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Regulatory T cell function correlates with serum 25-hydroxyvitamin D, but not with 1,25-dihydroxyvitamin D, parathyroid hormone and calcium levels in patients with relapsing remitting multiple sclerosis☆

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ABSTRACT

Vitamin D is a potent immune modulator in multiple sclerosis (MS), but was primarily identified for its effects on calcium homeostasis. It is uncertain whether these calcaemic functions of vitamin D are critically involved in its immune modulating potential. We earlier reported a correlation between serum 25-hydroxyvitamin D (25(OH)D) levels and regulatory T cell (Treg) function. In the present study, the correlation of serum levels of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), intact parathyroid hormone (PTH), and total calcium with Treg number and functionality and the proportions of other T helper cell subsets was assessed in 29 relapsing remitting MS patients. In contrast to serum 25(OH)D levels, serum concentrations of neither $1,25(OH)_2D$, nor PTH and total calcium correlated significantly with Treg function or Th1/Th2 ratio. None of the parameters correlated with the relative and absolute number of Tregs. Interestingly, the serum levels of $1,25(OH)_2D$ correlated positively with the proportion of T helper type 17 (Th17) cells. These results suggest that the serum levels of $1,25(OH)_2D$, PTH, and total calcium are not critically involved in the correlation between vitamin D status and T cell regulation.

1. Introduction

Multiple sclerosis (MS) is a debilitating disease affecting young people, in which an auto-reactive T helper cell type 1 (Th1) and Th17 response initiates focal inflammation in the central nervous system [1]. In experimental studies, vitamin D inhibits Th1 and Th17 cells and promotes Th2 and regulatory T cells (Treg) [2,3]. Recently, we showed that vitamin D status, as reflected by serum levels of 25-hydroxyvitamin D (25(OH)D) correlated positively with Treg functionality, and with a more Th2 directed Th1/Th2 balance in patients with relapsing remitting MS (RRMS) [4]. Associative studies showed that vitamin D status was poorer in subjects developing MS in later life [5], and correlated negatively with disease activity [6]. Although 25(OH)D is the most abundant metabolite, the biologically activemetabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) is also present in the circulation. The serum levels of $1,25(OH)_2D$ are primarily derived from the hydroxylation of 25(OH)D in the kidneys upon signals from calcium metabolism, e.g. calcium and parathyroid hormone (PTH) [7]. An increase in serum $1,25(OH)_2D$ results in increasing serum calcium levels, by promoting calcium resorbtion in kidney and intestine, and osteoclast activation. It is uncertain whether these calcaemic effects of vitamin D are also critically involved in its interaction with the immune system.

The data on T cell characteristics and 25(OH)D values have been reported in detail before [4]. In the present study, we assessed whether serum levels of $1,25(OH)_2D$, PTH, and total calcium, all related with the calcaemic function of vitamin D, correlated with Treg function, the absolute and relative number of Tregs in the circulation, and with T helper cell subsets in patients with MS.

2. Materials and methods

A cohort of 29 Caucasian RRMS patients was included. The cohort comprised 19 females (66%), the median age was 39 years (23–59), the median disease duration was 3.5 years (0.7–4.9),

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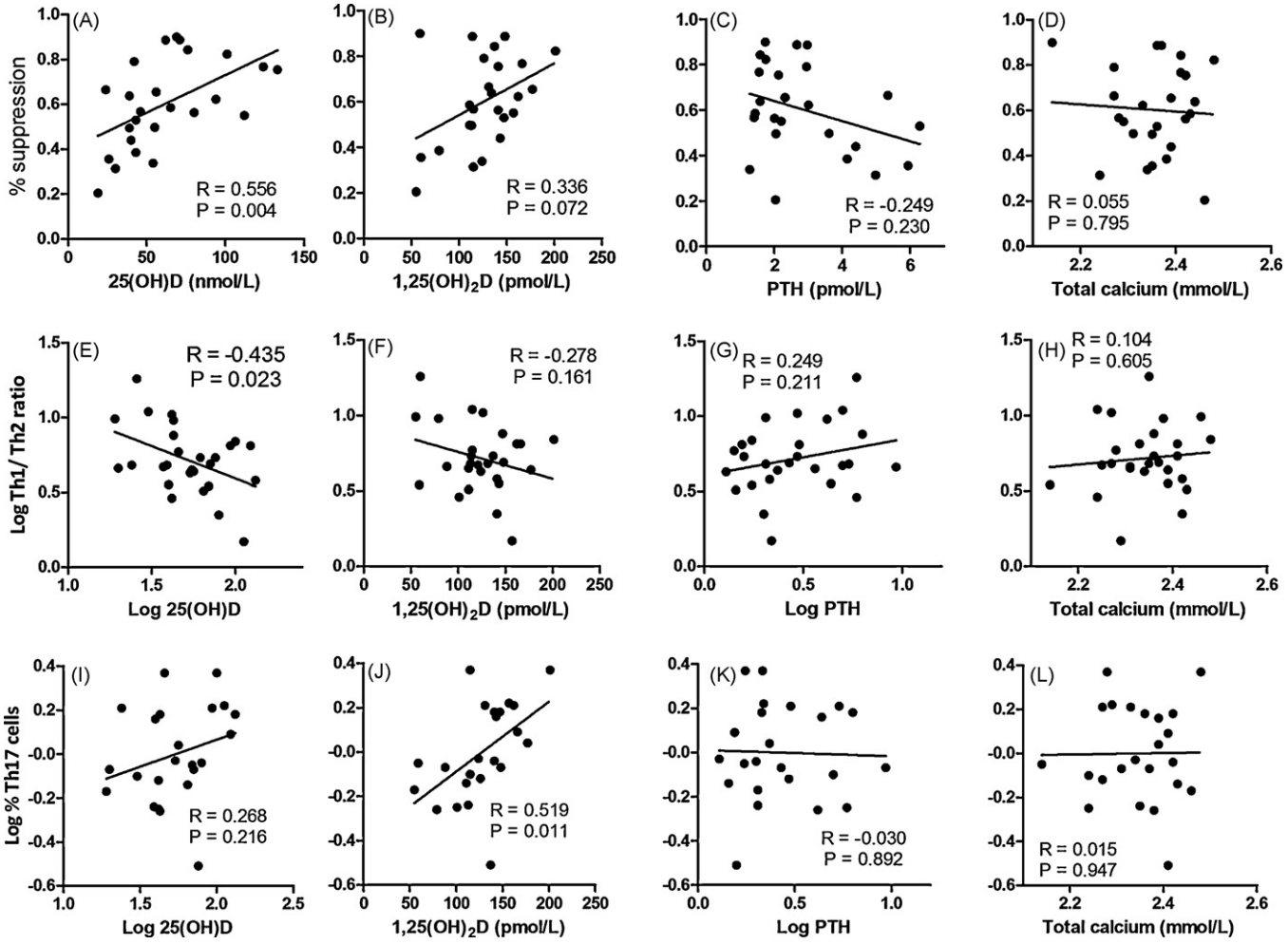

Fig. 1. Correlation of the serum levels of 25(OH)D, 1,25(OH)2D, PTH, and total calcium with T cell characteristics. T cell characteristics comprise: (A–D) the amount of suppression (%) of $CD4^+CD25^-$ responder T cell proliferation achieved by an equal amount of $CD4^+CD25^+CD127^-$ regulatory T cells in co-culture, (E–H) the ratio of the proportions of IFN-γ^+ and IL-4^+ $CD4^+$ T cells (Th1/Th2 ratio) in a PBMC culture stimulated with PMA and calcium ionomycine, and (I–L) the proportion of IL-17^+ $CD4^+$ T cells in a PBMC culture stimulated with PMA and calcium ionomycine. Dependent on the distribution of the variables, either the Spearman (A–D) or Pearson (E–L) correlation coefficient is provided. A regression line is inserted to illustrate correlation (A and E are adapted from Smolders et al. [4]).

the median relapse rate was 0/year (0–3), and the median expanded disability status scale (EDSS) score was 2 (0–6). Twenty-five (86%) patients were treated with beta interferon, and the remainder received no immune modulation. All patients signed informed consent, and the study was approved by the Local Medical Ethics Committee according to the Declaration of Helsinki.

Peripheral blood was drawn during the period from September 2008 to February 2009. Serum values of 25(OH)D and $1,25(OH)_2D$ were determined with commercially available radioimmuno assays (Immunodiagnostic Systems, Boldon, UK) [6]. Serum total calcium levels were determined with the Immunolite 2000™ (Siemens Healthcare Diagnostics, Deerfield, IL, USA), and intact PTH levels were determined with the Synchron LX™ (Beckman Coulter, Woerden, The Netherlands). Reference values of the clinical chemical laboratory are provided.

T cell variables were determined as described extensively before [4]. In short, the proportions of $CD4^+$ Tregs and T helper cell subsets in the circulation were determined directly ex vivo by flow-cytometry. Tregs were defined as $CD4^+$ T cells being either $CD25^+FoxP3^+$ or $CD25^+CD127^-$, and T helper cells were defined by intracellular cytokine expression of $CD4^+$ T cells on 5-h stimulation with phorbol 12-myristate 13-acetate (PMA) and calcium ionomycine (Th1: interferon-γ (IFN-γ), Th2: IL-4, Th17: IL-17, and IL-10 producing cells). To calculate absolute cell numbers, lymphocytes were counted on a hematological cell counter (Beckman Coulter). The ability of Tregs to suppress the proliferation of polyclonal activated responder T (Tresp) cells was assessed in a carboxyfluorescein succinimidyl ester (CFSE) based proliferation suppression assay. In brief, $CD4^+CD25^+CD127^-$ Tregs and $CD4^+CD25^-$ Tresps were sorted on a FacsAria™ cell sorter (BD Biosciences, Breda, The Netherlands). After CFSE labeling, Tresps were activated with anti-CD3 monoclonal antibody and cultured with irradiated autologous feeders in the presence of varying amounts of Tregs (Treg/Tresp ratio is 0/1, 0.25/1, 0.50/1, 1/1, and 1/2). After 5 days, proliferation in each culture condition was evaluated by flow-cytometric analyses of the CFSE signal and expressed relative to proliferation in the Tresp monoculture. Suppression of proliferation was then calculated. Samples were measured on a FacsCalibur™ flow-cytometer and analyzed with CellQuest™ software (both from BD Biosciences).

Statistical analysis was performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and figures were constructed with GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Continuous variables are presented as median value with corresponding range (min–max). When normally distributed (Shapiro–Wilk test $P > 0.05$), the Pearson correlation coefficient is provided to describe the relationship between two continuous variables. In case of an

abnormal distribution, persistent after logarithmic transformation, the Spearman correlation coefficient is given. An uncorrected *P*-value <0.05 was considered to be significant.

3. Results

3.1. Serum levels of vitamin D metabolites, PTH and calcium

The median 25(OH)D value was 54 nmol/L (19–133), the median $1,25(OH)_2D$ value was 126 pmol/L (55–203). The two vitamin D metabolites correlated positively (Pearson $R = 0.631$, $P < 0.001$). The median PTH-value was 2.32 pmol/L (1.28–9.36) (reference 1.3–6.8 pmol/L), the median total calcium value 2.36 mmol/L (2.14–2.48) (reference 2.10–2.55 mmol/L). Serum levels of PTH correlated negatively with total calcium (Pearson $R = -0.382$, $P = 0.041$) and 25(OH)D levels (Pearson $R = -0.582$, $P = 0.001$). Total calcium levels correlated positively with $1,25(OH)_2D$ levels (Pearson $R = 0.385$, $P = 0.039$).

3.2. Correlation with Treg number and functionality

The median suppression of proliferation in the 1:1 Treg/Tresp ratio was 60% (20–92). As described before [4], serum 25(OH)D values correlated positively with Treg suppressive function (Fig. 1A). Serum $1,25(OH)_2D$, PTH and calcium did not correlate significantly with Treg functionality (Fig. 1B–D). This was the same for all individual Treg/Tresp ratio culture conditions. The percentage of $CD25^+CD127^-$ Tregs within the $CD4^+$ T cell compartment was 6.6% (2.9–12.3) and of $CD25^+FoxP3^+$ Tregs was 5.6% (2.7–15.8). The absolute and relative numbers of Tregs in the circulation did not correlate with any of the parameters studied (data not shown).

3.3. Correlation with T helper cell subset percentages

The median percentage of Th1 cells within the $CD4^+$ T cell compartment was 12.1% (2.6–26.2), of Th2 2.1% (0.9–4.9), of Th17 0.9% (0.3–2.4), and of IL-10 producing cells 0.8% (0.4–1.5). As described earlier [4], serum 25(OH)D correlated negatively with the Th1/Th2 ratio (Fig. 1E). The other calcium metabolism parameters did not correlate significantly with Th1/Th2 ratio (Fig. 1F–H). There was also no correlation of any of the markers with the individual percentages of Th1, Th2 and IL-10 producing $CD4^+$ T cells (data not shown). Interestingly, the proportion of Th17 cells correlated positively with serum $1,25(OH)_2D$ levels, despite the absence of a correlation with 25(OH)D, PTH or total calcium levels (Fig. 1I–L).

4. Discussion

In the present study, the relationship between several T cell characteristics and serum $1,25(OH)_2D$, PTH and total calcium was studied in a cohort of RRMS patients. In contrast to the earlier reported positive correlation of serum 25(OH)D levels with Treg function and negative correlation with Th1/Th2 ratio [4], the serum values of $1,25(OH)_2D$, PTH, and total calcium did not correlate significantly with these T cell parameters. Serum $1,25(OH)_2D$ did correlate positively with the proportion of Th17 cells.

The role of calcium metabolism in the interaction between vitamin D and MS is at present unclear. In experimental autoimmune encephalomyelitis (EAE), an animal-model of MS, dietary calcium intake and the subsequent elevation of serum calcium concentration were critical factors in the clinical and immunological effects of $1,25(OH)_2D$ therapy [8]. Calcitonin, a protein released during hypercalcaemia, enhanced the clinical effectiveness of $1,25(OH)_2D$ in EAE [9], but was not mandatory [10]. This proposed importance of calcium in EAE, led to the notice that calcium might also be a critical factor in the relationship between vitamin D and MS. In a longitudinal study, Soilu-Hanninen et al. found that MS patients had lower serum total calcium and higher intact PTH levels during spring and winter compared to healthy controls, despite similar 25(OH)D levels [11]. Hereby, intact PTH and 25(OH)D levels were, respectively, higher and lower during relapse than remission.

Contrastingly, our data suggest that serum calcium, PTH, and $1,25(OH)_2D$ are not critically involved in the interaction between vitamin D status and T cell regulation in MS patients. The trend towards correlation of $1,25(OH)_2D$ with Treg suppressive function is likely to be secondary to the strong relationship between 25(OH)D and $1,25(OH)_2D$. Likewise, the apparent negative correlation between disability and $1,25(OH)_2D$ levels in MS patients that we earlier reported, appeared to be dependent on the correlation of EDSS with serum 25(OH)D in a multiple regression analysis [6]. A patient control study also suggested that the contribution of serum $1,25(OH)_2D$ levels to the risk for developing MS is limited [12]. Several authors proposed a model for interaction between vitamin D status and the immune system, in which activated immune cells, including macrophages and $CD4^+$ T cells, form high levels of $1,25(OH)_2D$ out of 25(OH)D at sites of immune activation [2]. These local levels of $1,25(OH)_2D$ inhibit auto-reactive T cell activation, promote T cell regulation, and might subsequently result in a reduced disease activity of MS and a lower risk for developing MS. A recent case report described 3 cases of vitamin D dependent rickets type 1 in combination with MS within one family [13]. Additionally to a deficiency of serum $1,25(OH)_2D$ levels in these patients, the defect in the 25(OH)D-1α-hydroxylase gene also abolishes $1,25(OH)_2D$ formation by activated immune cells. Although speculative, our data suggest the latter mechanism to be most important in the reported association. Royal 3rd et al. reported that the ratio between $1,25(OH)_2D$ and 25(OH)D correlated with the proportion of Tregs in the $CD4^+$ T cell compartment [14]. We could not reproduce these results in our patient cohort.

The positive correlation of $1,25(OH)_2D$ with the percentage of Th17 cells seems contradictory. Th17 cells have been proposed to be the most important pathogenic cells in MS [1]. Therefore, a positive correlation of serum $1,25(OH)_2D$ levels and these cells suggests a negative effect of vitamin D on MS, which has, however, not been observed in clinical studies [6,12]. Additionally, the exact significance of IL-17 producing T cells for autoimmune diseases, in the context of $CD4^+$ T cell plasticity and IL-17 producing Tregs, is at present uncertain [15]. Alternatively, several autoimmune diseases, including MS, have been associated with a loss of bone mineral density (BMD) [16]. Th17 cells have been proposed to promote osteoclast function via IL-17 production, and to establish hereby loss of BMD [17]. Therefore, elevated $1,25(OH)_2D$ values against a background of uncorrelated 25(OH)D levels might also be a result rather than a cause of an increased population of IL-17 producing Th17 cells.

Our study has several limitations. We assessed only a limited amount of markers of calcium metabolism in our cohort. Serum total rather than ionized calcium levels were measured. However, the cohort comprised no severely ill, cachectic or obese patients. Therefore, it is not likely that correction for serum albumin would dramatically affect our results. Lastly, the cohort is, although homogeneous, fairly small.

In conclusion, we found that the earlier reported correlations of Treg function and Th1/Th2 balance with serum 25(OH)D levels [4] were not accompanied by correlations of these T cell parameters with the serum values of $1,25(OH)_2D$, PTH, and calcium. These results support the hypothesis that serum 25(OH)D is important for peripheral T cell regulation in MS in a direct manner, and not via modulating calcium metabolism. At present, intervention studies supplied calcium together with vitamin D. From our study, we conclude that it is questionable whether an elevation of serum calcium levels is critical for the effects of vitamin D on immune regulation and subsequent disease activity of MS. However, to preserve bone

mineral density, a sufficient dietary intake of calcium in patients treated with high doses of vitamin D is required. Additionally, our data provide rationale to supplement MS patients with vitamin D, rather than $1,25(OH)_2D$.

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