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A longitudinal study of serum 25-hydroxyvitamin D and intact PTH levels indicate the importance of vitamin D and calcium homeostasis regulation in multiple sclerosis

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Abbreviations: [25(OH)D], 25-hydroxyvitamin D or calcidiol; iPTH, intact parathyroid hormone; IFNB-1a, interferon-beta-1a; MS, multiple sclerosis; TSH, thyroid stimulating hormone

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ABSTRACT — Background: Past sun exposure and vitamin D3 supplementation have been associated with a reduced risk of multiple sclerosis (MS). There are no previous longitudinal studies of vitamin D in MS. **Objectives:** To compare regulation of vitamin D and calcium homeostasis between MS patients and healthy controls. To study correlation of parameters of vitamin D metabolism with MS activity. **Methods:** We measured 25-hydroxyvitamin D, intact PTH, calcium, phosphate, magnesium, chloride, alkaline phosphatase, albumin and TSH in serum every three months and at the time of relapses during one year in 23 MS patients and in 23 healthy controls. MRI BOD and T2 activity was assessed every 6 months. **Results:** Vitamin D deficiency [S-25(OH)D ≤ 37 nmol/L] was common affecting half of the patients and controls at some time of the year. Seasonal variation of 25(OH)D was similar in the patients and in the controls, but the 25(OH)D serum levels were lower and the iPTH serum levels were higher during MS relapses than in remission. All 21 relapses during the study occurred at serum iPTH > 20 ng/L (2.2 pmol/L), whereas 38% of patients in remission had iPTH ≤ 20 ng/L. MS patients had a relative hypocalcaemia and a blunted PTH response in the winter. There was no correlation between serum 25(OH)D and MRI parameters**. Conclusions:** The endocrine circuitry regulating serum calcium may be altered in MS. There is an inverse relationship between serum vitamin D level and MS clinical activity. The role of vitamin D in MS must be explored further.

INTRODUCTION

Multiple sclerosis is generally believed to be an immune-mediated disorder that occurs in genetically susceptible people.[1] The reasons for the variation in the prevalence and incidence of multiple sclerosis worldwide are not understood, but genetic and environmental explanations have been offered.[1] In addition to its role in calcium and bone metabolism, vitamin D regulates cell proliferation and differentiation and can regulate immune responses. Receptors for vitamin D are expressed widely on the cells of the immune system. Diminished capacity of vitamin D to regulate immune responses as a consequence of lower serum concentrations of 25-hydroxyvitamin D [25(OH)D] during the winter in tempered climates could be one environmental explanation for the peculiar geographical distribution of MS.[2,3] A growing body of evidence is supporting this hypothesis. Intake of vitamin D is associated with a lower incidence of MS.[4] The risk on MS is reduced in association with past exposure to sun.[5] Moreover, a recent retrospective case-control study showed that the risk of MS is decreasing with increasing serum vitamin D levels, most notably in those with highest vitamin D levels (≥100 nmol/L) .[6] In an earlier cross-sectional study we showed that newly diagnosed MS patients have lower serum levels of 25(OH)D during MS relapses than in remission.[7] There are no previous longitudinal studies on serum 25(OH)D levels in relapsing-remitting MS.

The first hypothesis of this study was that serum concentrations of 25(OH)D would decrease during periods of increased disease activity. Therefore we measured the serum concentrations of 25(OH)D serially every three months and at the time of relapses in 23 patients during the first year of the PRISMS Study with subcutaneous interferon-beta-1a (IFNB-1a) in MS.[8] Serum 25(OH)D levels were compared with the clinical and MRI disease activity and with the therapy received. The second hypothesis was that MS patients have vitamin D insufficiency in comparison with healthy persons. Intact parathyroid hormone (iPTH) is a functional indicator of vitamin D sufficiency and has been used in several studies to define vitamin D deficiency more accurately than measurement of 25(OH)D.[9,10] Therefore, we included measurement of iPTH, as well as an exploratory analysis of other clinical chemistry parameters closely related to vitamin D metabolism, namely serum levels of calcium, phosphate, magnesium, chloride, alkaline phosphatase and albumin as well as thyroid stimulating hormone (TSH), in the MS patients and in age-and sex-matched healthy controls in our study.

METHODS

Patients

In the PRISMS Study, the patients were randomly assigned to receive subcutaneous recombinant IFNB-1a (Rebif®) 22 μg, or 44 μg, or, placebo three times a week for 2 years.[8] In the PRISMS-4 study, patients initially receiving placebo were randomized to IFNB-1a, 22 or 44 μg, others continued with their original dose.[11] In the present study, 23 Finnish patients, 17 women and 6 men, from Turku University Hospital district (60.13º Northern and 22.19º Eastern latitude) participating in the PRISMS and PRISMS-4 studies were included. Ethical approval was obtained from the Commission of Ethics of the Turku University and the Turku University Central Hospital (§ 34, 1.3.1994 and Appendix 1 § 191, 1.11.1994 and Appendix 1 § 62, 16.3.2004). All patients gave written informed consent. MRI was assessed every 6 months. The MRI parameters studied were burden of disease (BOD [mm^2]) and T2 activity (mean active new and/or enlarging

lesions/scan).[10] The patients had neurological examinations every 3 to 6 months and within 7 days of relapse. Morning blood samples were collected within 2 weeks before and 4 weeks after exacerbation onset were determined as "relapse" and others as "remission" samples. Three out of the 21 relapses were treated with iv methylprednisolone.

At the PRISMS study baseline, the mean±SEM age of the MS patients was 34.1±1.5 years and the mean expanded disability status scale (EDSS) was 2.3 (range 0-5). Time from the diagnosis of definite MS ranged from 6 months to 15 years (mean 5.6 years). The mean number of relapses during the preceding 2 years was 2.6 (range 2-6). Eight patients were treated with placebo, seven with Rebif 22 μg and eight with Rebif 44 μg. Blood was collected at PRISMS study baseline and at weeks 12, 26, 36, 52 and 64 as well as within 7 days of the onset of a relapse. Serum was separated rapidly from red cells by centrifuging at 1000xg for 10 min after clotting and stored frozen in aliquots of 1 ml at -40ºC until the analyses.

Controls

Morning blood samples from apparently healthy laboratory personal living in the same area as the MS-patients (60.13º Northern and 22.19º Eastern latitude) were collected at the same months of the year as samples from the MS patients and were similarly processed and stored until the analyses.[12] The mean±SEM age of the controls was 32.0±2.2 years and 17 were women and 6 were men.

25(OH) D analysis

The serum samples were stored at -40ºC and protected from direct exposure to sunlight until the analysis. For the quantitative determination of 25(OH)D in the serum samples, a commercially available 25-hydroxyvitamin D ^{125}I RIA Kit (DiaSorin Catalog No. 68100E, Stillwater, Minnesota, USA) was used according to the instructions provided by the manufacturer. All the determinations were performed in the Turku University Hospital Central Laboratory. Two quality control samples were included in each assay series and the specimens and controls were assayed in duplicates. Using this method, values less than 20 nmol/L (8 mcg/L) indicate severe hypovitaminosis D, 20-37 nmol/L (8-15 mcg/L) moderate hypovitaminosis D and levels above 37 nmol/L (15 mcg/L) adequate vitamin D stores.[9]

Intact parathyroid hormone (iPTH) analysis

The biologically active form of PTH, intact PTH, was measured from the serum samples using Elecsys® immunochemiluminometric assay and Modular E 170 analyzer (Roche, Mannheim, Germany). The reference values for iPTH using this assay method are 15 ng/L (1.7 pmol/L) lower limit and 65 ng/L (7.2 pmol/L) upper limit.

Analysis of albumin, calcium, magnesium, alkaline phosphatase, chloride, phosphate and thyroid stimulating hormone (TSH)

Analyses of serum albumin, calcium, magnesium, alkaline phosphatase, chloride and phosphate were performed with a Modular P800 analyzer and TSH analysis with a Modular E170 analyzer both from Roche Ltd (Mannhein, Germany). The laboratory reference values for each parameter are expressed in Table 1.

All the analyses were performed in the Turku University Hospital Central Laboratory according to the Finnish Accreditation Service (FINAS) standard EN ISO/IEC 17025.

Statistical analyses

Longitudinal data were analyzed using analysis of variance for repeated measurements (the mixed procedure ANOVA) using a confidence interval of 95%. Due to positively skewed distributions, 25(OH)D, PTH and TSH values were log-transformed for statistical analysis. Student's t-test was used for comparison between relapse and remission samples. Pearson analysis was used for testing correlation. SAS System for Windows (version 9.1, SAS Institute Inc., Cary, NC) was used for the analyses.

RESULTS

Comparison of serum levels of 25(OH)D and iPTH between MS patients and healthy controls

To examine the hypothesis that MS patients have vitamin D insufficiency in comparison with healthy persons, we examined the differences in summer, autumn, winter and spring 25(OH)D and iPTH serum levels between MS patients and healthy controls. Interaction between time and group was not significant in a variance analysis of serum 25(OH)D between MS patients and controls (p= 0.1507). Figure 1 panel A shows that seasonal variation of the serum 25(OH)D levels was almost identical in the MS patients and in the healthy controls with lowest levels in then winter and spring (December to May) and highest in the summer (June to August). The mean±SD level of serum 25(OH)D during the whole year in MS patients was 57.6±20.5 nmol/L (23.0±8.2 mcg/L) and in healthy controls 55.3±22.4 nmol/L (22.1±8.9 mcg/L, p-value for overall difference between groups 0.81, not significant). Interaction between time and group was significant (p=0.0385) in a variance analysis of serum iPTH between MS patients and controls demonstrating a blunted PTH response in the MS patients in the autumn and most notably in the winter (Figure 1, panel B). The mean±SD winter level of iPTH in the MS patients was 29.1±12.9 ng/L (3.2±1.4 pmol/L) and in the healthy controls 40.7±14.9 ng/L (4.5±1.6 pmol/L, p=0.0042). The mean±SD autumn level of iPTH in the MS patients was 26.8±11.1 ng/L (2.9±1.2 pmol/L) and in the controls 35.2±13.4 ng/L (3.8±1.5 pmol/L, p=0.0141). The mean summer and spring iPTH values in MS patients and controls did not significantly differ (Figure 1B). The mean±SD summer level of iPTH in MS patients was 23.5±10.8 ng/L (2.6±1.2 pmol/L) and in the controls 27.9±12.3 ng/L (3.1±1.3 pmol/L, p=0.3418). The mean±SD spring level of iPTH in MS patients was 24.0±11.6 ng/L (2.6±1.2 pmol/L) and in the controls 27.5±12.4 ng/L (3.0±1.3 pmol/L, p=0.2379).

Vitamin D deficiency [25(OH)D] ≤ 37 nmol/L (14.8 mcg/L) was detected in 43% of MS patients and 53% of controls. When a higher cutoff value for vitamin D deficiency, 50 nmol/L (20 mcg/L),[13] was used, only 17% of the MS patients and 22% of the controls had sufficient vitamin D levels throughout the year. Elevation of iPTH above the upper limit of the laboratory reference range of 65 ng/L (7.2 pmol/L) was detected in 15% of MS patients and 17% of controls.

Correlation of serum levels of 25(OH)D and iPTH with clinical and MRI activity of MS

To examine the hypothesis that serum concentrations of 25(OH)D would decrease during periods of increased disease activity, we examined differences in 25(OH)D serum concentration levels between periods of relapse and remission. Figure 2 shows that serum levels of 25(OH)D were significantly lower and serum levels of iPTH significantly higher during MS relapses than in remission. Mean±SD concentration of 25(OH)D at relapse was 47.4±14.4 nmol/L (18.9±1.6 mcg/L) and at remission 60.0±21.8 nmol/L (24.0±2.4 mcg/L, *p*-value for difference 0.012). Mean±SD concentration of iPTH at relapse was 33.1±11.9 ng/L (3.6±1.3 pmol/L) and at remission 26.4±11.6ng/L (2.9±1.3 pmol/L, *p*-value for difference 0.009). All relapses occurred at PTH levels above 20 ng/L (2.2 pmol/L) , whereas 38% of patients in remission had PTH less or equal to 20 ng/L (Figure 2). None of the relapses but 11% of remissions occurred at serum concentrations of 25(OH)D above 85 nmol/L (9.4 pmol/L) (Figure 2). There was no correlation with the serum 25(OH)D or iPTH levels and MRI BOD or T2 activity (not shown). Gadolinium enhanced images were not included in the study. Serum levels of 25(OH)D or iPTH were not significantly different in patients treated with placebo in comparison with patients treated with either dose of IFNB-1a (not shown).

When looking at longitudinal patterns of 25(OH)D and iPTH, and relapse, in individual patients, we found a pattern of 14 out of the 21 relapses occurring at the peak of increasing levels of iPTH and 16 of the 21 relapses occurring at the lowest or decreasing levels of 25(OH)D in 10 out of the 12 relapsing patients (Supplemental Figure). Only 4 out of the 21 relapses in 2 patients (panels H and L in the Supplemental Figure) occurred at the peak or increasing serum 25(OH)D levels.

Patients were then grouped into quintiles by their winter, spring, summer and autumn serum 25(OH)D levels. The pre-study relapse rate, relapse rate during the study and confirmed EDSS progression during the study in these vitamin D quintiles were determined (Supplemental Table). The EDSS increase during the study and the relapse rate before and during the study were lower in the highest quintile than in the lowest quintile, but there was no statistically significant inverse correlation between vitamin D nutrition and EDSS or relapse rate (p=0.065 for EDSS, p=0.660 for relapse rate during the study and p=0.119 for pre-study relapse rate).

Exploratory analysis of clinical chemistry parameters related to vitamin D metabolism in MS patients and controls

To examine the hypothesis that MS patients have vitamin D insufficiency in comparison with healthy persons, that would be reflected in the clinical chemistry parameters related to vitamin D metabolism, we measured serum albumin, calcium, magnesium, alkaline phosphatase, chloride, phosphate and TSH levels in MS patients and healthy controls. All these values in both patients and controls were within the laboratory reference range (Table 1). Analysis of variance for repeated measurements revealed a seasonal pattern in serum calcium, phosphate and albumin such that serum calcium and phosphate levels were significantly lower in the winter and spring in MS patients in comparison with controls, whereas serum albumin levels were significantly higher in MS patients in the summer and spring (Figure 1C, Table 1). Serum alkaline phosphatase and serum chloride levels were significantly lower and serum magnesium levels were significantly higher in

the MS patients than in the controls throughout the year, whereas serum TSH was similar in the patients and in the controls at all times (Table 1).

There were no significant differences in any of the clinical chemistry parameters or TSH between MS relapse and remission (data not shown). However, there was a trend towards lower serum calcium concentration in the relapse samples in comparison with the remission samples (0.24±0.02 mmol/L versus 0.28±0.01 mmol/L, p=0.06). There were no significant differences in any of the clinical chemistry parameters or TSH between patients treated with placebo and patients treated with IFNB-1a (data not shown).

DISCUSSION

The serum concentration of 25-hydroxyvitamin D [25(OH)D, calcidiol] is a reflection of the intake of vitamin D in food and its synthesis from pro-vitamins in the skin under the influence of UV light.[9] The cellular effects of vitamin D are mediated through the binding of the active metabolite of vitamin D, 1,25-dihydroxyvitamin-D_3 [1,25 $(OH)_2D_3$, vitamin D hormone] into the vitamin D receptor expressed on a wide variety of cell types, including cells of the immune system and neuronal and glial cells in human brain.[14, 15] The conversion of calcidiol into the vitamin D hormone takes place in the kidney and serum levels of calcidiol closely reflect the levels of the hormonally active form of vitamin D in subjects with normal kidney function.[16]

We showed that seasonal variation of 25(OH)D in serum is similar in Finnish MS patients and age- and sex-matched healthy persons. This suggests that vitamin D may be functioning as a disease modifier in genetically susceptible individuals, rather than as a disease determinant in the general population. Vitamin D deficiency [S-25(OH)D $\leq$ 37 nmol/L] was common in this longitudinal study, affecting half of the patients and controls at some time of the year. Similar high prevalence of vitamin D deficiency has earlier been detected in Finnish medical in-and outpatients and healthy army recruits during the winter.[10,17] In our earlier cross-sectional study 30% of newly diagnosed Finnish MS patients had vitamin D deficiency.[7]

Experimentally, vitamin D deficiency results in the increased incidence of autoimmune diseases.[18] The mechanism is likely to be related to the development of self-tolerance, since vitamin D hormone regulates T helper cell and dendritic cell function and induces regulatory T-cells resulting in a decrease in the Th1-driven autoimmune responses and tolerance instead of vigorous immune responses.[18,19] In a careful epidemiological study, risk of MS was shown to be higher in persons born in the month of May.[20] One explanation for this could be lower levels of circulating vitamin D available to the fetus during winter time pregnancies. Immunological tolerance to self-antigens is a process that occurs during development, rather than being genetically pre-programmed.[21]

In Finland, the incidence of MS is among the highest and type I diabetes the highest in the world. The incidence of both diseases have been increasing in parallel with a decrease in the recommended dose of vitamin D supplementation to infants from 4000-5000 IU until 1964, to 2000 IU until 1975 and to 1000 IU until 1992, when it was reduced to the current level of 400 IU/day.[22, 23] Vitamin D supplementation to infants in Finland at our latitude of 60º to 70º North is stopped at the age of 3 years. It has been suggested that at our latitude vitamin D supplementation should be continued from that age onward as prophylaxis for osteoporosis.[17]

There are two alternative interpretations of our results showing lower levels of circulating vitamin D during MS relapses than in remission: either increasing circulating vitamin D reduce the risk for MS relapses or MS relapses reduce serum vitamin D levels. In a recent pilot study with a 2.5 mcg/d of oral calcitriol (vitamin D hormone) in 15 relapsing-remitting MS patients, the on-study exacerbation rate was less than baseline.[24] This is in favor of the first interpretation. We also found a trend for correlation of increasing serum vitamin D status with less relapses and less EDSS progression, but possibly due to the sample size of our study statistical significance was not reached. Our results also suggest that the previously observed increased risk of MS relapses in the spring could be related to lower serum levels of vitamin D after the winter.[25] Unfortunately, Gadolinium-enhanced images where not included in the PRISMS trial and therefore we could not evaluate the previously presented hypothesis that serum vitamin D levels inversely correlate with Gadolinium enhanced brain images in MS patients two months later.[26, 27]

The current adult recommendations for vitamin D, 200-600 IU/day, are very low when one considers that a 10-15 min whole-body exposure to peak summer sun will generate and release up to 20,000 IU vitamin D_3 into the circulation.[28] Using functional indicators of vitamin D sufficiency such as calcium absorption, bone mineral density and iPTH, several studies have more accurately defined vitamin D deficiency as circulating levels of 25(OH)D $\leq$ 80 nmol/L (32 mcg/L).[28] Recent studies reveal that current dietary recommendations for adults are not sufficient to maintain circulating 25(OH)D levels at or above this level, especially in pregnancy and lactation, as there is hardly any vitamin D3 in diet.[28] In our study, none of the 21 serum samples that were collected during MS relapses but 13/122 samples collected in remission reached a serum 25(OH)D level of 85 nmol/L. Only four out of the 23 MS patients reached 85 nmol/L serum vitamin D level in the summer and two in the autumn or spring, but none in the winter. There was considerable overlap in the distribution of the serum vitamin D values in the participants in remission versus relapse. Intact PTH was a better predictor of MS relapse than vitamin D such that all patients in relapse had serum iPTH above 20 ng/L (2.2 pmol/L), whereas 38% of patients in remission had iPTH less than 20 ng/L (2.2 pmol/L).

The most important function of vitamin D in bone metabolism is maintenance of adequate calcium and phosphate supply to the bone be increasing their absorption from the gut.[29] We hypothesize that the observed lower winter calcium and phosphate levels in the MS patients than in the controls indicate a relative vitamin D deficiency in MS. Neither the MS patients nor the controls reported intake of vitamin D supplements. The serum albumin concentration has an effect on the serum concentration of total calcium such that an increase of 10 g/L in the serum albumin concentration leads to an increase of 0.2 mmol/L in the serum calcium concentration.[30] Since the serum albumin levels were higher in the MS patients than in the controls, the corrected difference between the serum concentrations of calcium in the MS patients and in the controls would be even greater than the reported one (0.12 mmol/L instead of 0.08 mmol/L). The observed winter hypocalcaemia and a blunted PTH response in MS patients compared to controls raises a possibility that the endocrine circuitry regulating serum calcium is altered in MS patients either as a cause or a consequence of their disease.

There were no statistically significant differences in any of the clinical chemistry parameters between the relapse and remission samples or between the IFNB-1a and

placebo treatment arms. Thence the differences cannot be explained by IFNB-1a or corticosteroid therapy. Higher magnesium levels in the MS patients than in the controls could be explained by frequent use of supplements containing magnesium by MS patients.[31] Alkaline phosphatase is stimulated by mobility and thence could be lower in MS patients as a consequence of lesser physical activity. Whatever the explanation for the observed differences in the clinical chemistry parameters closely related to vitamin D metabolism is, our results suggest that regulation of vitamin D and calcium homeostasis is likely to be of importance in MS. Our findings also raise a concern of bone health in MS patient. It is important to recognize that the pro-inflammatory cytokines IFN-gamma, IL-1-alpha and TNF-alpha, that are pathogenic in MS,[1] are also strong stimulators of osteoclastic bone resorption and inhibitors of bone formation.[30,32] The mechanism of the inflammation induced bone loss has been shown to be cytokine induced activation of the inducible nitric oxide (iNOS) pathway in bone cells.[32] In a recent work, dietary calcium and vitamin D hormone treatment directly and indirectly inhibited the TNF-alpha pathway and suppressed inflammatory bowel disease in vitamin D deficient knockout mice.[33]

Oral vitamin D3 supplementation is a safe and cost-effective way to increase circulating vitamin D levels. The risk of vitamin D3 supplementation is hypercalcaemia. It is usually asymptomatic at serum calcium concentrations below 2.8 mmol/l but at higher serum calcium concentrations may provoke disorientation, muscle weakness and cardiac arrythmias. However, the risk of severe hypercalcaemia only arises when high amounts of vitamin D (>1000 microg/day or > 40 0000 IU/d) are consumed.[30] There are also new vitamin D hormone analogs that effectively regulate the immune system without increasing serum calcium.[19] The therapeutic utility of vitamin D3 or vitamin D hormone analogs in MS should be addressed in randomized clinical trials. Based on our results we suggest choosing relapse rate reduction as a primary outcome measure for these trials. Analysis of other serum parameters of vitamin D metabolism including iPTH and calcium as well as measurement of bone density would be useful. Our results indicate that the dose of vitamin D3 or its analogs should target at suppressing iPTH levels to a minimum (<20 ng/L or 2.2 pmol/L). It remains to be determined whether vitamin D treatment in MS proves beneficial both for the health of the brain, the immune system, the muscles and the bones of the patients.

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Competing interests: None.

Ethical approval: This study was approved by the local research ethics committee. Informed consent was obtained from all the patients being studied.

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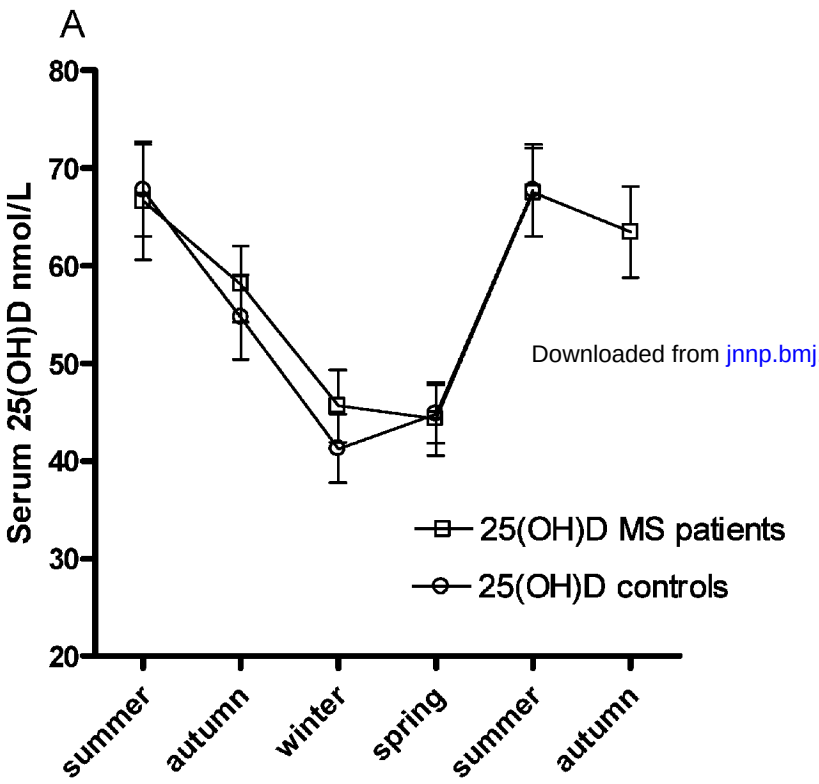
Figure Legends

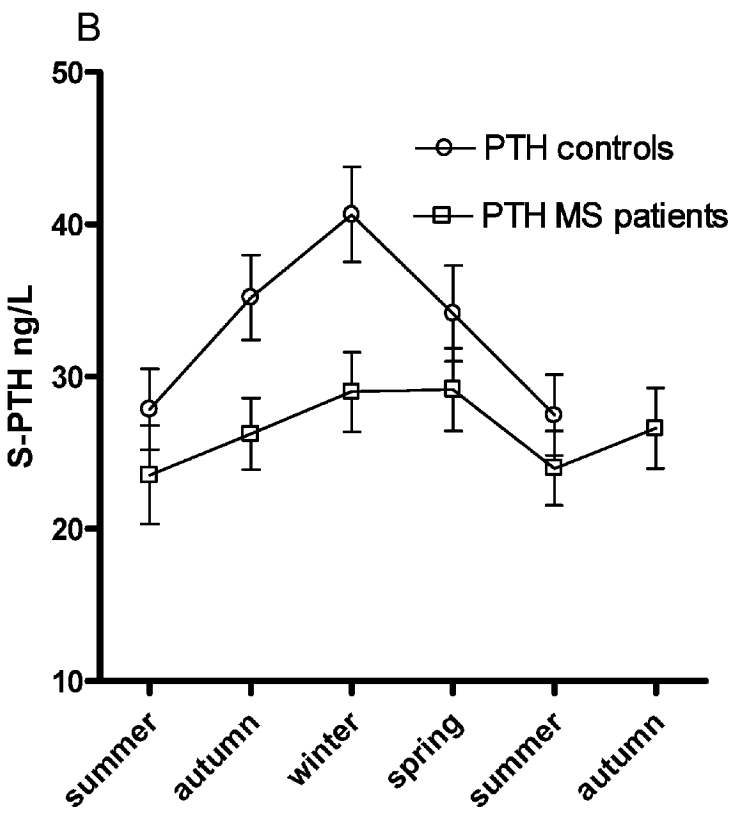
Figure 1 Seasonal variation of 25(OH)D, iPTH and calcium in serum in 23 MS patients and in 23 healthy controls. **A**: The mean serum concentration of 25(OH)D was similar in MS patients and in the healthy controls throughout the year (*p*-value for overall difference between groups 0.81; *p*-value for interaction between time and group 0.1507, not significant). **B**: There was a blunted PTH response in the MS patients in the autumn and most notably in the winter (*p*-value for interaction between time and group 0.0385*; *p*-value for the difference between MS and control values in the winter 0.0042* and in the autumn 0.0141*). **C**: There was relative winter and spring hypocalcaemia in MS patients in comparison with healthy controls (*p*-value for the interaction between time and group 0.0149*; *p*-value for the difference between MS and control values in the winter <0.0001* and in the spring 0.0013*). Error bars represent standard errors of mean.

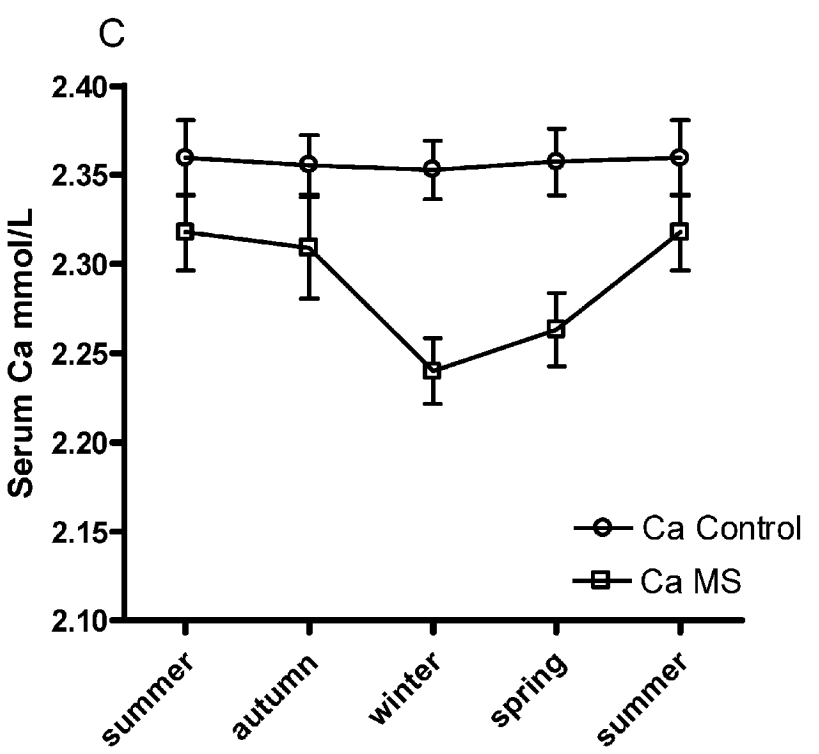
Figure 2 Serum levels of 25(OH)D and iPTH during MS relapse and remission. Serum levels of 25(OH)D and iPTH were measured every 3 months and at the time of relapses during one year in 23 MS patients. Samples collected within 2 weeks before and 4 weeks after exacerbations were determined as "relapse" and others as "remission" samples. **A**: Mean±SEM. concentration of 25(OH)D at relapse was 47.4±3.1 nmol/L (18.9±1.2mcg/L) and at remission 60.0±1.9 nmol/L (24±0.8 mcg/L, *p*-value for difference 0.012*). None of the 21 relapses but 13/122 (11%) remissions occurred at serum 25(OH)D concentrations above 85 nmol/L (34 mcg/L). **B**: Mean±SEM concentration of iPTH at relapse was 33.1±2.6 ng/L and at remission 26.4±1.1 ng/L (*p*=0.009*). All relapses occurred at PTH levels above 20 ng/L (2.2 pmol/L), whereas 38% of patients in remission had PTH less or equal to 20 ng/L.

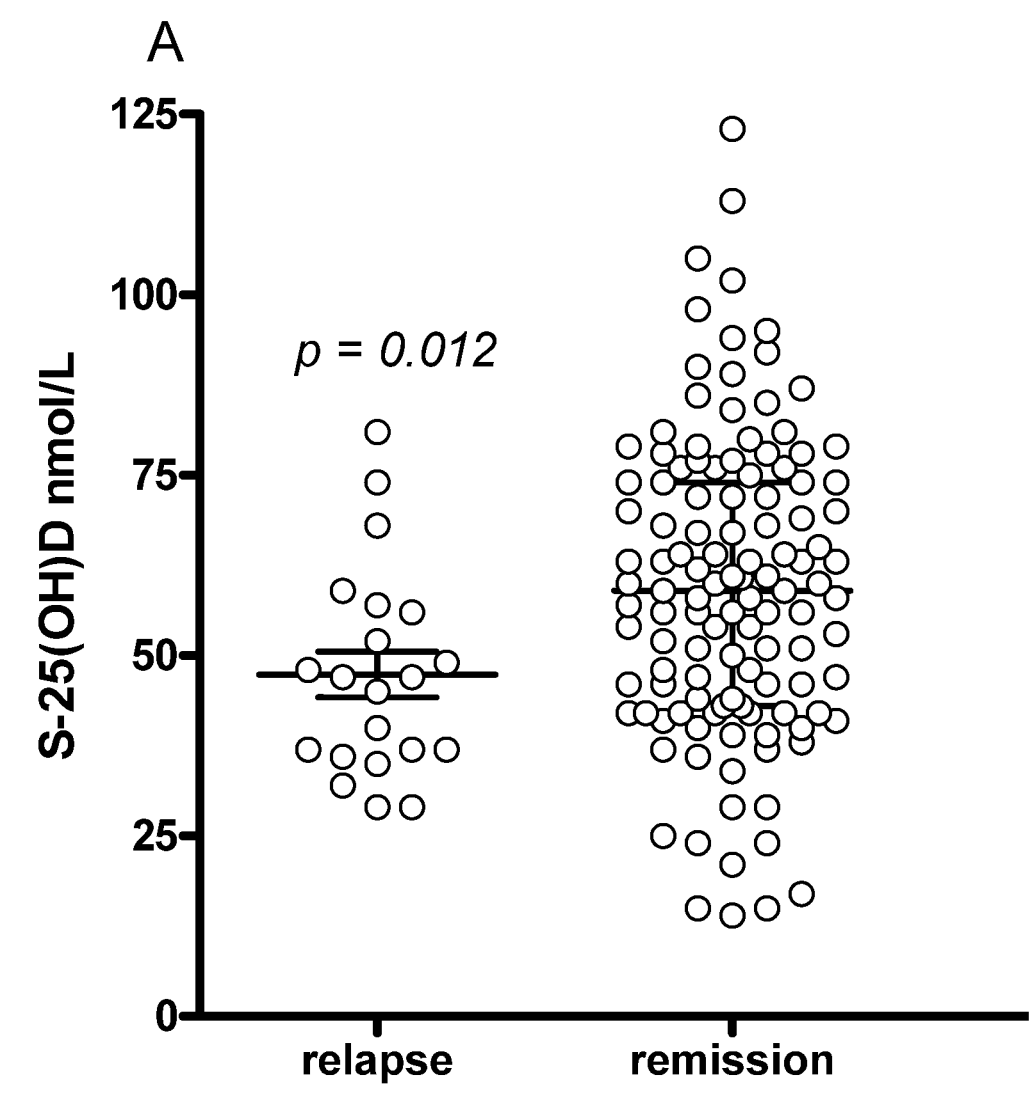
Parameter studied	Albumin	Calcium	Phosphate	AFOS	Magnesium	Chloride	TSH
Normal values	36-48 g/L	2.15-2.51 mmol/L	0.76-1.41 nmol/L female 0.71-1.53 nmol/L male	35-105 U/L	0.7-1.1 mmol/L	100-108 mmol/L	0.3-4.2 mU/L
Mean±SD MS patients	44.36± 3.12 g/L	2.28±0.11 mmol/L	1.01±0.16 nmol/L	43.00± 11.31 U/L	0.85±0.05 mmol/L	104.82± 1.75 mmol/L	2.21± 1.04 mU/L
Mean±SD controls	42.64± 3.48 g/L	2.36±0.09 mmol/L	1.19±0.21 nmol/L	58.50± 14.46 U/L	0.81±0.05 mmol/L	106.18± 1.72 mmol/L	2.55± 1.40 mU/L
Time*group interaction	0.0035*	0.0149*	0.011*	0.0778 n.s.	0.0922 n.s.	0.5677 n.s.	0.2851 n.s.
p-value for overall difference				0.0002*	0.0009*	0.0016*	0.2574 n.s.
summer	0.0086*	0.1699	<0.0001*				
autumn	0.1553	0.1670	0.0644				
winter	0.6733	<0.0001*	0.0030*				
spring	0.0276*	0.0013*	0.0103*				

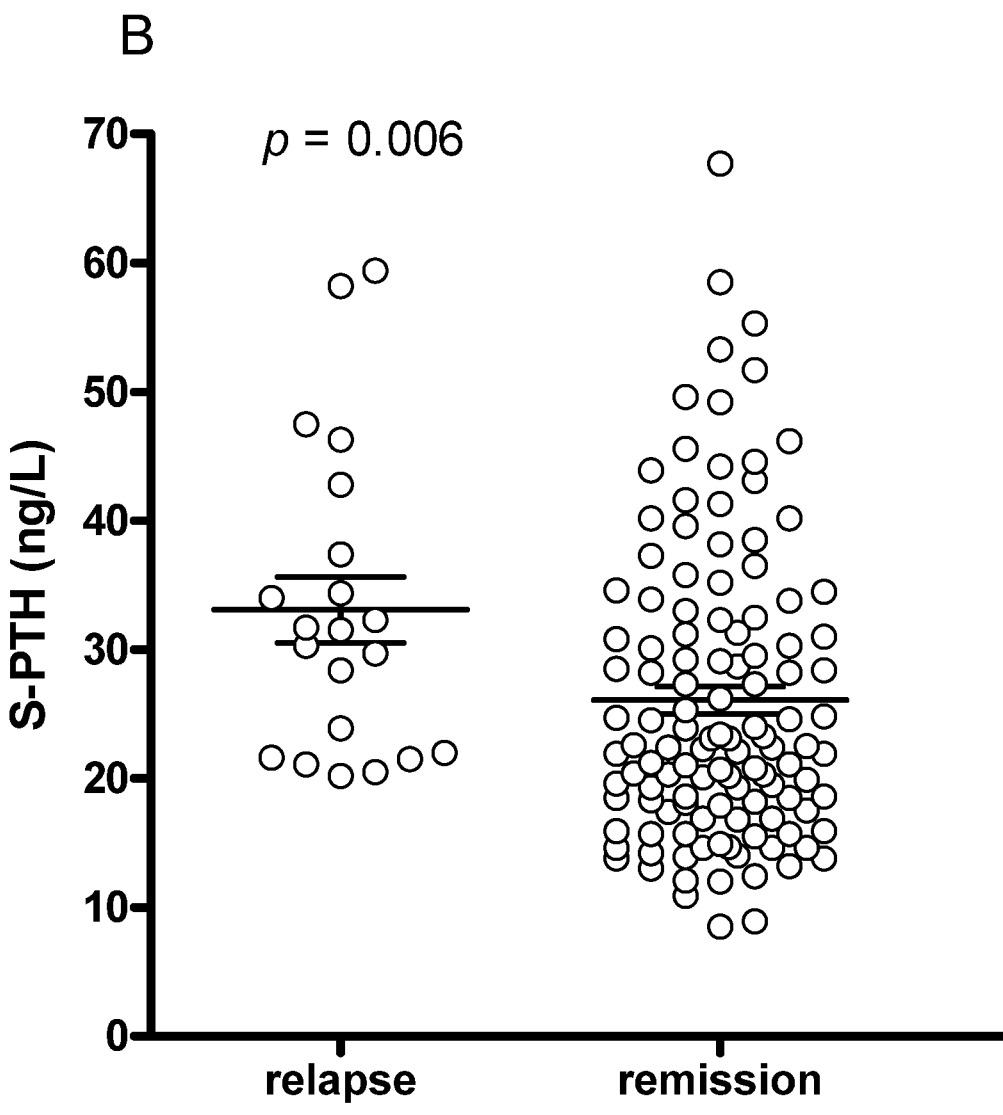
Table 1. Exploratory analysis of clinical chemistry parameters related to vitamin D metabolism. Values represent mean±SD of 23 MS patients and 23 age-and sex-matched healthy controls during the whole year. For each patient and control, four 3-monthly samples were included. When interaction between time and group was statistically significant (*), the *p*-values for each time point are given. When interaction between time and group was not significant, the *p*-values for the overall difference between MS patients and controls during the whole year are given.

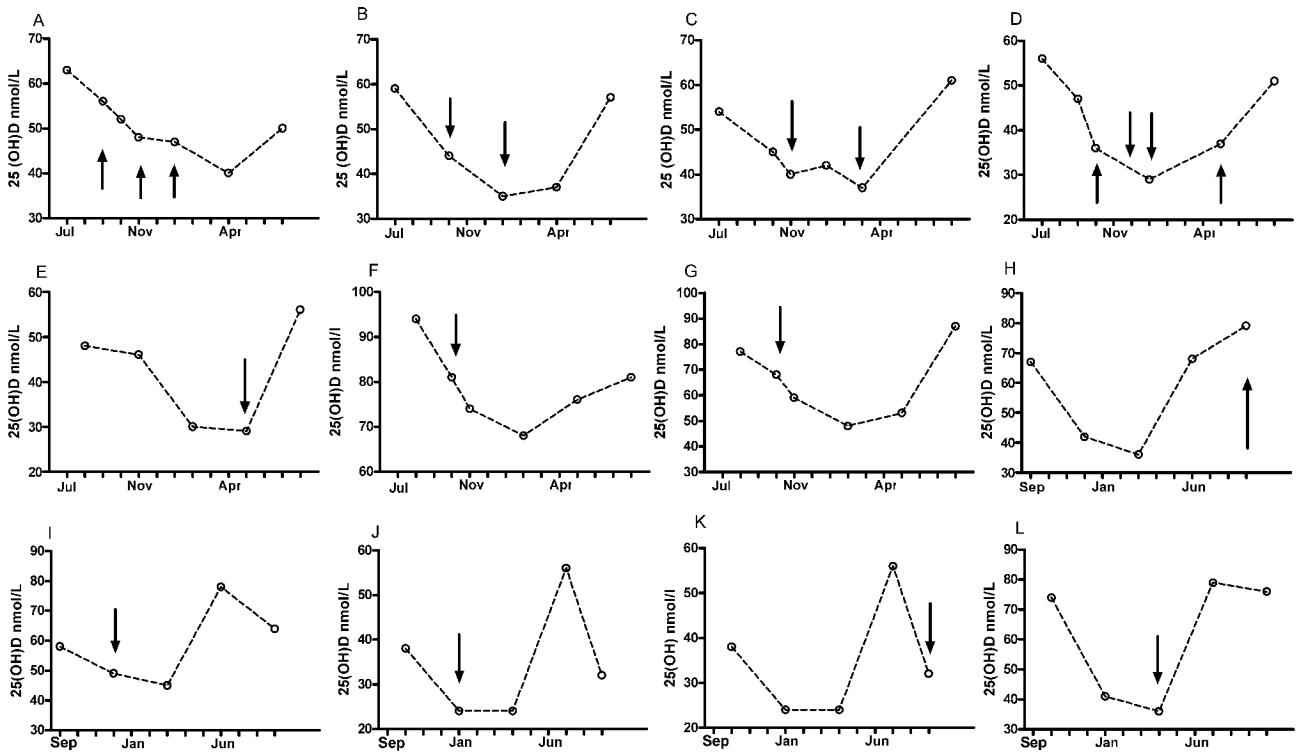







A
125
100
75
50
25
0
S-25(OH)D nmol/L
p = 0.012
relapse
remission


B
p = 0.006
S-PTH (ng/L)
70
60
50
40
30
20
10
0
relapse
remission


A
25 (OH)D nmol/L
70
60
50
40
30
Jul
Nov
Apr
B
25 (OH)D nmol/L
70
60
50
40
30
Jul
Nov
Apr
C
25 (OH)D nmol/L
70
60
50
40
30
Jul
Nov
Apr
D
25(OH)D nmol/L
60
50
40
30
20
Jul
Nov
Apr
E
25(OH)D nmol/L
60
50
40
30
20
Jul
Nov
Apr
F
25(OH)D nmol/l
100
90
80
70
60
Jul
Nov
Apr
G
25(OH)D nmol/L
100
90
80
70
60
50
40
30
Jul
Nov
Apr
H
25(OH)D nmol/L
90
80
70
60
50
40
30
Sep
Jan
Jun
I
25(OH)D nmol/L
90
80
70
60
50
40
30
Sep
Jan
Jun
J
25(OH)D nmol/L
60
50
40
30
20
Sep
Jan
Jun
K
25(OH) nmol/l
60
50
40
30
20
Sep
Jan
Jun
L
25(OH)D nmol/L
90
80
70
60
50
40
30
Sep
Jan
Jun