UK) and inhomogeneity correction routine embedded into the MedX v.3.4.3 software package (Sensor Systems Inc., Sterling, VA, USA).

The MedX v.3.4.3 software package was used for measuring the white matter lesion load and lesion count of T2w scans, the number and volume of contrast-enhancing and hypointense lesions on T1w scans. Segmentation of brain lesions was performed semi-automatically using the lesion segmentation toolbox (LST) (40) lesion probability algorithm on FLAIR images with subsequent manual correction using ITK-SNAP (41). Normalized brain volume (NBV) and percentage brain volume change (PBVC) were obtained with SIENA (FMRIB library) (40).

Statistical Analyses

Results for continuous variables are expressed as median with 25 and 75% quartiles [25, 75%] for non-normally distributed data and as mean ± standard deviation (SD) for age and disease duration. Results for categorical variables are given as absolute numbers and relative frequencies (%). Due to the non-normally distributed continuous data and small sample sizes, exact Mann–Whitney *U*-test was used for the comparison of independent groups. Simple linear regression analyses were used for the association between EDSS and serum vitamin D levels. Multivariate linear regressions were applied in order to adjust this association for possible confounding factors such as age, disease duration, and sex. We also tested for collinearity between the independent variables. Non-parametric analysis of covariance was applied to test T2-weighted lesion counts and volume for differences between groups of vitamin D sufficiency (< 30 vs. ≥ 30 ng/ml), adjusted for the covariates age, gender, and disease duration. A *p*-value of < 0.05 was considered significant. All other tests should be understood as constituting exploratory data analysis, such that no adjustments for multiple testing have been made. Statistical analyses were performed in R version 3.4.2 (2017-09-28), IBM® SPSS® Statistics, Version 24, © Copyright 1989, 2016 SPSS Inc., an IBM Company and SAS version 9.4 [TS1M3] Copyright © 2002 by SAS Institute Inc., Cary, NC, USA.

RESULTS

Patients and Serum 25(OH)D Levels

Data from all 51 RRMS and 2 CIS patients recruited into the EVIDIMS trial were used for this study. The mean age of patients was 43 ± 10.2 years with 37 (69.8 %) females and a mean disease duration of 9 ± 9.3 years.

The median (25, 75% Q) serum 25(OH)D was 18 ng/ml (12, 24), signifying overall VD deficiency (Table 1). After dichotomization by serum 25(OH)D < 30 vs. ≥ 30 ng/ml, 48 out of 53 patients (91%) had 25(OH)D < 30 ng/ml with a median (25, 75% Q) of 15 ng/ml (10, 21) in the < 30 ng/ml group while

TABLE 1 | Baseline characteristics of 51 RRMS and 2 CIS patients recruited in the EVIDIMS Trial.

Variable	Total
Female <i>n</i> [%]	37 (69.8)
Age in years [mean ± SD]	43 ± 10.2
Disease duration in years [mean ± SD]	9 ± 9.3
Serum 25 (OH) [med (25, 75% quartiles)] ng/ml	18 (12, 24)
EDSS [med (25,75% quartiles)]	2.0 (1.5, 3.0)
T2 lesion count [med (25,75% quartiles)]	55 (33, 84)
T2 lesion vol (ml) [med (25,75% quartiles)]	4 (2, 9)

TABLE 2 | Characteristics of patients with 25(OH)D levels < 30 vs. ≥ 30 ng/ml.

Subgroup analysis of 25(OH)D levels < 30 vs. ≥ 30 ng/ml			
Variable	< 30 ng/ml (<i>n</i> = 48)	≥ 30 ng/ml (<i>n</i> = 5)	<i>p</i> -value ¹
Age in years [mean ± SD]	42.9 ± 10.1	39 ± 12.8	0.46
Disease duration in years [mean ± SD]	9.6 ± 6.7	5.7 ± 5.4	0.15
Serum 25(OH) [med (25, 75% quartiles)] ng/ml	15 (10, 21)	33 (33, 35)	<0.001
EDSS [med (25, 75% quartiles)]	2.5 (1.5, 3)	1.0 (1, 2)	0.04
T2 lesion count [med (25, 75% quartiles)]	60 (36, 84)	25 (24,33)	0.03
T2 lesion vol (ml) [med (25, 75% quartiles)]	4 (2, 10)	2 (2, 3)	0.13
Gray matter vol (ml) [med (25, 75% quartiles)]	597 (553, 620)	594 (557, 652)	0.63
White matter volume(ml) [med (25, 75% quartiles)]	536 (509, 591)	567 (547, 570)	0.49
Brain volume(ml) [med (25, 75% quartiles)]	1126 (1063, 1209)	1161 (1114, 1222)	0.53

¹Mann–Whitney test.

the remaining 5 out of 53 patients (9%) had 25(OH)D ≥ 30 ng/ml with a median of 33 (33, 35) ng/ml (*p* < 0.001) (Table 2).

Association of Serum 25(OH)D and Disability Scores

In the entire cohort, there was an inverse association between EDSS score and serum 25(OH)D level. The results remained the same after adjustment for age, sex, and disease duration in a multivariate analysis (simple: $R^2 = 0.10$, $p = 0.02$; adjusted: $R^2 = 0.34$, $p < 0.001$) (Figure 1). EDSS was higher in patients with deficient (< 30 ng/ml) 25(OH)D: 2.5 (1.5, 3) compared to 1.0 (1, 2) for vitamin levels ≥ 30 ng/ml ($p = 0.04$) (Table 2). There was no collinearity between the predictor variables tested.

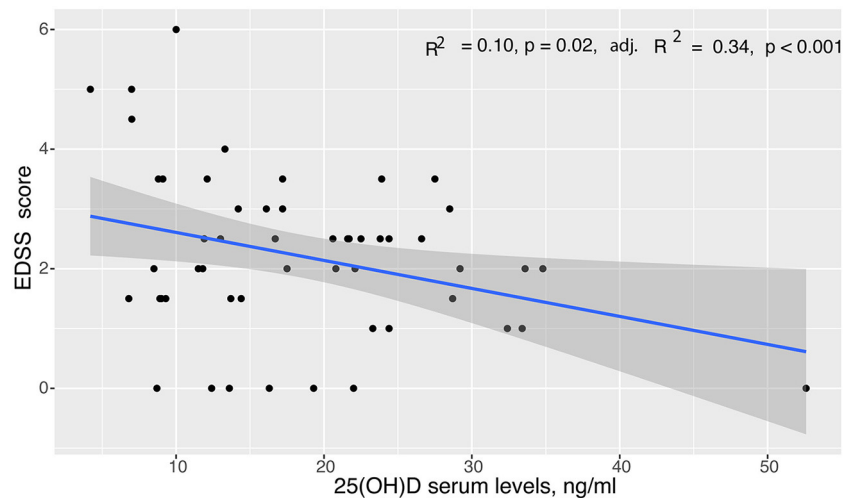


FIGURE 1 | Linear regression analysis of EDSS scores to serum vitamin D levels in the entire cohort with 95% confidence limits. Simple Linear regression showed a significant association between EDSS scores and serum 25(OH)D levels ($p = 0.02$), which was confirmed after adjustment for age, sex, and disease duration in multiple linear regression analysis (adjusted $p < 0.001$).

Association of Serum 25(OH)D and MRI Parameters

The median (25, 75% Q) T2 weighted lesion count was 25 (24, 33) in the ≥ 30 ng/ml group, compared to 60 (36, 84) in the < 30 ng/ml group ($p = 0.03$; adjusted for age, gender, 25(OH)D levels, and disease duration: $p < 0.001$). The numerical difference in median T2 lesion volume [2 (2, 3) ml in the ≥ 30 ng/ml group; 4 (2, 10) ml in the < 30 ng/ml group] was not significant [$p = 0.13$; adjusted for age, gender, 25(OH)D levels, and disease duration: $p = 0.06$] (Table 2). We found no association of serum vitamin D levels with gray matter, white matter, and total brain volumes (Table 2).

DISCUSSION

Using baseline characteristics of patients recruited in the EVIDIMS trial, we show that according to the definitions of the American Society of Medicine and the Japan Endocrine Society (22, 23, 27), VD deficiency is common in RRMS/CIS patients living in the north-eastern part of Germany. Moreover, the range of serum levels and the proportions of serum VD levels is in line with previously published MS cohorts. In this cohort, VD deficiency showed an inverse association with measures of disability and was furthermore linked to a higher T2w lesion count but was not associated with T2 lesion volume, total brain volume as well as gray and white matter volumes.

A high prevalence of VD deficiency in MS patients has been demonstrated in other studies: In an earlier study on a different patient cohort, we reported VD deficiency already in very early phases of MS (5). A cross-sectional study of 50 RRMS patients revealed a VD deficiency with a mean of 22.3 ng/ml/ml (42). In a Moroccan study of 113 MS patients, 97.3% of patients were VD deficient with a mean of 11.69 ng/ml (43). There is an ongoing debate whether VD deficiency represents a risk factor for MS or

whether this association is rather due to reverse causality, i.e., low VD levels are a result of MS, for example as a consequence of reduced outdoor activities. The fact that the cohort investigated here was relatively young with a rather mild disability status may be an argument against reverse causality. Moreover, in an earlier study on a different cohort of early MS or CIS patients we also observed VD deficiency already in very early phases of MS (5) which also supports the interpretation that VD deficiency is rather a cause than a consequence of MS.

Also, the inverse association between disability measures and serum 25(OH)D levels demonstrated here are in line with previous reports: a population-based study on 136 MS patients from Australia showed that patients with higher disability ($EDSS > 3$) had a higher probability of insufficient vitamin D levels (44). Similarly, a cross-sectional study on 267 MS patients revealed an inverse association between EDSS scores and serum 25(OH)D levels in the entire cohort (45).

The association of deficient serum vitamin D levels with MRI activity is controversial. While some studies could not show any associations between serum 25(OH)D levels and MRI activity (46) others showed that the development of T2w or contrast enhancing lesions is correlated to the VD serum level (47). Our MRI data support the association of MRI activity and VD status. We, however, did not detect any association between VD levels and measures of both global and regional brain atrophy. Although speculative, this might well be due to the small sample size and the low power to detect such associations.

Taken together, our results confirm the high prevalence of VD deficiency in MS patients and the possible associations it has with MRI and clinical disease activity. Of note, causality or directionality of these associations cannot be inferred from ours and other cross-sectional studies reporting similar associations.

Specifically, it is possible that reduced VD leads to a more severe disease course. Alternatively, it may also be possible that higher disability may lead to lower VD levels, e.g., by less physical activity and reduced sunlight exposure. Previously, we reported reduced VD levels in a cohort of very early MS/CIS patients with low disability, which makes the latter less likely, but not impossible. Our study, however, has some important limitations: first, the overall small sample size and particularly the very small number of patients in the VD sufficient group result in a low power to detect associations. Secondly, our analyses are based solely on the cut-offs for deficient and sufficient serum VD levels defined for osteoporosis and normal health which may not be generalized to the MS population. Additionally, we only investigated patients from a particular area of Germany which increased the homogeneity of our sample on the one side but on the other side might not be representative for MS patients in general. Finally, as serum samples were taken throughout the year, seasonal variations of VD levels may confound data.

In conclusion, the EVIDIMS cohort is representative of a typical RRMS cohort with VD deficiency, we confirm previous associations of low serum VD with clinical and disease activity which provides further support for the role of VD in the development and progression of MS.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

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ETHICS STATEMENT

The study was reviewed and approved by the German Federal Institute for Drugs and Medical Device (BfArM, 4037578) and the local ethics committees (11/0386-ZS EK 13). All patients gave written informed consent before entering the study.

AUTHOR CONTRIBUTIONS

PB-K collected, processed, cleaned, and prepared the data for statistical analysis, performed the statistical analysis, interpreted the data, and drafted the manuscript. JB-S recruited patients and collected clinical data. MS acquired and processed MRI data. JW acquired and processed MRI data. K-DW was a responsible biometrician, performed the statistical analyses, and interpreted the data. JD designed the trial, drafted the study protocol, recruited patients and generated data, and drafted the manuscript. AB and FP designed and conceptualized the study, interpreted the data, and revised the manuscript for intellectual content.

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5.2 Publication 2- VD_interventional (Project 2)

Dörr J*, **Bäcker-Koduah P***, Wernecke KD, Becker E, Hoffmann F, Faiss J, Brockmeier B, Hoffmann O, Anvari K, Wuerfel J, Piper SK, Bellmann-Strobl J, Brandt AU and Paul F. High-dose vitamin D supplementation in multiple sclerosis - results from the randomized EVIDIMS (efficacy of vitamin D supplementation in multiple sclerosis) trial. *Mult Scler J Exp Transl Clin.* 2020;6(1):2055217320903474. Published 2020 Jan 24. doi:10.1177/2055217320903474
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High-dose vitamin D supplementation in multiple sclerosis – results from the randomized EVIDIMS (efficacy of vitamin D supplementation in multiple sclerosis) trial

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Abstract

Background: Epidemiological, preclinical, and non-interventional studies link vitamin D (VD) serum levels and disease activity in multiple sclerosis (MS). It is unclear whether high-dose VD supplementation can be used as an intervention to reduce disease activity.

Objectives: The study aimed to compare the effects of every other day high- (20,400 IU) versus low-dose (400 IU) cholecalciferol supplementation on clinical and imaging markers of disease activity in patients with relapsing–remitting MS or clinically isolated syndrome.

Methods: The EVIDIMS (efficacy of vitamin D supplementation in multiple sclerosis) trial was a multicentre randomized/stratified actively controlled explorative phase 2a pilot trial with a double-blind intervention period of 18 months, add on to interferon- β 1b.

Results: Fifty-three patients were randomized, and 41 patients completed the study. Cholecalciferol supplementation was well tolerated and safe in both arms. After 18 months, clinical (relapse rates, disability progression) and radiographical (T2-weighted lesion development, contrast-enhancing lesion development, brain atrophy) did not differ between both treatment arms. Post-study power calculations suggested that the sample size was too low to prove the hypothesis.

Conclusions: The results neither support nor disprove a therapeutic benefit of high-dose VD supplementation but provide a basis for sound sample size estimations in future confirmatory studies. www.clinicaltrials.gov/NCT01440062

Keywords: Multiple sclerosis, clinical trial, vitamin D, efficacy, treatment, supplementation

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Introduction

Preclinical and epidemiological studies link 25-hydroxy (OH) vitamin D, hereafter referred to as vitamin D, serum levels to disease activity of multiple sclerosis (MS). In particular, associations of higher vitamin D levels in patients with MS with favourable clinical and magnetic resonance imaging (MRI) parameters of disease activity have been reported.^{1–3} Based on these data, MS clinicians commonly boost vitamin D levels in patients by oral cholecalciferol or ergocalciferol supplementation.

While safety data are rather unequivocal and suggest that vitamin D supplementation up to 280,000 IU/week is fairly safe,⁴ prospective data from interventional treatment trials of vitamin D supplementation on clinical efficacy, doses needed, and optimal serum levels in MS are sparse, inconsistent, and in part conflicting.^{5,6} A phase I/II randomized controlled open-label study comparing continuous intake of up to 40,000 IU/day or 4000 IU/day for 52 weeks in 49 patients with MS showed a significant reduction of annualized relapse rate in the

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high-dose arm.⁷ In contrast, two randomized, placebo-controlled and double-blind phase II trials from Finland and Norway, applying 20,000 IU/week cholecalciferol for 48 or 96 weeks, demonstrated no effect of vitamin D on relapse rates.^{8,9} In the Finish study, however, patients in the treatment group had significantly fewer gadolinium enhancing (gd+) lesions and a tendency to reduced disability accumulation.⁸ The placebo-controlled SOLAR study¹⁰ showed no effect of 14,000 IU cholecalciferol per day on the primary endpoint ‘no evidence of disease activity’ but a significant beneficial effect on the development of new lesions on cranial MRI and a trend towards a reduction of relapse rate.

The ‘efficacy of vitamin D supplementation in multiple sclerosis’ (EVIDIMS) clinical trial was an investigator-sponsored randomized actively controlled double-blind phase II trial of vitamin D supplementation in patients with MS. The detailed protocol has been published previously.¹¹ Here, we report the major clinical and MRI results.

Patients and methods

Study design and ethics statement

The EVIDIMS clinical trial was a multicentre, randomized and stratified, active controlled, double-blind, explorative phase II trial. Two doses of vitamin D3 (cholecalciferol) were compared: in the high-dose arm participants received 20,400 IU (1 ml cholecalciferol oil (20,000 IU) + 1 tablet (400 IU)) every other day for 18 months, while in the low-dose arm 400 IU (1 ml placebo oil (0 IU) + 1 tablet (400 IU)) were given every other day. To rule out confounding effects of variable disease-modifying treatments, all participants were required to be on stable treatment with interferon-beta-1b, started at least 3 months prior to inclusion. Interferon-beta treatment was continued throughout the study.

Patients with relapsing–remitting (RR)MS or clinically isolated syndrome (CIS) were recruited at seven German study centres (Berlin (four centres), Potsdam, Halle, Teupitz; for details see supplementary information). After a short screening period eligible patients were stratified according to gender and vitamin D level (< or ≥20 ng/ml (50 nmol/l)) and then randomized 1:1 to either high-dose or low-dose treatment as an add-on to continuous treatment with IFN β-1b. Clinical evaluations including Expanded Disability Status Scale (EDSS)¹² and MS Functional Composite (MSFC)¹³ were performed at

screening and then every 6 months. Standardized MRI was performed at screening and after 12 and 18 months using identical hardware for all patients (at the Charité study centre, Berlin). Pharmacovigilance laboratory testing was done after 4 weeks and thereafter every 3 months.

The primary endpoint was the number of new T2-weighted (T2w) hyperintense lesions on brain MRI at month 18 compared with baseline. Major secondary endpoints were relapse rate and disability progression on the clinical side, T2w lesion volume, number of gd+ lesions, and brain atrophy on the MRI side, and number of adverse events on the safety side. More detailed information on the design and conduct of the EVIDIMS trial has been published previously,¹¹ and additional information is provided as supplementary information. The study protocol (in German) is available from the corresponding author upon qualified request.

The study was approved by the German Federal Institute for Drugs and Medical Devices (BfArM, 4037578), as well as by the local ethics committees (11/0386-ZS EK 13) and is registered at European Clinical Trials Database (EudraCT 2011-002785-20) and ClinicalTrials.gov (NCT01440062). It was conducted strictly adhering to the study protocol and to applicable national laws (Arzneimittelgesetz, 14. Novelle, 2005), the Harmonized Tripartite Guideline for Good Clinical Practice (ICH GCP), and the principles of the Declaration of Helsinki in its applicable version. All participants gave written informed consent at screening prior to any study-related procedures.

Study population

The main inclusion criteria were: male and female patients with either CIS or a definite diagnosis of MS according to the 2005 revised McDonald criteria¹⁴ and a relapsing–remitting disease course; age at randomization 18–65 years; EDSS score 0–6; continuous treatment with IFN β1b for at least 3 months; and no relapse within 30 days prior to randomization. Important exclusion criteria were: relevant hepatopathy or renal dysfunction; history of sarcoidosis, nephrolithiasis, pseudo hypoparathyroidism; vitamin D supplementation >500 IU/day within 6 months prior to randomization; pregnancy; hypercalcaemia or urine calcium/creatinine ratio >1; concomitant medication with hydrochlorothiazide, digitoxin, digoxin, phenytoin, barbiturates; inability to provide informed consent; incompatibility with MRI procedures. A comprehensive listing of inclusion

and exclusion criteria is provided in the supplementary information.

Outcome measures

Clinical evaluations and safety laboratory tests.

All clinical evaluations and scorings were performed by trained study personnel blinded to the patients' treatment allocations according to standardized operating procedures. MS relapses were defined as: (re)occurrence of new or previous central nervous system dysfunction in the absence of infections or hyperthermia, duration ≥ 24 h, time-lag from the onset of previous relapse ≥ 30 days. Disability was assessed by EDSS and MSFC.^{12,13} EDSS rating was performed by trained (Neurostatus) and board-certified physicians otherwise not involved in the management of study participants. MSFC scoring was done by trained study personnel. All study-related safety laboratory tests were done by Bioscientia laboratories (Bioscientia Institut für Medizinische Diagnostik GmbH, Ingelheim, Germany).

MRI. Standardized MRI was performed in all participants in one study centre at Charité - Universitätsmedizin Berlin, using a Magnetom TIM TRIO 3 Tesla MRI (Siemens Healthineers, Erlangen, Germany). A sagittal 3D SPACE T2w sequence (TR 5,000 ms, TE 502 ms, TI 900 ms, flip angle 9 degree, isotropic resolution 1 mm^3 , GRAPPA 2) was used to obtain T2w images. In addition, we applied a 3D fluid-attenuated inversion recovery sequence (SPACE-FLAIR, TR 6,000 ms, TE 388 ms, TI 2,100) with identical spatial parameters, and a high-resolution 3-dimensional T1w sequence (MPRAGE, TR 1,900 ms, TE 3.03 ms, TI 900 ms, flip angle 9 degree, isotropic resolution 1 mm^3 GRAPPA 2) with identical spatial parameters. A 3D T1w sequence (VIBE, TR 4.8 ms, TE 2.2 ms, flip angle 9 degree, isotropic resolution 1 mm^3 GRAPPA 2) with identical spatial parameters was applied 5 min after injection of 0.1 mmol/kg gad-DTPA (Gadovist, Bayer, Berlin, Germany). Raw data were transferred to a Linux workstation and processed following a semi-automated procedure, including an image coregistration (FMRIB's Linear Image Registration Tool, FMRIB Analysis Group, University of Oxford, Oxford, UK) and inhomogeneity correction routine embedded into the MedX v.3.4.3 software package (Sensor Systems Inc., Sterling, VA, USA). T2w white matter lesion load, as well as number and volume of contrast-enhancing and hypointense lesions on T1w scans were routinely measured using the MedX v.3.4.3 software

package. Brain atrophy (percentage brain volume change (PBVC) and normalized brain volume (NBV)) was estimated with SIENA,¹⁵ part of FSL.¹⁶ Thalamic volumetry was performed using FIRST (FMRIB Analysis Group, University of Oxford, Oxford, UK).

Statistical analyses. Due to lack of data on the effect of vitamin D on T2w lesions, no statistical sample size calculation was performed in advance using a given error of the 1st kind and stipulated power. A sample size of 80 patients (40 in each arm) was planned, based mainly on feasibility.

Due to the explorative character of the study, statistical testing has to be understood as explorative, and data analyses were mainly descriptive for all endpoints. For univariate independent group comparisons exact Mann-Whitney *U* tests and exact Chi-Square tests were used. Survival data such as MS relapses were analysed by Kaplan-Meier curves with appropriate log-rank tests and multiple Cox-regressions. For time series data, a nonparametric multivariate analysis of longitudinal data (MANOVA) in a two-factorial design was applied (1st factor (independent): treatment groups, 2nd factor (dependent): study visits). When appropriate, multivariate nonparametric analysis of covariance (MANCOVA) using baseline values and additional parameters including age, gender, and disease duration as covariates was complemented. The PBVC was adjusted using baseline NBV. Missing values were replaced by multiple imputations. Statistical significance was assumed at the $p=0.05$ level. Because of the explorative nature of analyses, no adjustments for multiple comparisons were performed. Both intent-to-treat (ITT) and per-protocol (PP) analyses were carried out. The ITT group comprised 53 patients. The PP definition was regular study termination and protocol-conform study drug intake. The PP group comprised 38 patients. All calculations were performed using SAS Version 9.4 [TS1M3] Copyright 2002–2012 by SAS Institute Inc., Cary, NC, USA, IBM SPSS Statistics, Version 25, Copyright 1989, 2010 SPSS Inc., an IBM Company, Chicago, IL, USA and The R Project for Statistical Computing, Version 3.0.2 (2017-04-21).

Results

Study population

After starting recruitment, the study was terminated early. Fifty-three patients were randomized and

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included in the ITT analyses, 28 in the high-dose and 25 in the low-dose arm. Demographic baseline characteristics (ITT population) are shown in Table 1. T2w lesion volume was higher in the low-dose arm ($p = 0.01$), and thalamic volume was higher in the high-dose arm ($p = 0.048$). As shown in Figure 1, 41 participants completed the study. The number of dropouts did not differ between both arms. The reasons for dropping out are provided in the CONSORT flow chart (Figure 1). Two patients in the high-dose arm were excluded from analyses because of major protocol violations, which resulted in an insufficient increase of the serum vitamin D levels. In the low-dose arm, one patient was excluded because of missing MRI data at baseline and final visit (Figure 1). Thus, 38 patients were included in the PP analyses.

Vitamin D levels, MRI and clinical endpoints

Important explorative clinical and paraclinical outcome parameters (univariate, ITT population) are presented in Table 2. While on study medication, 25(OH)vitamin D levels increased from 19 (7–53) ng/ml at baseline to 62 (52–80) ng/ml at month 18 in the high-dose group and from 17 (4–35) to 23 (18–27) ng/ml in the low-dose group ($p < 0.001$).

The primary endpoint, number of new T2w hyperintense lesions on brain MRI at month 18 compared with baseline, was not different between arms, as were T2w lesion counts ($p = 0.15$) when including

baseline, age, gender, and disease duration as covariates in a MANCOVA for longitudinal data. Similarly, T2 lesion volume at month 18 compared with baseline was not different between arms ($p = 0.98$). A numerical but not significant ($p = 0.09$) higher number of cumulative new gd+ lesions in the low-dose arm was driven by only four patients. MANCOVA with baseline as covariate showed no differences in the thalamic volume ($p = 0.95$), PBVC ($p = 0.84$), and the brain parenchymal fraction ($p = 0.43$) (Table 2). Disease progression measured by median EDSS ($p = 0.64$) and mean MSFC z-score ($p = 0.31$) in a MANCOVA with baseline as covariate was not different in both arms (Table 2).

Safety endpoints

The numbers and severity of adverse events did not differ between groups ($p = 0.5$, exact Chi-Square test). In total, 41 adverse events were recorded; of these 23 were in the high-dose and 18 in the low-dose arm. The most often recorded adverse events were respiratory infections ($n = 25$) and musculoskeletal complaints ($n = 15$), but without differences between study arms. The majority of adverse events were mild or moderate and not considered related to the study medication. No serious adverse events and no vitamin D-related toxicity or safety issues were recorded.

Table 1. Baseline characteristics of patients (ITT).

	High dose	Low dose	<i>p</i> -value
Total (<i>n</i>) [within group (%)]	28 [53]	25 [47]	>0.99 ^a
Disease course (<i>n</i>)			
RRMS	26	25	0.49 ^a
CIS	2	0	
Female [within group (%)]	20 [71]	17 [68]	>0.99 ^a
Mean age at screening (years) [SE]	41 [2.1]	45 [1.8]	0.26 ^b
Mean disease duration onset to screening (months) [SE]	97 [14.4]	125 [16.8]	0.24 ^b
Mean BMI at screening [SE]	27.2 [1.3]	25.5 [0.9]	0.61 ^b
Median EDSS [range]	2.0 [5.0]	2.5 [6.0]	0.18 ^b
Mean 25OH vitamin D serum level (ng/ml) [SE]	18.8 [1.9]	17.8 [1.7]	0.9 ^b
Mean T2w lesion count (<i>n</i>) [SE]	52.6 [6.7]	76.1 [10.7]	0.08 ^b
Mean T2w lesion volume (ml) [SE]	4.6 [0.9]	10.4 [1.9]	0.01 ^b
Mean brain parenchymal fraction (ml) [SE]	1163.1 [25.8]	1121.3 [18.1]	0.4 ^b
Mean thalamus volume (ml) [SE]	15.5 [0.4]	14.4 [0.4]	0.05 ^b
Total gd+ lesions (<i>n</i>)	4	2	>0.99 ^b

BMI: body mass index; CIS: clinically isolated syndrome; EDSS: Expanded Disability Status Scale; gd+: gadolinium enhancing lesions; ITT: intention to treat; MSFC: Multiple Sclerosis Functional Composite; *n*: number; RRMS: relapsing remitting MS; SE: standard error.

^aexact Chi-Square tests; ^bexact Mann–Whitney *U* test

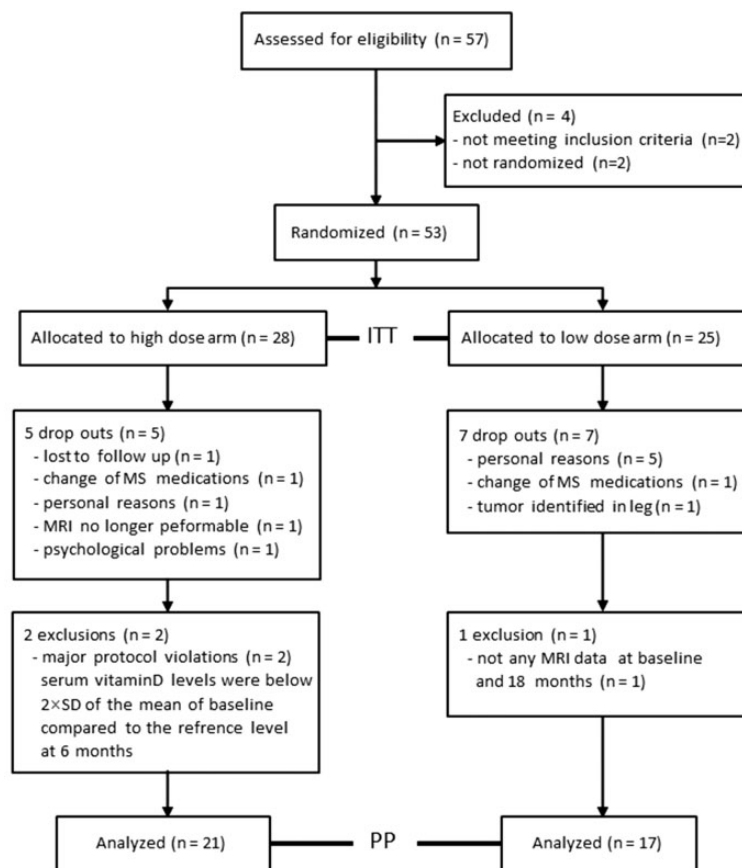


Figure 1. The CONSORT flow diagram.

Figure 1 shows the numbers of screened, randomized, and analysed patients in both treatment arms. The reasons for exclusion from randomization, drop out or exclusion from analysis are displayed.

ITT: intention to treat; PP: per protocol; *n*: number.

Table 2. Main clinical and MRI outcome parameters after 18 months and the changes from baseline (ITT).

	High dose	Low dose	<i>p</i> -value	Change from baseline		
				High dose	Low dose	<i>p</i> -value
Mean 25OH vitamin D serum level (ng/ml) [SE]	65.0 [5.5]	22.3 [1.4]	<0.001 ^c	45.9 [5.4]	5.9 [2.3]	<0.001 ^c
Cumulative number of relapses (n)	5	7	0.6 ^b			
Median EDSS [range]	2.0 [3.5]	2.0 [5.5]	0.26 ^a	0 [4]	0 [2.5]	0.64 ^c
Mean MSFC z-score change [SE]	-0.24 [0.14]	-0.44 [0.28]	0.13 ^a	0.12 [0.07]	-0.09 [0.16]	0.31 ^c
Mean T2w lesion count (<i>n</i>) [SE]	53.4 [7.3]	84.1 [13.5]	0.05 ^a	1.3 [0.1]	2.1 [1.4]	0.15 ^c
Mean T2w lesion volume (ml) [SE]	3.6 [0.5]	11.9 [2.2]	<0.001 ^a	0.1 [0.1]	0.2 [0.3]	0.98 ^c
Mean brain parenchymal fraction (ml) [SE]	1167.7 [25.9]	1126.6 [20.7]	0.97 ^a	-9.3 [3.7]	-7.3 [2.6]	0.43 ^c
Mean brain volume changes (%) [SE]	-0.61 [0.12]	-0.52 [0.10]	0.98 ^a	na	na	0.84 ^c
Mean thalamus volume (ml) [SE]	15.7 [0.3]	14.4 [0.5]	0.048 ^a	0.41 [0.6]	-0.12 [0.1]	0.95 ^c
cumulative number of new gd+ lesions (V0–V6) (–)	2	14	0.09 ^a	na	na	na

EDSS: Expanded Disability Status Scale; gd+: gadolinium enhancing lesions; ITT: intention to treat; MSFC: Multiple Sclerosis Functional Composite; *n*: number; SE: standard error; na: not applicable.

^aexact Mann–Whitney *U* tests; ^bKaplan–Meier analysis, log-rank tests and multiple Cox regression; ^cmultivariate analysis of covariance (MANCOVA) when including baseline values, age, gender and disease duration as covariates in a longitudinal analysis.

Discussion

Upon 18 months of continuous vitamin D intake we did not detect a different effect of high-dose versus low-dose supplementation of MS patients on important clinical and MRI parameters. We also did not record any relevant vitamin D-related adverse events. The study was carefully planned to account for heterogeneity of patients and variability of data (i.e. stratification, monocentric standardized MRI for all patients, standardization of evaluation, separation of treating and evaluating personnel). We chose an actively controlled paradigm because we expected a high incidence of vitamin D deficiency, and it was considered ethically not justifiable to deprive participants of any vitamin D supplementation. In fact, at baseline, the mean serum vitamin D levels were < 20 ng/ml in both arms (Table 1). The higher dose (corresponding to 10,200 IU per day) was chosen empirically under consideration of safety aspects and was higher than the doses given in the majority of previous trials,^{8,9} but was lower than the treatment arm dose (14,007 IU) in the SOLAR trial.¹⁰ The lower dose was derived from recommendations of the German Nutrition Society at the time when the study was designed, which were 200 IU per day. One might speculate that the difference between both doses was too low, or that already the low dose was immunologically active. But the observation of a rather small increase of the mean vitamin D serum level in the low-dose arm (from 18 to 22 ng/ml) in contrast to the pronounced rise in the high-dose arm argues against an insufficient difference between both doses. The rather mild disease activity in the entire cohort, which might be explained by the disease-modifying pretreatment with IFN β and the add-on treatment paradigm to an approved disease-modifying drug, may also have contributed to the negative results. But from an ethical point of view it was considered unacceptable to 'treat' MS patients with active disease solely by vitamin D and to withhold an approved treatment.

Finally, the rather small sample size may well account for the lack of differences in both study arms during therapy with a drug that significantly reduces relapses and MRI activity. The targeted sample size of 80 participants was mainly based on feasibility and did not result from a statistically supported sample size calculation. Nevertheless, unforeseen recruitment difficulties such as the contemporaneous approval of oral MS drugs and a highly comparative environment with a large number of recruiting clinical trials may explain

why, despite a fairly long recruitment period of 45 months, only 53 patients were randomized. A substantial dropout rate further reduced the power to detect primary endpoint changes with $\alpha=0.05$ (two-sided) to 11% (PP population) and 4% (ITT population), respectively. However, these figures provide important information for future trials on vitamin D supplementation in MS. To detect a difference in the T2w lesion count with a power of 80% and a type 1 error (α) of 0.05 (two-sided) 252 participants (PP population) or 613 (ITT population) per arm would be necessary. The corresponding numbers for the hypothetical endpoint T2w lesion volume would be 3147 or 33,202 participants per arm. These power considerations suggest the in principle feasibility to demonstrate (or disprove) a disease-modifying effect of vitamin D supplementation in MS patients, at least for the endpoint T2w lesion count.

Tolerability and safety even of the high-dose were excellent in this study. The number of adverse events was similar in both arms, and adverse events recorded were not considered related to vitamin D intake. Most importantly, we did not observe hypercalcaemia. Thus, the EVIDIMS trial suggests that a mean daily intake of about 10,000 IU cholecalciferol is safe, at least under the premises of the cohort studied, i.e. in patients aged 25 to 62 without any relevant kidney dysfunction and exclusion of certain co-medications.

In conclusion, the results of the EVIDIMS study neither support nor disprove a beneficial effect of vitamin D supplementation in MS. However, the explorative trial results provide valuable information for sample size calculation and feasibility of future confirmatory trials on this topic. The data further support the safety and tolerability of a mean daily dose of 10,200 IU cholecalciferol in patients with MS.

Author Contributions

All authors approved the final version of the manuscript. JD designed the trial, drafted the study protocol, treated patients, generated data, and drafted the manuscript. PBK collected and processed data, performed statistical analyses, and drafted the manuscript. KDW was the responsible biometrician, performed the statistical analyses, and drafted the manuscript. EB, FH, JF, BB, OH and KA were principal investigators of a study site, treated patients and generated data. JW was in charge of acquisition and processing of MRI data. SKP supported the statistical analyses. JBS treated patients, generated data and coordinated as well as supervised the process of data collection. AB coordinated and supervised the process of data collection,

compilation and evaluation and drafted the manuscript. FP designed the trial, drafted the study protocol, generated data, and drafted the manuscript.

Conflict of Interests

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Supplemental Material

Supplemental material for this article is available online.

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5.3 Publication 3- VD_immunological (Project 3)

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RESEARCH ARTICLE

Effect of vitamin D supplementation on N-glycan branching and cellular immunophenotypes in MS

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Abstract

Objective: To investigate the effect of cholecalciferol (vitamin D3) supplementation on peripheral immune cell frequency and N-glycan branching in patients with relapsing-remitting multiple sclerosis (RRMS). **Methods:** Exploratory analysis of high-dose (20 400 IU) and low-dose (400 IU) vitamin D3 supplementation taken every other day of an 18-month randomized controlled clinical trial including 38 RRMS patients on stable immunomodulatory therapy (NCT01440062). We investigated cholecalciferol treatment effects on N-glycan branching using L-PHA stain (*phaseolus vulgaris leucoagglutinin*) at 6 months and frequencies of T-, B-, and NK-cell subpopulations at 12 months with flow cytometry. **Results:** High-dose supplementation did not change CD3+ T cell subsets, CD19+ B cells subsets, and NK cells frequencies, except for CD8+ T regulatory cells, which were reduced in the low-dose arm compared to the high-dose arm at 12 months. High-dose supplementation decreased N-glycan branching on T and NK cells, measured as L-PHA mean fluorescence intensity (MFI). A reduction of N-glycan branching in B cells was not significant. In contrast, low-dose supplementation did not affect N-glycan branching. Changes in N-glycan branching did not correlate with cell frequencies. **Interpretation:** Immunomodulatory effect of vitamin D may involve regulation of N-glycan branching *in vivo*. Vitamin D3 supplementation did at large not affect the frequencies of peripheral immune cells.

Introduction

Multiple sclerosis (MS) is considered a T-cell-mediated disease, but other immune cells have been implicated in its pathology, most notably B and NK cells.^{1–3} Characteristics are a disruption of T, B, and NK regulatory cells^{4–6} with reduced levels of T regulatory cells (Tregs),⁷ and impaired B regulatory cells (Bregs) function.⁸ Genetic and environmental factors contribute to risk, age at onset, and

progression of MS.^{9–11} Among the latter are sunlight exposure and 25-hydroxyvitamin D (25(OH)D).^{9,10,12–17} Epidemiological, retrospective, and a few interventional studies have investigated vitamin D in MS. But larger interventional studies are scarce^{18,19} and are increasingly difficult to conduct as patients self-supplement vitamin D.

Exact mechanisms by which vitamin D influences MS and its clinical efficacy when supplemented remain to be elucidated. Animal studies and *in vitro* assays proposed an

immunomodulatory role of 25(OH)D.²⁰⁻²² *In vitro*, the active metabolite, 1,25(OH)2D3 downregulates CD4+ T cell interleukin-17 (IL-17) while upregulating interleukin-10 (IL-10)²³ production. Furthermore, 1,25(OH)2D3 inhibits antibody production by plasma cells²² and promotes differentiation of CD4+ T cells into immunomodulatory T helper 2 (Th2) cells.²¹ Human *in vivo* studies investigating 25(OH)D in MS have produced varying results regarding its effect on proinflammatory and anti-inflammatory factors.^{24,25} A recent randomized controlled trial in 40 RRMS patients showed a dose-dependent reduction in the proportion of IL-17+ CD4+ T cells.¹⁹

One known effect how vitamin D regulates immune cells is through effects on cell-surface protein glycosylation, that is, by regulation of enzymes of the glycosylation pathway.^{11,26} Cell-surface proteins are co and posttranslationally modified by N-glycosylation, a process that adds sugars to proteins on asparagine (N) residues through N-glycosidic bonds (N-glycans).²⁷⁻²⁹ Complex N-glycans are branched via the addition of N-acetyl-D-glucosamine (GlcNAc) by N-acetylglucosaminyltransferases (Mgat) enzymes in the Golgi apparatus.^{29,30} Four branching enzymes act sequentially with declining efficiency to generate N-glycans with up to four GlcNAc branches, namely Mgat 1, 2, 4, and 5.³¹ Upregulation or downregulation of Mgat 1 activity reduces N-glycan branching by limiting the substrate, uridine diphosphate N-acetylglucosamine, UDP-GlcNAc.¹¹ Interestingly, vitamin D seems to upregulate N-glycan branching, as exposure of activated mouse or human T cells to 1,25(OH)2D3 raises N-glycan branching by increasing Mgat1 mRNA, whereas it lowers N-glycan branching in deficient mice.¹¹ Hence, 1,25(OH)2D3 and UDP-GlcNAc availability determine N-glycan synthesis by Mgat enzymes.^{11,32}

Reduced N-glycan branching lowers T-cell activation threshold, drives proinflammatory Th1 and Th17 differentiation, and inhibits anti-inflammatory Treg responses.^{29,33,34} Conversely, T-cell activation increases N-glycan branching in T cells blasts, which serve as negative feedback to terminate T-cell responses.^{33,35} On the other hand, genetic variations in IL-2R and IL-7R signaling increases the risk of MS by reducing branching on T-cell blasts.^{11,32} In mouse models, reducing N-glycan branching promotes spontaneous autoimmunity including inflammatory demyelination,^{28,29,36} whereas raising branching attenuates immune responses.^{37,38}

Thus, activated or partially activated T cells could hypothetically be suppressed by vitamin D supplementation in MS via increase in N-glycan branching. But human interventional studies investigating the effect of vitamin D supplementation on N-glycan branching have not been performed to date, leaving it unclear if this mechanism is of actual physiological relevance *in vivo* in humans.

In the 'Efficacy of Vitamin D supplementation In Multiple Sclerosis' (EVIDIMS) trial, (NCT01440062) patients with RRMS received either high-dose (20 400 IU) or low-dose (400 IU) cholecalciferol every other day over 18 month.³⁹ Patients were monitored clinically and with brain magnetic resonance imaging (MRI).^{39,40} The primary endpoint of cumulative new T2w hyperintense lesions was missed with no serious adverse event.⁴⁰ A high prevalence of 25(OH)D deficiency was recorded at baseline and this was associated with increased disease activity prior to supplementation.⁴¹ In this exploratory substudy, we investigated the immunomodulatory properties of vitamin D3 on (a) frequencies of immune cells associated with MS pathology and (b) N-glycan branching on immune cells.

Materials and Methods

Subjects

Detailed inclusion/exclusion criteria have been published elsewhere.^{39,40} In brief, patients with RRMS according to the 2005 McDonald criteria,⁴² 30 days of no relapse before study entry, age of 18-65 years, EDSS score between 0.0 and 6.0 were included. Patients were on stable IFN β -1b treatment at least 3 months before study entry. Exclusion criteria were the presence of other autoimmune diseases or immunomodulatory therapy besides IFN β -1b.

Patients were recruited from a single region in north-eastern Germany and were randomized in 1:1 to either high or low-dose arm stratified according to gender and serum 25(OH)D levels (< or \geq 20 ng/mL (50 nM)) at screening.⁴⁰ Patients were examined at baseline, 3, 6, 12, and 18 months, and at each visit blood samples were collected for serum and peripheral blood mononuclear cells (PBMCs).

In this substudy, we included 38 patients (Table 1). Patients were excluded if they violated the study protocol: lost to follow-up, change in MS medication, personal reasons, MRI no longer performable, psychological problems, or if their serum 25(OH)D levels were below 2 \times the baseline mean compared to the reference level at 6 months for the high-dose arm and vice versa for the low-dose arm. Two patients from the high-dose arm were excluded because serum 25(OH)D levels indicated nonresponse or noncompliance according to this rule.⁴⁰ The trial successfully raised 25(OH)D serum levels with a significant increase in the high-dose arm compared to the low-dose arm ($p < 0.001$) (Fig. S1), supporting the validity of this exploratory analysis.

Ethics approval

This study is an ancillary study of the EVIDIMS trial (NCT01440062), a German multicenter, stratified,

Table 1. Baseline Demographics and univariate analysis of clinical parameters at 6 and 12 months.

Variable	Experiment 1 (n = 29)						Experiment 2 (n = 38)					
	Baseline			12 months			Baseline			6 months		
	High dose	Low dose	P value	High dose	Low dose	P value	High dose	Low dose	P value	High dose	Low dose	P value
Male n (%)	6(54.5)	5 (45.4)	0.999 ¹				6 (46.2)	7 (53.8)				
Female n (%)							15 (60.0)	10 (40.0)	0.502 ¹			
Age in years mean (SD)							41.2 (12.1)	43.1 (9.6)	0.527 ²			
Serum 25(OH) mean (SD) ng/mL	20 (11)	17 (7)	0.678 ²	76 (18)	23 (5)	<0.001 ²	18.7(9.7)	16.5 (8.1)	0.428 ²	73.9 (18.4)	23.3 (4.81)	<0.001 ²
EDSS, median [interquartile range]	2 [1.2]	2.5 [2.0]	0.112 ²	2 [1.0]	2.5 [2.4]	0.261 ²	2.3 [0.6]	2.0 [2.0]	0.634 ²	2.5 [1.1]	2.3 [2.1]	0.711 ²

¹Exact χ^2 -test.²Exact Mann–Whitney-test.

randomized, controlled, and double-blind clinical phase II pilot study. The study was approved by the German Federal Institute for Drugs and Medical Devices and the Ethics Committee of the State of Berlin at the Office for Health and Social Affairs. All patients gave written informed consent.

Serum 25(OH)D measurement

Serum 25(OH)D levels were measured by Bioscientia Institute for Medical Diagnostics, GmbH Berlin, Germany, using the LIAISON® Fa. DiaSorin chemiluminescence analyzer (DiaSorin, Dietzenbach, Germany).

Isolation of PBMCs

PBMCs were isolated using 10 mL of Biocoll separating solution (Biochrom GmbH, Darmstadt, Germany) and 10 mL blood by gradient centrifugation. Aliquots of 10 million cells were frozen in liquid nitrogen with cryoprotection until immunological assays were performed.

Investigators were blinded to the different treatment arms during the conduction of the experiments and analysis until after the first preliminary data analyses.

Immunophenotyping for frequencies of effector and regulatory immune cells

We followed the protocol and antibody panel as previously published by the Sys4MS (Systems medicine approach For MS) study.⁴³ The Sys4MS study is a European consortium, which aims to personalize healthcare in MS using a systems medicine approach.

Briefly, three customized single-batch lyotubes (BD, USA) with antibody cocktails were used for identifying, T

effector cells (Teff), Treg, B, and NK effector/regulatory (B/NK) cells.

The B/NK lyotubes contained antibodies for staining CD19, CD3, CD24, CD16, CD38, CD14, CD56, the Treg lyotubes contained antibodies for staining CD3, CD4, CD8, CD28, CD25, CD45RA, CD127, and the Teff lyotubes for staining, CD3, CD4, CD8, CD161, CXCR3, CCRA, and CCR6.

Frozen PBMCs were thawed in a water bath at 37°C and suspended in 2 mL Ca²⁺- and Mg²⁺-free PBS. Cells were washed and resuspended in 5 mL cold FACS buffer (PBS + 1% Fetal calf serum, FCS) and counted. The pellets were resuspended in FACS buffer to 1 million cells/100 μ L.

Lyotubes were rehydrated using 100 μ L of Brilliant stain buffer (BD, Cat. #563794) for 5 minutes at 4°C and 1 million PBMCs added to each lyotube.

PBMCs and antibodies were incubated for 30 minutes at 4°C and washed. Dead cells were identified using 500 μ L of Fixable viable stain, FVS 520 (2.5 μ L in 50 mL PBS) (BD, Cat. #564407) for 10 minutes at 4°C and washed. Cells were acquired same day without fixation using the FACSCanto II with Diva 6.1.3.8 (BD Pharmingen, Franklin Lakes, NJ, USA).

Storage events were set to the immune cell population of interest, 15,000 events were recorded for the Treg tubes and Teff tubes and 5000 for the B/NK tubes.

Flow cytometric analysis for β 1,6 N-glycan expression on immune cells

A cocktail of antibodies for L-PHA-FITC (2 μ g/mL of staining volume) (Vector Laboratories, Cat. #FL-1111), CD45 PerCP (1:100, BioLegend, Cat. #368506), CD3 APC (0.5:100, BioLegend, Cat. #317318), CD4 APC-Cy7 (0.5:100, BioLegend, Cat. #317418), CD8a PB (0.5:100,

BioLegend, Cat. #301033), CD19 BV510 (1:100, BioLegend, Cat. #302242), CD16 APC/Cy7 (0.5:100, BioLegend, Cat. #302018), CD56 PB (0.5:100, BioLegend, Cat. #362520) was prepared in PBS (- Ca+ and - Mg+).

PBMCs (1×10^6 cells) were incubated for 30 minutes at 4°C with the antibody cocktail after Fc blockade (0.25 mg BD, Cat: #564220). Cells were resuspended in 500 μ L FACS buffer and proceeded to acquisition immediately. Cells were stained in duplicates and a total of 20,000 events recorded for each tube. For duplicate acquisitions, the average of the two recorded events was used in the data analysis.

Statistics

Data distribution was checked using the Shapiro–Wilk test. Results for continuous variables are presented as median [interquartile range] and as mean (SD) for non-normally and normally distributed data, respectively. For categorical variables, we present data as absolute numbers and relative frequency (%). Immunology data are presented as percentage fold change. Serum 25(OH)D levels over the entire study period were analyzed using a nonparametric multivariate covariance analysis for longitudinal data with baseline as a covariate in a two-factorial design (1st factor: groups, 2nd factor: time). To compare the changes from baseline to successive visits in the immune cell data, we used a nonparametric analysis of covariance (ANCOVA) for longitudinal data with baseline as a covariate. Univariate comparisons between high- and low-dose arms for particular visits were performed using the nonparametric exact Mann–Whitney test. For experiments with duplicates, the average of the duplicates was used in the analysis. Correlation analyses were performed using Spearman's Rho. Statistical significance was set as $P < 0.05$. All tests should be understood as constituting exploratory data analysis, such that no adjustments for multiple testing have been made. Data were analyzed in R 3.6.0 (2019-04-26) and SAS 9.4 [TS1M3] (SAS Institute Inc., Cary, NC, USA).

Results

Vitamin D3 supplementation and immune cell frequency (Experiment 1)

To investigate the effect of vitamin D3 supplementation on the frequencies of broad, effector/ regulatory T, B, and NK cells, we compared the percentage fold change at 12 months without L-PHA stain. The experimental design is illustrated in (Fig. 1).

In group-wise analyses, high- and low-dose arms did not differ in terms of the frequencies of total CD19+ B cells, B-mature (CD19+CD24lowCD38low), B- memory

(CD19+CD24highCD38low), Breg (CD19+CD24highCD38-high), B-memory atypical (CD19+CD24highCD38-), and B-plasma (CD19+CD24-CD38high), cells at 12 months (Fig. 2B–G). High-dose vitamin D3 did not affect the frequencies of CD56dim cells (CD16+CD56low) and CD56bright cells (CD16+ CD56high) at 12 months (Fig. 2H,I).

Likewise, frequencies of CD3+ T cells, CD3+CD4+ T cells, CD3+CD8+ T cells and naïve Tregs (CD45RA + CD25low) did not differ between high- and low-dose arms at 12 months (Fig. 3B–E). In the low-dose arm, the frequency of CD8+ Tregs (CD8+ CD28-CD127-) was reduced compared to the high-dose arm at 12 months ($P = 0.030$), whereas that of CD4 + Treg (CD4+CD25+CD127-) cells did not differ between arms at 12 months (Fig. 3F–G). The proportions of Teffs; Th17 cells (CD3+CD4+CCR6+CD161+CXCR3-CCR4+), Th1 classic (CD3+CD4+CCR6-CD161-CXCR3+) and Th1 nonclassic (CD3+CD4+CCR6-CD161+CXCR3+) cells were not affected by high- or low-dose supplementation at 12 months (Fig. 4B–D).

In analyses combining both arms, we found no correlations between immune cell frequencies and serum 25 (OH)D levels at 6, 12, or 18 months (data not shown). An association between CD8+CD28-CD127- and serum 25(OH)D concentration was not confirmed in the correlation analysis ($P = 0.166$).

Vitamin D3 supplementation and N-glycosylation (Experiment 2)

We investigated the effect of vitamin D3 supplementation on the intensity of β 1,6 N-glycan expression on broad T, B, and NK cells measured by MFI of L-PHA, at baseline, 6, 12, and 18 months. L-PHA binds to β 1,6 branched N-glycans produced by Mgat5 and serves as an overall measure of N-glycan branching.²⁹ The gating strategy is shown in Figure S2.

First, we tested for correlations between MFI of L-PHA on immune cells and serum 25(OH)D levels at baseline, 6, 12, and 18 months. The time point that showed strong negative correlations of MFI of L-PHA with serum 25 (OH)D levels was 6 months (Fig. S3A–D). No strong correlations were found at baseline, 12 or 18 months (Fig. S3A–D). As a result, we focused our analyses at the 6 months' time point.

In group-wise analyses, comparing high-dose vs. low-dose arms, high-dose vitamin D3 supplementation led to a decrease in the MFI of L-PHA at 6 months on CD3+ ($P = 0.007$), CD4+ ($P = 0.005$), CD8+ ($P = 0.026$) T cells (Fig 5A–C). This decrease lasted up to 18 months compared to the low-dose arm, which did not change significantly over time. MFI of L-PHA on CD19+ B cells also showed a reduction although not significant ($P = 0.178$)

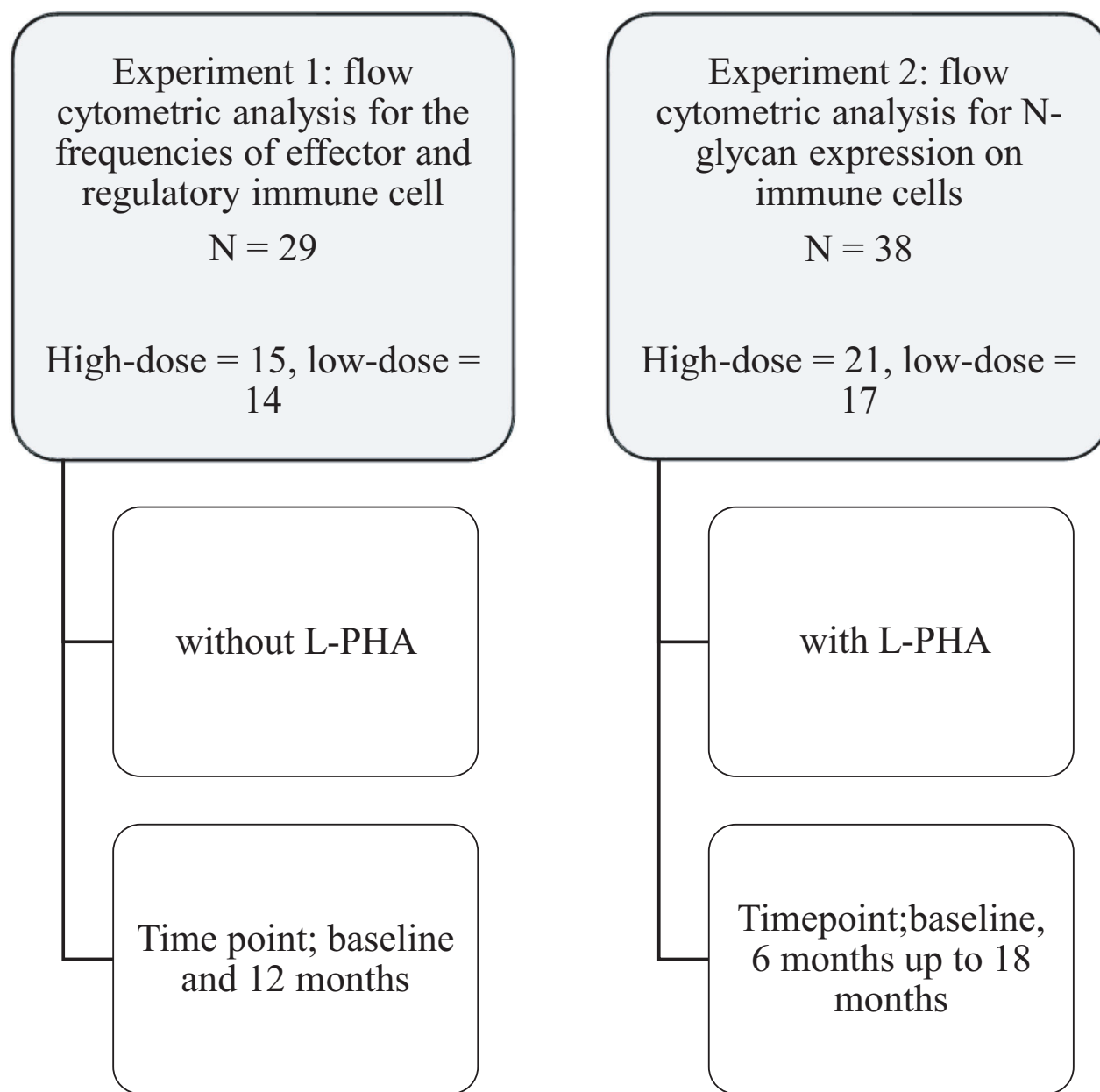


Figure 1. Experimental design flowchart.

(Fig. 5D). In NK cells, CD56dim cells showed reduced branching at 6 months in the high-dose arm ($P = 0.028$) and continuously decreased until 18 months, whereas the low-dose arm remained stable over time. In CD56bright cells, branching was reduced in the high-dose arm at 6 months ($P = 0.020$) and decreased steadily until 18 months. The low-dose arm, on the other hand, continuously increased up to 18 months (Fig. 5E,F).

In correlation analyses combining both arms, we found negative correlations between L-PHA MFI and serum 25 (OH)D levels in CD3+, CD4+, CD8+ T cells, CD19+ B

cells, CD16 + CD56low (CD56dim) and CD16+CD56high (CD56bright) NK cells. This further confirmed reduced N-glycan branching intensity observed in the group-wise analyses (Fig. 6A–F).

N-glycan branching and immune cell frequency

To investigate if the observed effect of vitamin D3 on N-glycan branching was due to differences in immune cell frequency, we tested correlations between cell frequencies

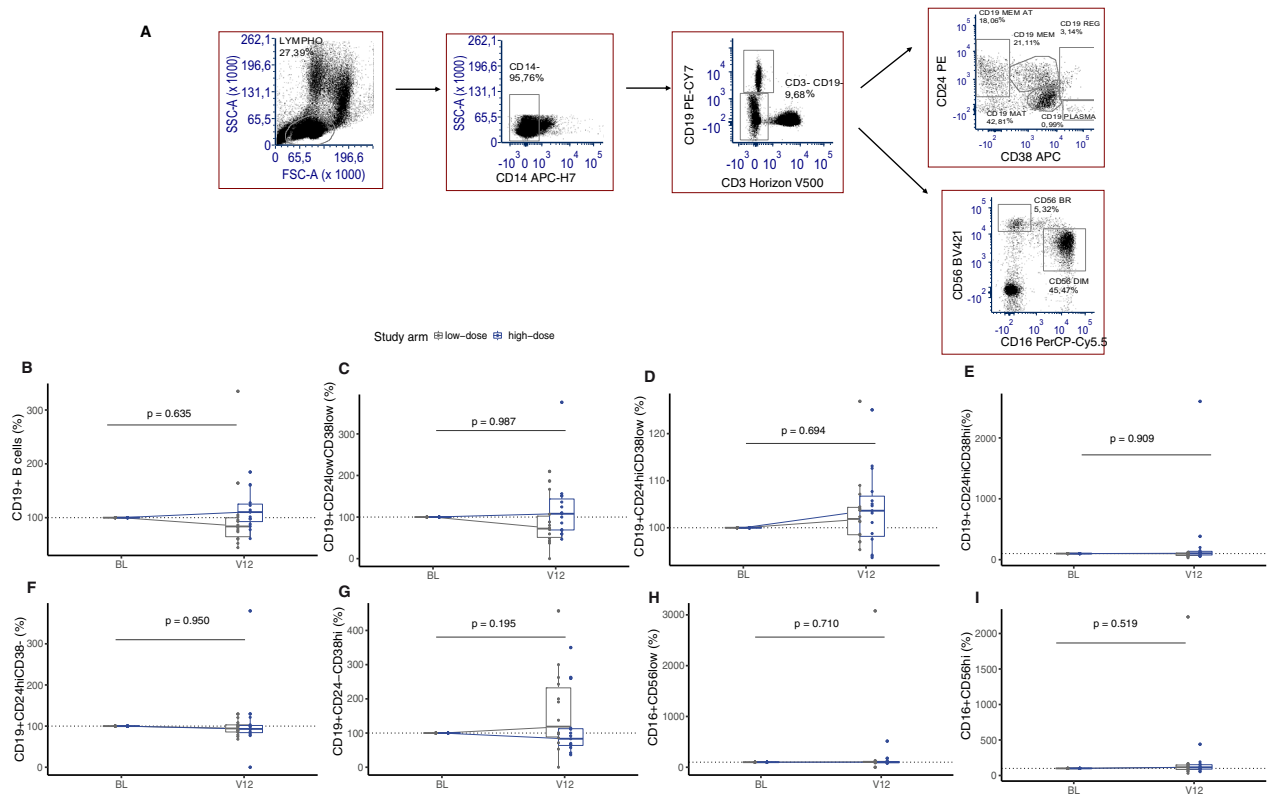


Figure 2. Immune cell frequencies expressed as percentage fold change at 12 months in B and NK cells (B/NK). The figure shows the gating strategy (A) and boxplot of the frequencies of immune cells (percentage fold change) between high-dose (blue) and low-dose (gray) arms in: (B) CD19+ B cells+ (C) CD19+ CD24lowCD38low (B-mature), (D) CD19+CD24highCD38low (B-memory), (E) CD19+CD24highCD38high (B-regulatory), (F) CD19+CD24highCD38- (B-memory atypical), (G) CD19+CD24-CD38high (B-plasma), (H) CD16+CD56low (CD56dim), and (I) CD16+CD56high (CD56bright). Nonparametric analysis of covariate (ANCOVA) with baseline as covariate (n = 29). Abbreviations: BL, baseline; V12, 12 months.

and their corresponding L-PHA MFI. There was no association between cell frequencies and the expression of GlcNAc on CD3+ T cells, CD4+ T cells, CD8+ T cells, CD19+ B cells, CD56dim, and CD56bright NK cells at 6 months (Fig. 7A–F), as well as 12 months or 18 months (data not shown).

Discussion

Vitamin D3 supplementation did not affect the proportions of Th/Teff cells, Beff/Breg cells, and NK cells subpopulations. Specifically, the proportions of Th1 and Th17, which are known to be disturbed in MS remained stable. We observed a reduction in CD8+ Treg proportions in the low-dose arm, which remained unchanged in the high-dose arm. CD8+ Treg is a type of natural Tregs, which lack FoxP3 expression.^{44,45} Similar to CD4+CD25+ Tregs, they induce and maintain peripheral tolerance by regulating harmful and autoreactive T cells.^{46,47} This indicates that high-dose supplementation may support maintaining CD8+ Treg levels. Although this is an interesting

finding, this was not correlated with serum 25(OH)D levels nor with effector T cells raising the likelihood that this exploratory result is false positive.

Vitamin D3 supplementation did not affect naïve Treg proportions (CD45RA+CD25low). This is not surprising as naïve T cells do not express the vitamin D receptor (VDR) and hence respond weakly to the stimulation of the T-cell receptor by vitamin D.⁴⁸ *In vitro* studies have shown that activated naïve CD4+ T cells cannot convert 25(OH)D to 1,25(OH)D3 when cultured in serum or with vitamin D-binding protein (DBP).⁴⁹ In contrast, in DBP-null mice, although 1,25(OH)D3 blood levels were significantly reduced, levels in target tissue were significantly higher compared to wildtype.⁵⁰ This suggests that DBP may have limited impact on distribution, uptake, and biological activity in target tissues.⁵⁰ In comparison, in our study cells were not cultured or prestimulated, hence the effects of DBP or serum on the action of 25(OH)D cannot be directly applied to our results. Moreover, studies that have shown significant effects of vitamin D supplementation in MS, prestimulated immune cells

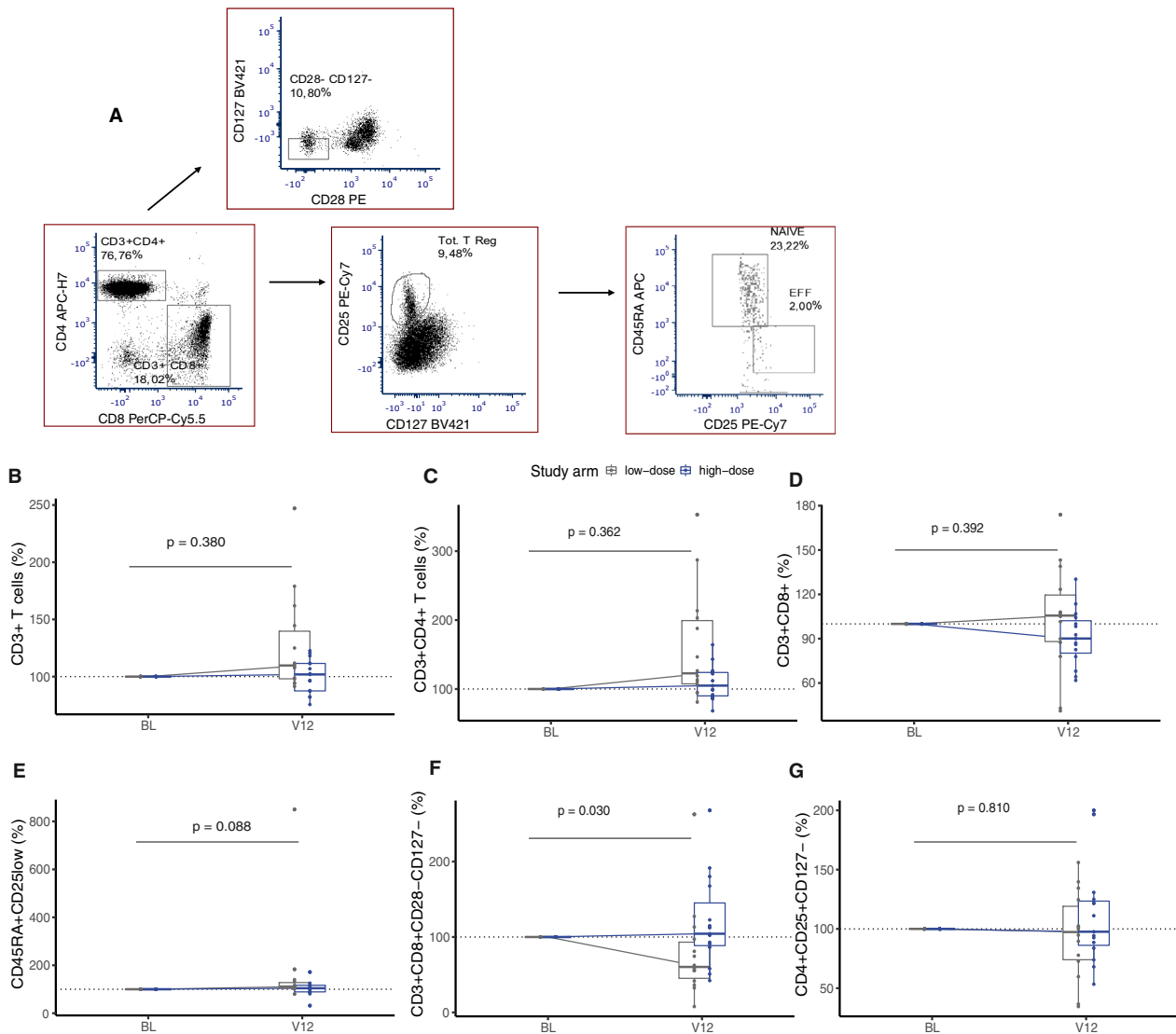


Figure 3. Immune cell frequencies expressed as percentage fold change at 12 months in T regulatory cells (Tregs). The figure shows the gating strategy (A) and boxplot of the frequencies of immune cells (percentage fold change) between high-dose (blue) and low-dose (gray) arms in; (B) CD3+ T cells (C) CD3+CD4+ T cells (T helper), (D) CD3+CD8+ T cells (T cytotoxic), (E) CD45RA+CD25low (naïve T regulatory), (F) CD3+CD8+CD28-CD127- (CD8+ T regulatory), and (G) CD3+CD4+CD25+CD127- (CD4+ T regulatory). Nonparametric analysis of covariate (ANCOVA) with baseline as covariate (n = 29).

before immunostaining.^{19,51} VDR expression on immune cells is upregulated upon activation,⁵²⁻⁵⁴ and this may explain why studies that prestimulated immune cells before vitamin D treatment show varying results compared to those without prestimulation.

A randomized controlled trial with cholecalciferol supplementation showed no difference in the degree of change from baseline in CD4+CD161+ cells, which are markers for Th17 cells in both high- and low-dose arms. However, a significant decrease was observed in CD4+ IL-17+ cells proportions in the high-dose arm.¹⁹

We did not observe a reduction in the CD161+CD4+ T cells fraction with high-dose supplementation as reported by Sotirchos et al.,¹⁹ potentially due to the differences in methodology, time of analysis and supplementation plan. In their, study, patients received additionally 1 000 mg calcium and multivitamins.¹⁹ Calcium is known to enhance both vitamin D absorption and half-life of 25 (OH)D.⁵⁵ While we investigated the effect of vitamin D after 12 months of supplementation, Sotirchos et al., investigated vitamin D effects after 6 months. In a previous trial, supplementation with 2000 to 8000 IU

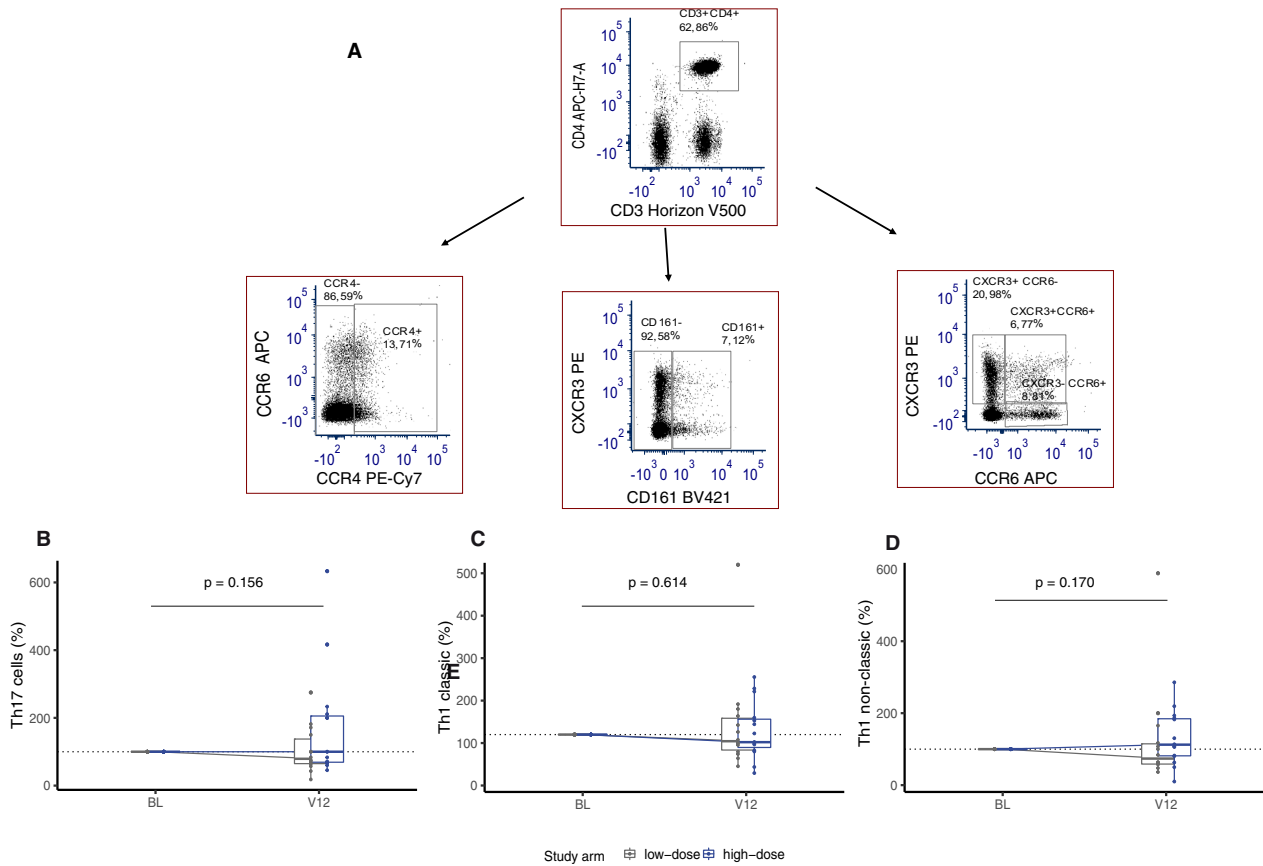


Figure 4. Immune cell frequencies expressed as percentage fold change at 12 months in T effector cells (Teff). Gating strategy (A) and boxplot of immune cell frequencies (percentage fold change) between high-dose (blue) and low-dose (gray) arms in; (B) CD3+CD4+CCR6+CD161+CXCR3-CCR4+ (Th17), (C) CD3+CD4+CCR6-CD161-CXCR3+ (Th1), and (D) CD3+CD4+CCR6-CD161+CXCR3+ (Th1 nonclassic). Nonparametric analysis of covariate (ANCOVA) with baseline as covariate (n = 29).

cholecalciferol daily for 12 weeks reduced frequencies of CD4 + IL-17 + cells in prestimulated peripheral T cells.⁵¹ However, no response was observed on B and T cells in a linear-dose-dependent manner.⁵¹

Other studies also could not detect any association or effect between vitamin D and frequencies of Th17, naïve T cells, or Bregs in MS patients.^{24,56,57} The SOLAR study recently reported on a lack of effect of vitamin D on the proportions of Breg and Tregs or on IL-17 cytokines production between high-dose and placebo arms.⁵⁷ Similarly, in two randomized placebo-controlled trials with high-dose vitamin D supplementation as add-on to IFNβ, the levels of IL-17 cytokines were not different between groups.^{58,59} We recently investigated the impact of treatment on immunophenotypes in different subtypes of 227 MS patients under different disease-modifying therapies (DMT). In this study, we found no changes in frequencies of Th17, Th1, CD4+ Tregs nor Breg/Beff cells in RRMS patients compared to healthy controls. The only DMT that showed differences in lymphocyte

populations was Fingolimod, whereas patients on IFN showed no differences in the frequencies of immunophenotypes.⁴³

The vitamin D response index; the efficiency with which one responds to vitamin D at the molecular level could also explain the different outcomes on the frequencies of immune cells.⁶⁰ Molecular-based (gene expression and chromatin accessibility) analyses revealed three types of responders; high, mid, and low responders, implying that individuals may need different amounts of vitamin D to show biologically relevant response.⁶⁰⁻⁶³

Our study is the first to investigate N-glycan branching in the context of vitamin D supplementation trial.

Ex-vivo cells and animal studies have shown that prestimulated human CD4+ T cells treated with 40 ng/ml (100 nM) 1,25(OH)2D3 enhanced Mgat1 mRNA levels with a concurrent increase in N-glycan branching.¹¹ In EAE mice, reduced dietary supplementation decreased N-glycan branching, whereas injection of 1,25(OH)2D3 inhibited autoimmunity.^{11,32}

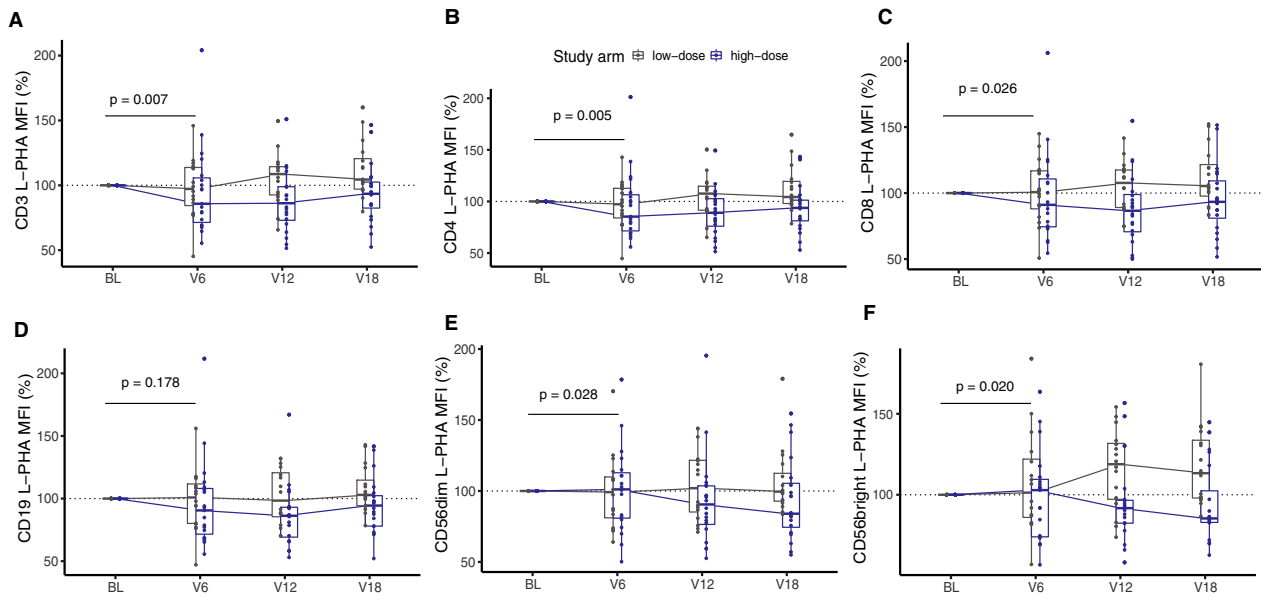


Figure 5. Immune cells L-PHA MFI over different time points. Boxplots of L-PHA MFI on immune cells expressed as percentage fold change in T, B, and NK cells. The change in MFI of L-PHA was compared between baseline and 6 months in the high-dose arm (blue) and the low-dose arm (gray) in (A) CD3+ ($P = 0.007$), (B) CD4+ ($P = 0.005$), (C) CD8+ ($P = 0.026$) T cells, (D) CD19+ B cells ($P = 0.178$), (E) CD56bright ($P = 0.020$), and (F) CD56dim, ($p = 0.028$) NK cells. Nonparametric analysis of covariate (ANCOVA) with baseline as covariate ($n = 38$). Abbreviations: BL, baseline, V6, 6 months, V12, 12 months, V18, 18 months.

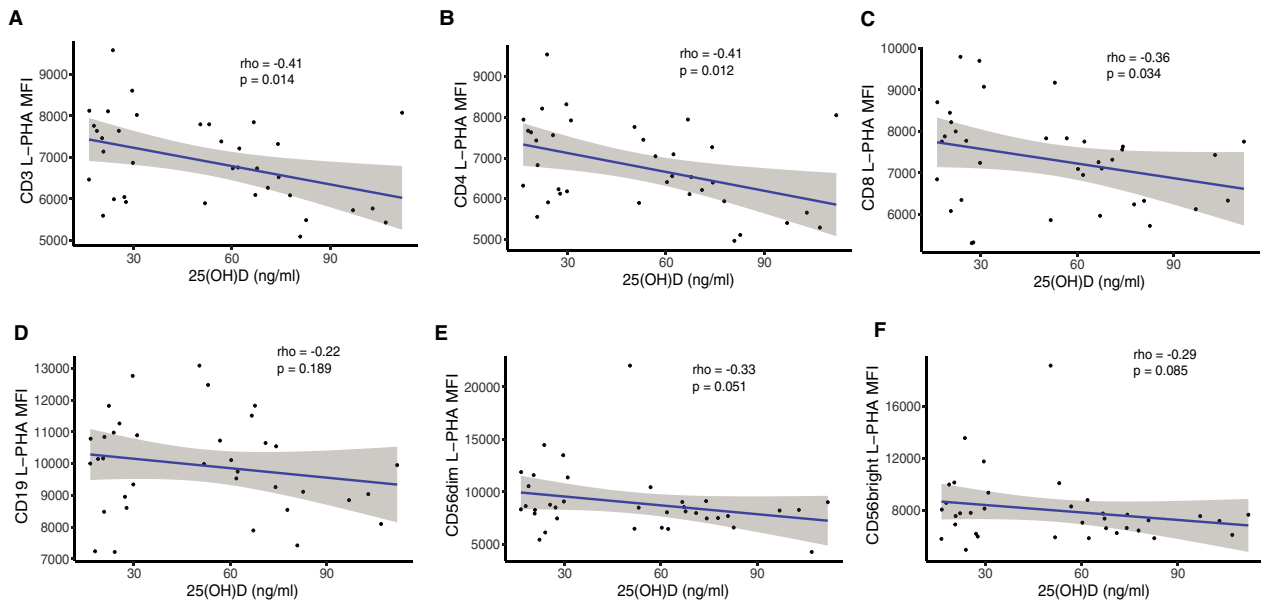


Figure 6. Correlational analyses at 6 months between L-PHA MFI and serum 25(OH)D levels. Association of serum 25(OH)D levels in both arms with the L-PHA MFI of (A) CD3+, (B) CD4+, (C) CD8+ T cells, (D) CD19+ B cells, (E) CD56dim, and (F) CD56bright NK cells. Spearman's Rho ($n = 38$).

On the basis of these data, we expected higher N-glycan branching with high-dose supplementation, however, we found the opposite effect. In *ex vivo* human PBMCs, high-dose oral cholecalciferol intake reduced branching in T cells and NK cells. This effect was not dependent on

immune cell frequencies, indicating that vitamin D3 downregulated branching via Mgat enzymes.

Resting T cells minimally express the VDR compared to activated T cells,⁵² therefore, activated T cells will primarily respond to vitamin D *in vivo*. Indeed, previous studies

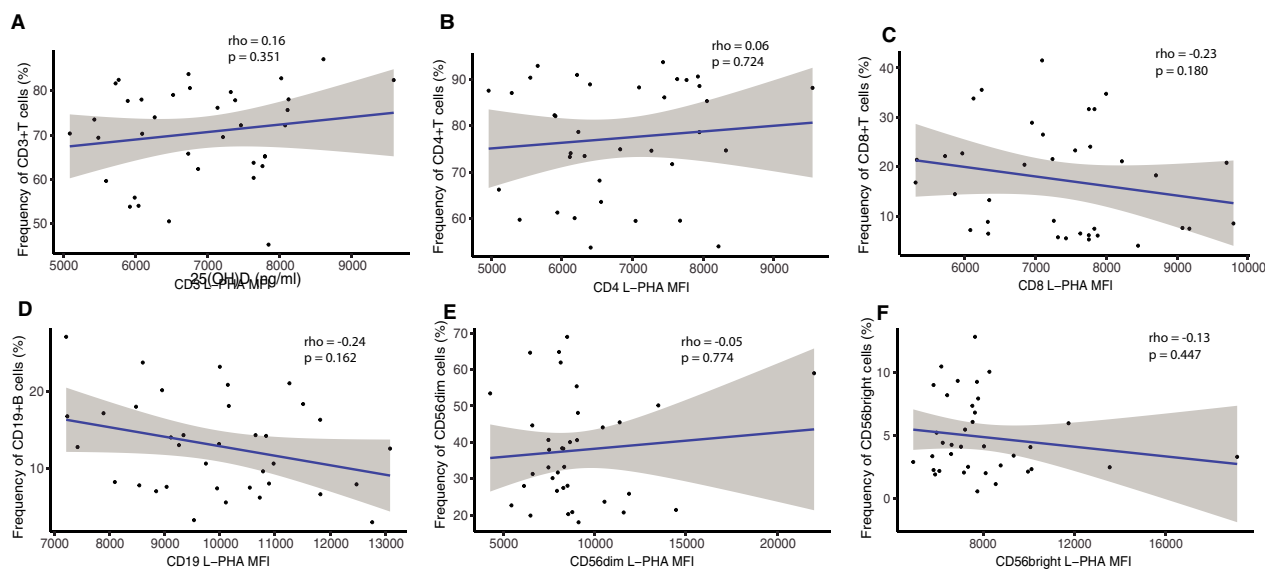


Figure 7. Correlational analyses at 6 months between L-PHA MFI and the corresponding cell frequencies. The plot of L-PHA MFI with the corresponding cell frequencies at 6 months in both arms, (A) CD3+, (B) CD4+, (C) CD8+ T cells, (D) CD19+ B cells, (E) CD56dim, and (F) CD56bright NK cells. Spearman's Rho ($n = 38$).

showed that 1,25(OH)2D3 increased N-glycan branching in preactivated, but not resting human/mouse T cells.^{33,35} As most of the patients at enrolment in our study were 25(OH)D deficient; this might have induced a high activation state within the immune system, which might have already increased branching shortly (days to weeks) after supplementation. Thus, vitamin D3 supplementation over time would be expected to lower branching in the activated cells as they revert to a quiescent resting state, with a net effect of decreased branching observed in our study in the high-dose arm. The effects of vitamin D on N-glycan branching in activated T cells occur over days *in vitro*,¹¹ however, we evaluated changes in branching at 6 months after supplementation. Evaluation at earlier time points would be needed to confirm this hypothesis.

Thus, the effect of vitamin D on N-glycan branching depends on VDR expression, the activation state of cells, Mgat 1 enzymes, substrate availability, and genetic factors.^{11,33,37,52}

Vitamin D3 supplementation in low- and high-dose arms raised serum 25(OH)D levels from deficient to sufficient levels (>20 ng/mL (50 nM)).⁶⁴⁻⁶⁷ This affirms that patients included in this study were able to successfully supplement and metabolize vitamin D. Nonetheless, our study lacks the power to perform further subgroup analyses, that is, regarding high, mid, and low responders. The EVIDIMS study was aborted after the availability of oral drugs and the increasing trend to self-medicate with vitamin D made continuation unfeasible. Another limitation is the restriction of analysis to certain time points due to

limited source material. For the same reason, we could not address differential glycosylation effects on effector/regulatory subpopulations.

In our study, both treatment arms involved patients who were on stable IFN β -1b before study entry, thus eliminating confounding effects due to interactions of IFN β -1b with vitamin D. IFN β is generally known to increase anti-inflammatory factors while reducing pro-inflammatory cytokines.⁶⁸ Vitamin D and IFN β are considered to have synergistic effects in modulating MS.⁶⁹⁻⁷² Combining IFN β with an analogue of 1,25(OH)2D3 prevented autoimmune encephalitis in animal models of MS.⁷³ Additionally, IFN β enhanced the synthesis of 25(OH)D from sun exposure which was associated with reduced relapse risk.⁶⁹

Conclusion

We show in an interventional human study that vitamin D affects N-glycan branching. This is consistent with *ex vivo* and animal studies suggesting that the immunomodulatory effects of vitamin D are associated with regulating N-glycan branching. We did not observe consistent effects of vitamin D supplementation on immune cell frequencies, which is in support of some but not all previous studies.

Declaration

Data used in this study are available from the corresponding author upon reasonable request.

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Conflict of Interest

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Serum 25-hydroxyvitamin D, 25(OH)D levels in the treatment arms over time. Boxplot of serum 25(OH)D levels at each visit in the high-dose (blue) and low-dose (gray) arms. Significant differences between both arms for group differences, time change, and interactions were tested using multivariate nonparametric analyses of longitudinal data $**P < 0.001$. Abbreviations: BL (baseline), V6 (6 months), V12 (12 months), V18 (18 months), (n = 38).

Figure S2. Gating strategy for T, B, and NK cells with L-PHA staining.

Figure S3. Heat map correlation matrixes of the MFI of L-PHA on immune cells with serum 25(OH)D. The figure illustrates the heat map correlation matrixes of the dependence of MFI L-PHA of immune cells on serum 25(OH)D levels. (A) Baseline, (B) 6 months, (C) 12 and (D) 18 months after supplementation. Each box shows the correlation coefficient between the MFI L-PHA of immune cells and serum 25(OH)D. The correlations are indicated by the color intensities from blue and from brown to red. Very strong positive correlations are given as 1, whereas strong negative correlations as -1 . (n = 38).

6.0. Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

My curriculum vitae is not published in the electronic version of my dissertation for data protection reasons.

7.0. Publication list

All publications are peer-reviewed.

Original Publications (first author)

Bäcker-Koduah P, Bellmann-Strobl J, Scheel M, Wuerfel J, Wernecke K-D, Dörr J, Brandt AU and Paul F. Vitamin D and Disease Severity in Multiple Sclerosis-Baseline Data From the Randomized Controlled Trial (EVIDIMS). *Front Neurol.* 2020;11:129. Published 2020 Feb 25. doi:10.3389/fneur.2020.00129

Dörr J*, **Bäcker-Koduah P***, Wernecke KD, Becker E, Hoffmann F, Faiss J, Brockmeier B, Hoffmann O, Anvari K, Wuerfel J, Piper SK, Bellmann-Strobl J, Brandt AU and Paul F. High-dose vitamin D supplementation in multiple sclerosis - results from the randomized EVIDIMS (efficacy of vitamin D supplementation in multiple sclerosis) trial. *Mult Scler J Exp Transl Clin.* 2020;6(1):2055217320903474. Published 2020 Jan 24. doi:10.1177/2055217320903474

Bäcker-Koduah P, Infante-Duarte C, Ivaldi F, Uccelli A, Bellmann-Strobl J, Wernecke K-D, Sy M, Demetriou M, Dörr J, Paul F, Brandt AU. *Effect of Vitamin D on N-glycan branching of MS patients from the randomized controlled EVIDIMS trial.* ACTN. 2020.

Original Publications (coauthor)

Cellerino M, Ivaldi F, Pardini M, Rotta G, Vila G, **Bäcker-Koduah P**, Berge T, Laroni A, Lapucci C, Novi G, Boffa G, Sbragia E, Palmeri S, Campi C, Piana M, Inglese M, Paul F, Harbo H, Villoslada P, Kerlero de Rosbo N* and Uccelli A*: *Impact of treatment on cellular immunophenotype in multiple sclerosis. A cross-sectional study.* *Neurol – Neuroimmunol Neuroinflammation.*2020;7(3):e693. doi:10.1212/NXI.0000000000000693

Oyama N†, Winek K†, **Bäcker-Koduah P**, Zhang T, Dames C, Werich M, Kershaw O, Meisel C, Meisel A and Dirnagl U. Exploratory Investigation of Intestinal Function and Bacterial Translocation After Focal Cerebral Ischemia in the Mouse. *Front Neurol*. 2018 Nov 19;9:937. doi: 10.3389/fneur.2018.00937. eCollection 2018. PubMed PMID: 30510535; PubMed Central PMCID: PMC6254134.

Winek K, Engel O, **Koduah P**, Heimesaat MM, Fischer A, Bereswill S, Dames C, Kershaw O, Gruber AD, Curato C, Oyama N, Meisel C, Meisel A, Dirnagl U. *Depletion of Cultivable Gut Microbiota by Broad-Spectrum Antibiotic Pretreatment Worsens Outcome After Murine Stroke*. *Stroke*. 2016 May;47(5):1354-63. doi:10.1161/STROKEAHA.115.011800. Epub 2016 Apr 7. PubMed PMID: 27056982; PubMedCentral PMCID: PMC4839545.

Review Articles

Koduah P, Paul F, Dörr J. *Vitamin D in the prevention, prediction and treatment of neurodegenerative and neuroinflammatory diseases*. *EPMA J*. 2017 Nov15;8(4):313-325. doi: 10.1007/s13167-017-0120-8. eCollection 2017 Dec. Review. PubMed PMID: 29209434; PubMed Central PMCID: PMC5700019

International Conferences

Bäcker-Koduah P, Infante-Duarte C, Ivaldi F, Uccelli A, Bellmann-Strobl J, Dörr J, Paul F, Brandt AU. *Effect of high-dose and low-dose vitamin D treatment on peripheral blood immune cells: immunological data from the EVIDIMS trial*. Poster ISNI Aug/Sept 2018

Koduah P, Brandt AU, Piper SK, Bellmann-Strobl J, Wuerfel J, Paul F, Dörr J. *Vitamin D supplementation in Multiple Sclerosis: Primary efficacy endpoint and safety of a randomized, controlled, double-blind phase II trial (EVIDIMS)*. ECTRIMS Oct/2018

I Zubizarreta, F. Ivaldi, M. Rinas, E. Hogestol, S. Bos, T. Berge, **P. Koduah**, M. Cellerino, M. Pardini, G. Vila, N. Kerlero de Rosbo, A. Brandt, F. Paul, H. Harbo, J. Saez-Rodriguez, A. Uccelli, P. Villoslada, *The Sys4MS project: personalizing health care in multiple sclerosis using systems medicine tools*. ECTRIMS Online Library. Zubizarreta I. 10/11/18; 228707; P864

Cellerino M, Pardini M, Campi C, Ivaldi F, Vila G, Berge T, **Koduah P**, Rotta G, Paul F, Harbo H, Villoslada P, Kerlero de Rosbo N, Uccelli A, *Personalizing health care in Multiple Sclerosis using systems medicine tools: presentation of the cytomics data from the Sys4MS project. ECTRIMS 2018.*

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