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CLINICAL STUDY

Decreased plasma levels of 25(OH)D in multiple sclerosis patients. Correlation with disease severity expressed by EDSS, MSSS, progression index and Herbert´s scale severity grade

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ABSTRACT
OBJECTIVES: *Multiple sclerosis* is a chronic inflammatory and autoimmune demyelinating disease of the brain and spinal cord. Vitamin D has anti-inflammatory and anti-Th1, Th17 activities, activates the function of regulatory T cells, shifts the immune response towards Th2, so it might be favorable for downregulation of the disease pathogenesis, and if inflammation and Th1 and Th17 immunity are hyperactivated. The aim of our study was to highlight the role of vitamin D in multiple sclerosis pathogenesis.
METHODS: We investigated 178 patients with multiple sclerosis. Plasma levels of 25(OH)D and HMGB1 were investigated.
RESULTS: Despite a regular use of VD by patients, the plasma levels of 25(0H)D were significantly decreased in 57% of them, 14.1% had VD deficiency (level of 25(OH)D < 20 ng/mL) and more than 6 % of patients had VD severe deficiency with the plasma level of 25(OH)D < 12 ng/mL. The level of 25(OH)D negatively correlated with the severity of the disease (EDSS, index of progression, duration of the disease) and negative association was found also with Herbert´s six severity grades. HMGB1 levels were higher in patients ($p < 0.0001$).
CONCLUSION: Our result showed that vitamin D deficiency plays a role in multiple sclerosis pathogenesis. We believe that administration of vitamin D to patients at a sufficient dose providing a physiological level of vitamin D could have a positive effect on the course of the disease. However, regular monitoring of vitamin D levels is required, which should be at least within 30-75 ng/mL, and even more, but below the toxicity limit *(Tab. 6, Ref. 66)*. Text in PDF *www.elis.sk.*
KEY WORDS: HMGB1, multiple sclerosis, MS, inflammation, vitamin D.

Introduction

Multiple sclerosis is a chronic inflammatory and autoimmune demyelinating disease of the brain and spinal cord with multifocal areas of focal lymphocytic infiltrations that lead to myelin sheath destruction, oligodendrocyte, axonal and neuronal damage. Pathological processes occur predominantly in white matter, although grey matter infiltrates are also detected (1–3). This leads to a significant disability with deterioration of the motor, sensible, autonomic and neurocognitive functions (1, 4, 5). Neurological symptoms include: sensory disturbances, optic neuritis, loss of vision, limb weakness, ataxia, bladder dysfunction, cognitive deficits and fatigue (6–9).

The cause of MS that affects more than 2.5 million people worldwide, predominantly young adults between 20–40 year, is multifactorial and still unknown (10). It is undeniable, that besides genetic background also environmental factors, stress, immune abnormalities and other factors play a role in MS development. The higher frequency of MS is seen in women, who are affected twice as often as men (11).

It has been long known, that latitude influences MS risk, the prevalence of the disease is minimal at the equator and increases with North or South latitude (12). MS prevalence may change also after migrations that occurred during the second decade of life, with a beneficial effect for people, who migrated from a high latitude region with a higher MS prevalence to a summer, lower latitude region with low MS prevalence (13, 14). Sloka et al (2011) reported that MS prevalence is inversely correlated to the level of solar radiation (15). It was also documented that people, who spent enough time outdoors during childhood and adolescence or people, who prac-

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ticed many outdoor activities during their youth, and thus received more UVB, have a significantly lower risk later for MS (16–18).

There are two important key pathogenic events in MS development – autoimmune mechanisms and neuroinflammation. The first one arises from the failure or loss of the tolerance and decreased number or function of regulatory T cells. Th1- and Th17 type of lymphocytes with their production of IL-2, IFN-gama / IL-17 or IL-22 respectively, as well as myelin-reactive $CD4^+$ T cells and autoreactive $CD8^+$ cytotoxic T-lymphocytes play an important role in development of MS (19–26).

In the recent years, the attention has been focused on the role of chronic low -grade inflammation, that accompanies myelin degradation and neurodegeneration and the role of pro-inflammatory cytokines has been studied. The process of inflammation in MS that represent the second key pathogenic mechanism in MS development is promoted by several pro-inflammatory cytokines produced by the immune cells themselves and local resident cells like activated microglia (27). Consecutive damaging pathways involve the transmigration of activated B-lymphocytes and plasma cells, which synthesize antibodies against the myelin sheath, boost the immune attack, and result in ultimate loss of myelin (28).

Vitamin D (VD) – a „sunshine“ vitamin was primarily known to regulate the bone calcium-phosphate homeostasis. Later it was found that it plays also a major role in many extra-skeletal metabolic processes, even it can modulate the function of both natural and adaptive immunity.

There are three major immune activities of this vitamin that can have an impact on MS pathogenesis. VD has 1) a direct anti-inflammatory activity; 2) suppresses the Th1 and Th17 differentiation and thus the Th1 and Th17 immunity; on the contrary: 3) VD promotes the activation of regulatory T cells, that also contributes to downregulation of Th1 and Th17 immunity and inflammation (29, 30). So, vitamin D may inhibit all most important pathogenic mechanisms that can potentiate the development of MS.

As we have mentioned above, chronic inflammation plays a great role in MS development and its progression. In our study, we decided to investigate the plasma level of 25(OH)D (25-hydroxyvitamin D), as a factor influencing many immunological mechanisms, alterations of which can potentiate the MS development. Moreover, we tested also the plasma levels of a modern inflammatory marker HMGB1 (high mobility group box 1 protein). It is a vital intranuclear protein with many intranuclear functions. However, after getting to extracellulary milieu it has a strong pro-inflammatory activity and can be passively secreted by necrotic cells or actively produced by activated monocytes, macrophages and also microglia (31). Finally, the aim of our study was to find an association between the evaluated plasma levels of 25(OH)D and MS, severity of MS defined by EDSS (expanded disability status scale) (32), MSSS (multiple sclerosis severity score) (33), PI (progression index) (34) and Herbert's six severity grades H1 – H6 (35).

Subjects and methods

The study was performed at the Institute of Immunology, Faculty of Medicine Comenius University in Bratislava in collaboration with three Slovak specialized neurological centers for multiple sclerosis therapy (two in Bratislava – the 1st Department of Neurology, Faculty of Medicine, Comenius University and University Hospital, the 2nd. Department of Neurology, Faculty of Medicine Comenius University and University Hospital in Bratislava and one in Presov – Centre for Therapy and Diagnosis of Multiple Sclerosis at Faculty Hospital of Jan Adam Raiman in Presov, Slovakia.

The blood samples were obtained during diagnostic procedures from 178 patients into a 9 mL tube with EDTA (ethylendiamine tetra acid) to prevent coagulation. Immediately after that, the blood was centrifuged (3000 rpm for 10 min), subsequently the plasma was sucked off and distributed pro rata into 5 – 8 small Eppendorf tubes and after that the plasmas and buffy coats (for DNA isolation) were frozen in a deep freezer box (–80 °C), where they were stored until analysis. The study was approved by the local Ethics Committee for Research of the Faculty of Medicine Comenius University and University Hospital Bratislava, Slovakia and each patient signed an informed consent.

178 patients were enrolled in the study (125 women (70.22 %) and 53 men (29.78 %). 162 of them had relapsing remitting form of MS (RR-MS), one primary progressive form (PP MS) and 15 patients had secondary progressive form of MS (SP MS). The diagnosis of MS was based on the revised Mc Donald criteria (36). Patients underwent a complex clinical investigation, laboratory and MRI (magnetic resonance imaging) investigation and corresponding physicians from above mentioned specialized MS centers were responsible for exact diagnosis and therapy of MS patients. The degree of neurological disability at the time of examination was quantified using Kurtzke´s extended disability status scale (EDSS) (32). EDSS is used to express the disability of MS patients and MSSS is used to express the severity of the disease using disability and disease duration (33). In addition, a progression index (PI) is used, that indexes progression, calculated as disability grade divided by duration of the disease (34). The latest MS classification system includes a patient's disability scale according to the severity of the course, relative to the MSSS score – Six degrees of disease severity, Herbert's six severity grades: H1-H6 (35).

The plasma level of 25(OH)D was evaluated by electrochemiluminescent binding test (Elecsys Vitamin D total-cobas; Roche Diagnostics GmbH, Mannheim, Germany) in Laboratoria Piestany Ltd., Piestany, Slovakia.

Moreover, in the cohort of 178 MS patients (125 women (70.22 %) and 53 men (29.78 %) the plasma level of HMGB1 (high mobility group box 1) protein was determined by a sandwich enzyme-linked immunosorbent assay (ELISA; Shino-test Corporation, Japan/IBL Technology USA) according to the manufacturer's protocol. Finally, the obtained results concerning 25(OH)D were compared within subgroups of patients and correlation analysis with EDSS, MSSS, PI, H1-H6 and plasma levels of HMGB1 were calculated.

31 healthy subjects (20 women and 11 men) were also enrolled into the study as a control group to test the plasma levels of HMGB1. All of them were healthy, without MS and any other neurological diseases, without autoimmune, allergic or oncological diseases and without any acute or chronic sickness.

Statistical analysis

The INSTAT 3 program (GraphPad Software Inc., San Diego, CA, USA) was used to statistically evaluate the measured parameters. The one-sample Kolmogorov–Smirnov test was used to determine whether the investigated population followed a normal distribution. Mean and medians were determined by an unpaired t-test with Welch correction, or non-parametric Mann–Whitney test. One-way ANOVA and Kruskal–Wallis test were used when comparing the median of three or more variable values. For correlation analysis, the Pearson's test (linear relationships), or non-parametric Spearman´s test were used. A significant difference was assumed if p value was < 0.05.

Results

Characteristics of baseline demographic and clinical data of investigated patients

We investigated 178 MS patients, the most represented form was the relapsing-remitting form of MS. The duration of the disease was higher in women (11.1±6.6; p = 0.03) than in men (7.9±6.59). The EDSS score was significantly higher in women with MS (EDSS: 3.10±1.29 *vs* 2.50±1.28, p = 0.0364). The MSSS score did not differ. The progression index was higher in men (p = 0.04932), with a shorter, but more severe duration of the disease (p = 0.03) (Tab. 1).

25(OH)D sufficiency, insufficiency, deficiency and severe deficiency in MS patients. Comparison of plasma level of 25(OH)D vitamin in men and women

Despite a regular use of VD by MS patients, only approximately 43 % of all investigated patients were VD sufficient (the plasma level of 25(OH)D ≥ 30 ng/mL), 57 % had decreased plasma level of 25(OH) vitamin D – 36.7 % had VD insufficiency (level of 25(OH)D between 20–29 ng/mL), 14.1 % had VD deficiency (level of 25(OH)D < 20 ng/mL) and more than 6 % of MS patients had VD severe deficiency with the plasma level of 25(OH)D < 12 ng/mL (Tab. 2). Men had higher levels of 25(OH)D than women, however, the difference was not statistically significant. We did not find the difference neither between individual forms of MS (Tab. 3), probably also due to uneven representation of the tested subgroups. The most represented form was the RR MS (162 patients), the others were less represented, which could affect the result.

Comparison of plasma level of 25(OH)D with disease severity according to Herbert's six severity grades H1-H6 in MS patients

The Kruskall–Wallis test revealed a statistically significant negative association between the plasma level of 25(OH)D and disease severity according to Herbert's six severity grades (p = 0.0446) (Tab. 4). Patients with the most severe disease grades (H5 and H6) had significantly lower levels of vitamin D than the patients with H1-H4 (p = 0.0032). The level of 25(OH) D was

Tab. 1. Characteristics of baseline demographic and clinical data of investigated MS patients.

Parameter	MS patients (n=178)	MS women (n=125)	MS men (n=53)
Age (mean±SD; years)	40.6±9.7	41.6±9.8	38.1±9.7
Sex, women/men	125/53	–	–
Age of disease onset (mean±SD; years)	28.3±8.5	27.7±8.6	27.3±8.5
MS form (RR/PP/SP)	162/1/15	114/1/10	48/0/5
Disease duration (mean±SD; years)	10.5±6.6	**11.1±6.6**	**7.90±6.59**
p (unpaired t-test)		**p = 0.03**	
EDSS (mean±SD)	2.94±1.28	**3.10±1.29**	**2.50±1.28**
p (unpaired t-test)		**p=0.0364**	
MSSS (mean±SD)	3.89±1.94	3.92±1.96	3.80±1.95
PI (mean±SD)	0.43±0.46	**0.40±0.46**	**0.52±0.46**
p (unpaired t-test)		**p=0.04932**	

MS – *multiple sclerosis*; RR – relapsing remitting form; PP – primary progressive form; SP – secondary progressive form; EDSS – expanded disability status score; MSSS – *multiple sclerosis* severity score; PI – progression index

Tab. 2. 25(OH)D sufficiency, insufficiency, deficiency and severe deficiency in MS patients.

	All MS patients	RR MS (n=162)	SP (n=15)	PP (n=1)
VD severe deficiency	6.2%	6.8%	0%	
VD deficiency	14.2%	13.6%	20.0%	
VD insufficiency	36.7%	37.6%	26.7%	
VD sufficiency	42.9%	42,0%	53.3%	100.0%

MS – *multiple sclerosis*; RR – relapsing remitting form of MS; SP – secondary progressive form; PP – primary progressive form; VD – 25(OH) vitamin D; VD severe deficiency – the plasma level of 25(OH)D < 12 ng/mL; VD deficiency – the plasma level of 25(OH)D <20 ng/mL; VD insufficiency – the plasma level of 25(OH)D between 20–29 ng/mL; VD sufficiency – the plasma level of 25(OH)D ≥ 30 ng/mL

Tab. 3. Comparison of plasma level of 25(OH)D in women and men.

	n	25(OH)D (Mean±SD) (ng/ml)	25(OH)D Median (ng/ml)	Unpaired t test Welch corrected p
Women	125	23.33±11.34	23.98	p=0.1222
Men	53	26.17±11.04	25.17	
RR MS	162	24.35±11.23	24.42	
PP MS	1	25.48	–	p=0.5322
SP MS	15	22.21±12.55	22.36	

N – number of patients; MS – multiple sclerosis; RR – relapsing remitting form of multiple sclerosis; PP – primary progressive form; SP – secondary progressive form

Tab. 4. Comparison of plasma level of 25(OH)D with disease severity in MS patients.

	n	25(OH)D Mean±SD (ng/ml)	25(OH)D Median (ng/ml)	p
H1	27	25.32±10.65	24.42	Kruskal-Wallis test p=0.0446
H2	50	23.71±9.53	23.71	
H3	56	26.58±12.60	26.23	
H4	28	23.97±11.07	23.17	
H5	14	17.28±10.55	17.21	
H6	3	11.02±8.65	7.90	
H1-H3	133	25.24±11.12	25.17	Unpaired t test p=0.0258
H4-H6	45	21.02±11.33	20.79	
H1-H4 vs. H5-H6	178	25.02±11.09 16.17±10.29	24.70 *vs.* 15.98	Unpaired t test p=0.0032

n - number of patients, H1-H6 - disease severity grades in MS patients

Tab. 5. Correlation of serum vitamin D level with MSSS and PI.

	Number of patients	25(OH) D Median (ng/ml)	Correlation analysis p
MSSS	178	24.24	Pearson´s test p=0.0292
PI	178		Spearman´s test p=0.0144

MSSS – *multiple sclerosis* severity score, PI – progression index

the lowest in two most aggressive disease groups (H5, H6), with the highest levels of vitamin D in the group of patients with mild disease. Subsequently, we compared the two groups of patients H1-H3 vs H4-H6 (p = 0.0258).

Correlation of 25(OH)D levels with MSSS and PI

The levels of 25(OH)D significantly negatively correlated also with disease progression – lower levels of vitamin D3 were associated with more serious course of the disease – higher MSSS scores (p = 0.0292) and progression index (p = 0.0144 sign) (Tab. 5).

Plasma levels of HMGB1 in patients with MS, men and women, different forms of MS and healthy controls

Plasma levels of HMGB1 in MS patients (median: 13,529 ng/mL; 2.330–113.45) were statistically significantly higher than in the healthy subjects (median: 2.999 ng/mL; 1.686–9.844; p < 0.0001) (Tab. 6). The values in MS patients were highly dispersed, whereas in the control group of healthy subjects the HMGB1 values were all low. Women had slightly higher levels of HMGB1 (median: 15.098 ng/mL) than men (median: 12.571 ng/mL), but the difference was not statistically significant.

Correlation of plasma levels of 25(OH)D with level of HMGB1

We did not find a significant correlation between the level of 25(OH)D and late proinflammatory cytokine HMGB1 in the plasma of MS patients (Spearman´s test: p = 0.3483).

Discussion

Vitamin D – the „sunshine“ vitamin, was primarily known as a micronutrient regulating bone calcium-phosphate homeostasis. Later it was found that it is a neurohormone that plays also a major role in myriad extra-skeletal metabolic processes, such as glucose metabolism, and in many aspects of cellular functions and imunomodulation (37–40). It has a direct effect on the function of both innate and adaptive immunity via VD receptor expressed on several immune cells (41–44). VD inhibits the maturation and function of DC, downregulates the presentation of antigen and development of adaptive immune response (45, 46). 1,25(OH)2D increases the number of Tregs and directly affects $CD4^+$ T cells (47–50).

Vitamin D deficiency is associated with various disorders such as: autoimmune diseases, infections, cardiovascular diseases, asthma bronchiale, chronic pulmonary diseases, severity and mortality of sepsis, and excess mortality in the general population (30, 51–55).

MS is the most common chronic autoimmune neuroinflammatory demyelinating disease affecting central nervous system (CNS) of young adults worldwide characterized by demyelination and axonal damage in CNS and spinal cord (1–3). Except inflammation, the loss of tolerance and increased Th1/Th17 immune response and downregulated regulatory T cells play a role in MS pathogenesis. Chronic inflammatory processes that continuously disturb neuroaxonal homeostasis drive neurodegeneration, so the clinical outcome probably depends on the balance of stressor load (inflammation) and any remaining capacity for neuronal self-protection (56).

There are three major immune activities of vitamin D, that have an impact on MS pathogenesis: suppression of inflammation, suppression of Th1 and Th17 immunity and activation of regulatory T cells, that also contributes to downregulation of Th1 and Th17 immunity and inflammation (29, 30). Downregulating Th1 immunity and increasing the activity of regulatory T cells, VD participates also in the shifting of T helper (Th) cell response from Th1 (adaptive cell mediated immunity accompanied by inflammation) to Th2 (adaptive humoral immunity). The production of Th1 cytokine IFN-γ together with proinflammatory cytokines is inhibited, the production of Th2 cytokines IL-4, IL-5 and IL-10 is elevated, which leads to the limitation of the potential tissue damage associated with excessive Th1 cellular immune responses associated with hyperinflammation (57, 58). VD has also been proven to promote self-tolerance (38, 59, 60). While mechanisms of adaptive Th1/Th17 immunity are depressed, mechanisms of natural immunity are potentiated (29, 61, 62).

Vitamin D has anti-inflammatory and anti-Th1, -Th17 activities, activates the function of regulatory T cells, shifts the immune response towards Th2, so it might be favorable for downregulation of MS pathogenesis, where inflammation and Th1 and Th17 immunity are hyperactivated.

Tab. 6. Plasma levels of HMGB1 in patients with MS and healthy controls.

STUDY GROUP	Number of patients	Median (HMGB1) (ng/ml)	Confidential interval	Mann-Whitney test (2-tailed) p
Controls	31	**2.999**	1.686-9.844	
MS (all patients)	165	**13.529**	2.330-113.45	**p<0.0001**
MS women	111 (67.27%)	15.098	2.330-113.45	p>0.05
MS men	54 (32.73%)	12.571	2.557-99.725	
RR MS	159	15.351	2.330-113.45	p=0.6872
SP MS	6	13.935	3.255-65.608	p>0.05

HMGB1 – high mobility group box 1; MS – *multiple sclerosis*; RR – relapsing remitting form of multiple sclerosis; SP – secondary progressive form

25(OH)D is the major circulating form of vitamin D, that has a half-life of approximately 2–3 weeks. It is a summation of both VD produced from sun exposure and VD intake and it is just that vitamin D metabolite used to determine whether a patient is vitamin D deficient, sufficient or intoxicated (63). There is no absolute consensus about normal range for 25(OH)D, but most experts now agree that VD deficiency should be defined as a 25(OH)D level of < 20 ng/mL (50 nmol/L). VD insufficiency is classified as a serum 25(OH)D level between 20 and 29 ng/mL (50–74 nmol/L). The preferred level for 25(OH)D is now recommended to be > 30 ng/mL (75 nmol/L) (63–65).

The aim of our study was to find association of VD plasma levels with MS and MS severity expressed by EDSS score, MSSS, PI and Herbert´s severity scale H1-H6 and proinflammatory cytokine HMGB1. In our study, we investigated 178 MS patients, in which the most represented form was the relapsing-remitting form of MS (162), which also reflects the real representation in the population. The duration of the disease was higher in women than in men ($p = 0.03$), the EDSS score was significantly higher in women ($p = 0.0364$), the progression index was higher in men ($p = 0.04932$), with a shorter, but more severe duration of the disease ($p = 0.03$) (Tab. 1).

Despite a regular use of VD by MS patients, only approximately 43 % of all our investigated patients were VD sufficient (the plasma level of 25(OH)D ≥ 30 ng/mL), 57 % had a decreased plasma level of 25(OH)D; 36.7 % had VD insufficiency (level of 25(OH)D between 20–29 ng/mL), 14.1 % had VD deficiency (level of 25(OH)D < 20 ng/mL) and more than 6 % of MS patients had VD severe deficiency with the plasma level of 25(OH) D < 12 ng/mL (Tab. 2).

A statistically significant negative association between the plasma level of 25(OH)D and disease severity expressed according to Herbert's six severity grades H1–H6 was found in MS patients ($p = 0.0446$) (Tab. 4). Patients with the most severe disease levels (H5 and H6) had significantly lower levels of vitamin D compared to patients with H1-H4 ($p = 0.0032$). The level of 25(OH) D was the lowest in two most aggressive disease groups (H5, H6), with the highest levels in the group of patients with mild disease. The levels of 25(OH)D significantly negatively correlated also with a disease progression – lower levels of vitamin D3 were associated with a more serious course of the disease – higher MSSS scores ($p = 0.0292$) and progression index ($p = 0.0144$) (Tab. 5). We believe that administration of vitamin D to patients with MS at a sufficient dose providing a physiological level of vitamin D could have a positive effect on the course of the disease. However, regular monitoring of vitamin D levels is required, which should be at least within 30–75 ng/mL, and even more, but below the toxicity limit.

The recent systematic review of Berezowska et al (2019) also supports our results and opinion. They selected ten studies, one trial found a significant effect on EDSS score, three demonstrated a significant change in serum cytokines level, one found benefits to current enhancing lesions and three studies evaluating the safety and tolerability of vitamin D reported no serious adverse events. Disease measures improved to a greater extent overall in those with lower baseline serum 25(OH)D levels (66).

Plasma levels of the late proinflammatory cytokine HMGB1 in MS patients were statistically significantly higher than in healthy subjects ($p < 0.0001$) (Tab. 6) and confirmed ongoing inflammatory process. Our results concerning higher HMGB1 levels in MS patients as compared to healthy controls were demonstrated also by other authors, but they were not correlated with the level of 25(OH)D.

Conclusion

Vitamin D has anti-inflammatory and anti-Th1, Th17 activities, activates the function of regulatory T cells, shifts the immune response towards Th2, so it might be favorable for downregulation of MS pathogenesis, where they are hyperactivated. Our results showed elevated levels of proinflammatory cytokine HMGB1, which points to an ongoing inflammation. Despite a regular use of VD by MS patients, the plasma levels of 25(OH)D were significantly decreased in 57 % of them, 14.1 % had VD deficiency (level of 25(OH)D < 20 ng/mL) and more than 6 % of MS patients had VD severe deficiency with the plasma level of 25(OH) D < 12 ng/mL. The level of 25(OH)D negatively correlated with severity of the disease expressed as EDSS, index of progression, duration of the disease, and negatively associated with a disease the severity expressed according to Herbert's six severity grades. We believe that the administration of vitamin D to patients with MS at a sufficient dose providing a physiological level of vitamin D could have a positive effect on the course of the disease. However, regular monitoring of vitamin D levels is required, which should be at least within 30–75 ng/mL, and even more, but below the toxicity limit.

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