Vitamin D as an immune modulator in multiple sclerosis

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Vitamin D as an immune modulator in multiple sclerosis

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ter verkrijging van de graad van doctor
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*How manifold it is, what thou hast made!
They are hidden from the face of man.*

Hymn to the sun god Aten,
attributed to pharaoh Akhenaten (1351 – 1333 BC)
(translation in Pritchard, 1958).

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Abbreviations

1,24,25(OH) ₃ D	1,24,25-trihydroxyvitamin D
1,25(OH) ₂ D	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
7AAD	7-aminoactinomycin D
Apa-I	Acetobacter pasteurianus restriction enzyme
APC	Antigen presenting cell
APC	Allophycocyanin
BMD	Bone mineral density
Bsm-I	Bacillus stearothermophilus restriction enzyme
CD	Cluster defining molecule
CFSE	Carboxy fluorescein diacetate succinimidyl ester
CNS	Central nervous system
CTLA-4	Cytotoxic T lymphocyte antigen 4
CYP	Cytochrome P
DBP	Vitamin D binding protein
DC	Dendritic cell
DNA	Desoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ED50	Half-maximal proliferation point
EDSS	Expanded disability status scale
FITC	Fluorescein isothiocyanate
Fok-I	Flavobacterium okeanokoites restriction enzyme
FoxP3	Forkhead box P3
GM-CSF	Granulocyte macrophage-colony stimulating factor
IFN-β	Interferon beta
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL-	Interleukin
IR40	Inhibitor ratio for 40% suppression
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis

NGF	Nerve Growth Factor
PBMC	Peripheral blood mononuclear cells
PE	R-Phycoerythrin
PEcy7	Conjugate of PE and cyanine dye 7
PerCP	Peridinin chlorophyll protein
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PPMS	Primary progressive multiple sclerosis
PTH	Parathyroid Hormone
Rag	Recombination activating genes
RNA	Ribonucleic acid
RRMS	Relapsing remitting multiple sclerosis
RXR	Retinoid X receptor
SNP	Single Nucleotide Polymorphism
SPMS	Secondary progressive multiple sclerosis
Taq-I	Thermus Aquaticus restriction enzyme
TGF- β	Tumour growth factor beta
Th1	T helper cell type 1
Th17	T helper cell type 17
Th2	T helper cell type 2
TNF- α	Tumour necrosis factor alpha
Tr1	T regulatory cell type 1
Treg	Regulatory T cell
Tresp	T responder cell
UVB	Ultra violet B radiation
VDR	Vitamin D receptor
VDRG	Vitamin D receptor gene
VLA-4	Very late antigen 4

General introduction and aims

Adapted from: Joost Smolders, Jan Damoiseaux (2011). Vitamin D as a T cell modulator in multiple sclerosis. In: Litwack G (ed.), Vitamins and hormones volume 86: vitamins and the immune system (Chapter 18, pp. 401–428), Elsevier, London (United Kingdom).

1. Multiple sclerosis

Multiple sclerosis is an inflammatory disease of the central nervous system (CNS). The prevalence of MS in the Netherlands is about 100 per 100.000 subjects. Similarly to most other autoimmune diseases, MS affects more females than males. The female/male-ratio has increased during the past decades, and is now estimated to be about 3.0 (Orton *et al.*, 2006). The age at disease onset is typically between 20 and 50 years of age, although MS can also sporadically have its onset during childhood ($\approx 5\%$) or in the elderly (Compston and Coles, 2008). The exact cause of MS is uncertain, but MS has been argued to arise from an unlucky interplay of several genetic and environmental risk factors. Several risk alleles within different genes have been identified in genome wide association studies, but the most prominent remain within the MHC class II region (International Multiple Sclerosis Genetics Consortium *et al.*, 2007). Many environmental risk-factors for MS have been proposed. The most well-consolidated, is the Epstein Bar Virus (EBV) infection. MS patients experienced prior to MS onset two times more symptomatic EBV infections (infectious mononucleosis) when compared with healthy individuals (Handel *et al.*, 2010). Higher anti-EBV antibody titers were found in MS patients prior to MS onset versus matched healthy controls (Ascherio *et al.*, 2010). Interestingly, EBV-negative MS patients were not to be found (Levin *et al.*, 2010). Additionally, smoking has been shown to increase the risk on MS especially in subjects with high anti-EBV titers (Simon *et al.*, 2010). Besides identification of new risk-factors, exploration of the exact interactions between these different genetic and environmental risk-factors is an important issue in MS research.

The diagnosis of MS was, until recently, exclusively based on clinical manifestations with the Schumacher and later the Poser criteria (Poser *et al.*, 1983; Schumacher *et al.*, 1965). Recently, newer diagnostic criteria became in use, integrating newer diagnostic modalities as MRI in the diagnosis of MS (McDonald *et al.*, 2001; Polman *et al.*, 2005). However, the keystone in the diagnosis of MS is the dissemination of symptoms arising from CNS dysfunction in space and time. At onset, patients most frequently experience sub-acute attacks of neurological impairment, which resolve spontaneously after several weeks. At this stage, the disease is called relapsing remitting MS (RRMS) (**Figure 1**). Although impairment resolves completely at disease onset, in later stages of disease recovery is not complete and impairment accumulates. Although some patients only experience a limited amount of disability throughout their disease course of MS, many will in time develop a gradually worsening of symptoms without attacks. In this stage the disease is called secondary progressive MS (SPMS). A small proportion of patients experiences progressive disability from disease onset onwards without exacerbations, and in these patients the disease is called primary progressive MS (PPMS) (Compston and Coles, 2002). At disease onset, there have been only few tools identified to predict the severity of

the clinical course of an individual patient. The impairment which patients experience is diverse in quantity and quality, and can arise from dysfunction of any component of the CNS. Although loss of motor function and subsequent loss of mobility are most well recognized, symptoms can also include visual impairment, sensory impairment, balance disorders, bowel dysfunction, sexual dysfunction, cognitive dysfunction, etc (Compston and Coles, 2002). Several clinimetric scales have been developed to monitor disability of MS. Although having clear limitations, the most widely used scale to express MS severity is the Expanded Disability Status Scale (EDSS) (Kurtzke 1983). This scale rates neurological impairment due to MS in a scale of 0 to 10 (**Table 1**).

Figure 1 Multiple Sclerosis disease course
Schematic presentation of the disease course of MS. During the relapsing remitting phase, the disease is mostly characterized by inflammation. In the later stages of MS, axonal loss and loss of brain volume are predominant features (Adapted with permission of Elsevier from: Compston A, Coles A. Multiple Sclerosis. *Lancet* 2002;359:1221–1231).

Table 1 Kurtzke's Expanded Disability Status Scale (EDSS)

0.0	Normal neurological examination
1.0	No disability, minimal signs in one functional system
1.5	No disability, minimal signs in more than one functional system
2.0	Minimal disability in one functional system
2.5	Mild disability in one functional system or minimal disability in two functional systems
3.0	Moderate disability in one functional system, or mild disability in three or four functional systems. Fully ambulatory
3.5	Fully ambulatory but with moderate disability in one functional system and more than minimal disability in several others
4.0	Fully ambulatory without aid, self-sufficient, up and about some 12 hours a day despite relatively severe disability; able to walk without aid or rest some 500 meters
4.5	Fully ambulatory without aid, up and about much of the day, able to work a full day, may otherwise have some limitation of full activity or require minimal assistance; characterized by relatively severe disability; able to walk without aid or rest some 300 meters
5.0	Ambulatory without aid or rest for about 200 meters; disability severe enough to impair full daily activities
5.5	Ambulatory without aid or rest for about 100 meters; disability severe enough to preclude full daily activities
6.0	Intermittent or unilateral constant assistance required to walk about 100 meters with or without resting
6.5	Constant bilateral assistance required to walk about 20 meters without resting
7.0	Unable to walk beyond approximately five meters even with aid, essentially restricted to wheelchair
7.5	Unable to take more than a few steps; restricted to wheelchair; may need aid in transfer
8.0	Essentially restricted to bed or chair or perambulated in wheelchair, but may be out of bed itself much of the day
8.5	Essentially restricted to bed much of day
9.0	Confined to bed; can still communicate and eat.
9.5	Totally helpless bed patient
10.0	Death due to MS

Adapted from: Kurtzke JF. Rating neurologic impairment in multiple sclerosis; An expanded disability status scale (EDSS). *Neurology* 1983;33:1444.

2. The T cell compartment in multiple sclerosis

Pathological and radiological studies of the CNS of MS patients revealed that pathogenesis is characterized by two phenomena: i) focalized inflammation of CNS tissue, and ii) loss of neurons (Compston and Coles, 2002). Although the causality of these phenomena has often been disputed, there is increasing consensus that inflammation is the primary driver of the disease process of MS. T cell-mediated inflammation in the CNS is most prominent at disease onset (**Figure 1**), and evidence of a

disturbed peripheral T cell homeostasis in MS patients at disease onset is mounting (Venken *et al.*, 2010). Additionally, large genetic screens revealed almost exclusively immune-related risk alleles for developing MS (International Multiple Sclerosis Genetics Consortium *et al.*, 2007). Furthermore, even in patients with progressive disease, compartmentalized inflammation of the CNS remains a key characteristic in the pathological image of MS (Frischer *et al.*, 2007). Many clinical and experimental studies have addressed the immune-pathogenesis of MS (reviewed in Bar-Or 2007; Goverman, 2009).

Figure 2 T cell response in MS.

Simplified model of the T cell response in patients with MS. Regulatory T cells (Treg) fail to maintain peripheral tolerance, and naïve T cells are primed in the periphery (1). Subsequently, activated auto-reactive T cells enter the circulation and cross the blood brain barrier (2). In the central nervous system, they are reactivated by APC (3), and contribute to the activation of several pro-inflammatory cells including other lymphocytes, macrophages and microglia cells (4). This activation results in an inflammatory response which attacks the myelin sheet surrounding the neurons. This inflammation results in loss of neuronal function and ultimately in loss of neurons (5). It has been postulated that fragments of degraded myelin fragments are presented again in the periphery to maintain the immune cascade (6).

The attack of the immune system on the CNS starts in the peripheral lymphoid organs, where antigen presenting cells (APC) present an epitope to naïve T cells (**Figure 2**). The nature of this epitope is uncertain, but a viral protein mimicking CNS proteins, soluble factors leaking from the CNS, and CNS proteins actively transferred by dendritic cells (DC) from the CNS have been proposed. Auto-reactive CD4⁺ T cells loose there tolerant state for self-antigens, are primed, and enter the circulation. These cells adopt a pro-inflammatory cytokine profile, including production of interferon gamma (IFN- γ) by T helper type 1 (Th1) cells, and interleukin 17 (IL-17) by

Th17 cells. Patient-control studies revealed that peripheral regulatory mechanisms to maintain T cell tolerance are impaired in MS patients. In a healthy T cell compartment, regulatory CD4⁺ T cells (Tregs) are capable of controlling the quality and quantity of the immune response (Sakaguchi *et al.*, 2008). In patients with a number of autoimmune diseases, including MS, these Tregs have been found to be less effective suppressors of T-cell responses, while their number in the circulation is not affected (Constantino *et al.*, 2008; Venken *et al.*, 2005; Viglietta *et al.*, 2004). This defective control mechanism enables the activation of auto-reactive T cells. The auto-reactive T cells attach to adhesion molecules on the endothelium of the blood brain barrier (including very late antigen 4 (VLA-4) receptors), and actively enter the CNS by secreting metalloproteinases. In the CNS, they are re-activated by resident APC, including macrophages and microglia cells. Subsequently, the T cells contribute to a focalized inflammatory reaction, in which the myelin sheet surrounding axons is degraded. This process leads to neuronal dysfunction, neuronal damage, and ultimately loss of neurons.

3. Treatment of multiple sclerosis

MS cannot be cured. The registered MS drugs so far are mostly either modulators or suppressors of the peripheral T cell compartment. Beta Interferons and Glatiramer Acetate skew the balance between pro-inflammatory Th1 and anti-inflammatory Th2 cells towards a Th2 phenotype, and promote the suppressive function of Tregs as well as the induction of naïve Tregs (Schrempf and Ziemsen, 2007; de Andres *et al.*, 2007). Therapies like mitoxantrone and cyclophosphamide are cytotoxic for T cells in the periphery (Neuhaus *et al.*, 2005). The newer therapy Nataluzimab (anti-VLA-4 antibodies) prevents activated T cells from entering the CNS (Miller *et al.*, 2003). Efficacy of these MS disease modulating therapies has only been shown in RRMS patients. Additionally, treatment with Interferon Beta was more efficacious when started early after the diagnosis of MS (Kappos *et al.*, 2007). Longitudinal studies show that patients vary in time-course until an EDSS-score of 3, but that the progression from EDSS-score 3 to 6 shows few variation between patients (Leray *et al.*, 2010). Therefore, modulation or suppression of the peripheral T cell compartment appears to be most efficacious at the start of MS, when disability is limited. Therapeutic trials in progressive MS patients have not delivered efficacious treatments so far. Regarding the severe side effects of the immune suppressive therapies, most patients start with immune modulating drugs. These drugs reduce the number of MS exacerbations with 30%, but at the cost of several side effects, including influenza-like illness, headache, nausea, and injection-site erythema. The last years, attention has been drawn on vitamin D as a potential therapy in MS.

4. Aim of this thesis

The aim of this thesis is to assess whether vitamin D supplementation can be developed as an additional treatment modality in MS. Therefore, this thesis addresses two main questions: i) is vitamin D₃ a modulator of MS disease activity? ii) which biological processes underlie this modulation?

5. Outline of this thesis

Chapter 2 describes a review of literature as was available at the start of the project, with some later updates on the experimental work. We show that vitamin D is a potent modulator of the adaptive immune response *in vitro* and in animal models of MS, but that it is uncertain whether vitamin D is also an *in vivo* modulator of the immune system in patients with MS. To address this problem, we firstly assessed in **chapter 3** an association of vitamin D status with disease severity/ activity of MS in our MS population in South Limburg, The Netherlands. Subsequently, we tested in **chapter 4** whether peripheral T cell homeostasis correlated in these patients with their vitamin D status, and extended these data in **chapter 5** by exploring the role of calcium homeostasis in this association. Additionally, the effect of supplementation of high doses of vitamin D₃ on the peripheral T cell compartment was assessed in the pilot study presented in **chapter 6**. In the pilot study, the safety of vitamin D₃ supplementation was also analyzed. As an addition to these studies, we explored in **chapter 7** the effect of a genetic polymorphism of the vitamin D receptor with structural and functional consequences on the risk on developing MS and vitamin D status per se. The results of these studies were all combined in the design of a large randomized placebo-controlled clinical trial, of which the study design is shown in **chapter 8**. The most important conclusions of this thesis will be discussed in **chapter 9**.

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Abstract

The role of vitamin D in calcium homeostasis is well known. More recently vitamin D has become a topic of interest in immune regulation and multiple sclerosis. The main reason for this is the observed geographical distribution of multiple sclerosis. Areas with high sunlight exposure, the principal inducer of vitamin D synthesis, have a relatively low prevalence of multiple sclerosis and vice versa. Furthermore, low levels of the principal vitamin D metabolite (25-hydroxyvitamin D) in the circulation are associated with a high incidence of multiple sclerosis. Other epidemiological evidence also supports the view that vitamin D metabolites have an immune and disease modulating effect in multiple sclerosis. Experimental research in vitro and in animal models has further clarified the interaction of vitamin D metabolites with the immune system. The evidence obtained from these studies strongly supports a model in which vitamin D mediates a shift to a more anti-inflammatory immune response, and in particular to enhanced regulatory T-cell functionality. In the current review we link the basic knowledge on vitamin D and immune regulation with the vitamin D related observations in multiple sclerosis. We conclude that there is a sound basis on which to initiate double-blind placebo-controlled trials that not only address the effect of vitamin D on the clinical outcome of multiple sclerosis, but also on the regulatory T-cell compartment.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) of unknown aetiology (Compston and Coles, 2006). Although a genetic susceptibility for the development of MS is well known, exposure to as yet undefined environmental risk factors is also required (Hauser and Oksenberg, 2006). Apart from viral infections and geographical variation in food intake, environmental supplies of vitamin D have been proposed as a possible risk factor for developing MS (Goldberg *et al.*, 1986). There is geographical, biological and immunological evidence that exposure to low environmental supplies of vitamin D is associated with an increased risk of developing MS. Although vitamin D is best known as a modulator of calcium homeostasis, it also has strong immune modulating potential. When considering MS as an autoimmune disorder, the idea of a beneficial immune modulating effect of vitamin D is logical. This has been investigated *in vitro*, in experimental allergic encephalomyelitis (EAE), an animal model of MS and *in vivo*, in MS patients.

The aim of this article is to review the evidence for the potential immune modulating effect of vitamin D in MS. We will take into account not only observations in the human MS population, but also experimental research *in vitro* and in animal models. A possible therapeutic application of vitamin D in MS patients will be discussed.

2. Vitamin D

Vitamins are defined as substances of whom we are dependent upon our environment and which are vital for biological processes in the body, apart from their caloric value. While most vitamins are acquired via dietary intake, vitamin D is a unique vitamin in this perspective. The largest part is synthesized in the skin under the influence of ultraviolet B (UVB) radiation (Ohmdahl *et al.*, 2005). Out of 7-dehydrocholesterol, UVB radiation forms pre-vitamin D, which transforms spontaneously as a result of body temperature to cholecalciferol or vitamin D₃. The vitamin D which is acquired via the diet contains both cholecalciferol, derived from animal products, and ergocalciferol (vitamin D₂), which is derived from plant products. It is uncertain whether these forms of vitamin D differ in biological function. The contribution of dietary vitamin D intake to the total daily amount of vitamin D acquired is modest. Total body sun exposure easily provides the equivalent of an oral intake of 250 µg (10 000 IU) vitamin D₃, whereas the Office of Dietary Supplements of the National Institute of Health (USA) indicates 5 µg (200 IU) as an adequate daily oral intake for adults (Vieth, 1999). However, depending on the geographical latitude, the amount of vitamin D effective UVB radiation is limited through winter in large

areas on the globe (Kimlin 2008). In British children and elderly, it was shown that vitamin D intake only predicts vitamin D status during winter, and not in the other seasons (Bates *et al.*, 2003; Davies *et al.*, 1999).

In the circulation, only small amounts of vitamin D are present. This molecule has a biological halflife of 12–16 hours (Smith and Goodman 1971), and fluctuates depending on the exposure to either sunlight or dietary intake (Hollis *et al.*, 2007). It is entirely bound to a specialized carrier protein, vitamin D binding protein (DBP). The vitamin D is almost instantly hydroxylated in the liver by 25-hydroxylases (CYP2R1, CYP27A1, and CYP3A4) towards 25-hydroxyvitamin D (25(OH)D) (**Figure 1**). This is the most abundant metabolite in the circulation and is most accepted to reflect the overall vitamin D status of an individual. It has a half-life of 20–90 days and is bound for about 90% to DBP (Smith and Goodman 1971; Mawer *et al.*, 1969). The other 10% is primarily bound to other serum proteins including albumin and only a small fraction of about 0.40% is freely available. Although 25(OH)D is the most abundant vitamin D metabolite, it is not biologically active. Another hydroxylation step by 1- α -hydroxylase (CYP27B1) is required to form the biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)₂D). The serum levels of 1,25(OH)₂D are mostly dependent on expression of 1- α -hydroxylase in the kidneys upon signals from calcium metabolism (Jongen *et al.*, 1984). The biological effects of 1,25(OH)₂D result from either direct actions at the cell membrane, or via modulation of gene transcription via intracellular binding to the vitamin D receptor (VDR). When bound to 1,25(OH)₂D, the VDR forms heterodimers with the retinoid X receptor (RXR), and moves to the nucleus of the cell. Here, it binds to a vitamin D response element, and causes transcription or transrepression of vitamin responsive genes (**Figure 2**) (reviewed in Smolders *et al.*, 2009c).

Figure 1 Vitamin D metabolism.

Vitamin D is acquired via diet and via photosynthesis in the skin, dependent on the amount of intake and sun-exposure, respectively. In the body, vitamin D is instantly hydroxylized in 25-hydroxyvitamin D (25(OH)D), the most abundant vitamin D metabolite. This metabolite can be further hydroxylized in the biologically active form, 1,25-dihydroxyvitamin D (1,25(OH)₂D). This metabolite interacts with the vitamin D receptor, which binds to vitamin response elements (VDRE) in the nucleus and induces transcription or transrepression of vitamin D responsive genes. This interaction affects calcium metabolism, cell proliferation and immune regulation.

Figure 2 Intracellular organisation of the VDR.

A) In the absence of 1,25(OH)₂D, VDR shuttles between cytoplasm and the nucleus. The VDR can dimerize with RXR, but the formed heterodimer is not stable and has a low affinity for the VDRE. This results in basal regulation of target gene transcription. B) Upon binding 1,25(OH)₂D, VDR is activated and translocated to the nucleus. The VDR/RXR heterodimer is stabilised, giving it a high affinity for the VDRE, which results in an increased transactivation or transrepression of the genes. The size of the arrows indicates the degree of the respective processes (dimerization, translocation, DNA binding or transcription). VDR: vitamin D receptor; RXR: retinoid X receptor (Adapted from: Smolders et al. 2009c).

3. Vitamin D and calcium homeostasis

The role of vitamin D in calcium metabolism is most well known. During the Industrial Revolution in the nineteenth century, rickets reached almost epidemic proportions in ill-fed children living in cities with little sunlight, in the northern countries. A beneficial effect of sunlight and cod-liver oil was observed, but it was not until the 1930's that vitamin D was identified as the therapeutic element in those therapies (Bouillon, 2005). Vitamin D is an essential component for maintaining a stable calcium level in the circulation. If the serum calcium level drops, chief cells in the parathyroid gland start secreting parathyroid hormone (PTH). Besides some direct effects on bone metabolism, PTH predominantly catalyses, via CYP27B1, the hydroxylation of 25(OH)D to 1,25(OH)₂D in the kidneys. Elevated serum 1,25(OH)₂D levels lead to an increased uptake of calcium from the intestines, increased resorption of calcium in the proximal tubules of the kidneys, and an increased osteoclast activity. The subsequent rise of serum calcium restores the balance between serum calcium and PTH. The availability of too little of the precursor 25(OH)D to be hydroxylized in 1,25(OH)₂D results in a decompensation of this system. Since a stable serum calcium level is vital for many processes in the body, calcium is retrieved from the skeleton to compensate for the loss of calcium via kidneys and intestine. Therefore, a poor vitamin D status has been associated with loss of bone mineral density (BMD) (Bischof-Ferrari *et al.*, 2004), and increased risk of fractures (Lopes *et al.*, 2009; Cauley *et al.*, 2010). Supplementation of vitamin D with or without calcium has been shown to improve BMD (Tang *et al.*, 2007) and to decrease fracture risk (Bischof-Ferrari *et al.*, 2005) in the elderly. The latter effect may be due to an increased BMD, but additionally supplementation of vitamin D also decreases the risk of falling (Bischof-Ferrari *et al.*, 2009). The decreased risk of falling may be attributable to a favorable effect of vitamin D on muscle strength (Stewart *et al.*, 2009).

Both the concentration where serum PTH concentration just starts to rise above normal values and the level at which effective fracture prevention is achieved, have been suggested as reliable measurements for the level of 25(OH)D required for a normal bone metabolism. An estimated optimal plasma 25(OH)D value of 50–80 nmol/L has been proposed (Dawson-Hughes *et al.*, 2005). Although these levels are required for a normal bone homeostasis, it has been argued that a higher 25(OH)D level is required to optimise other biological functions of vitamin D (Vieth, 2006).

4. Vitamin D in the central nervous system

In the CNS, expression of VDR has been described in different cell types (Garcion *et al.*, 2002). CYP27B1 (1 α -hydroxylase) and CYP24A1 (24-hydroxylase) are also expressed in the CNS (Overbergh *et al.*, 2000; Zehnder *et al.*, 2001). Therefore, the

CNS may be a site of action, metabolism and catabolism of vitamin D. Intra-uterine vitamin D depletion leads to brain development disorders and reduced levels of nerve growth factor (NGF) at birth in rats, underlining the importance of vitamin D in the brain (McGrath *et al.*, 2004). A rise in VDR transcription and NGF transcription and synthesis by astrocytes on $1,25(\text{OH})_2\text{D}$ stimulation has been reported (Garcion *et al.*, 2002; Neveu *et al.*, 1994). In microglial cells, APC's within the CNS, an inhibition of TNF- α , IL-6 and Nitric Oxide production was observed *in vitro* (Lefebvre d'Hellencourt *et al.*, 2003). These data suggest a paracrine function of vitamin D in the CNS.

5. Vitamin D and the immune system

5.1 Vitamin D receptor expression by immune cells

Numerous immune cells express the vitamin D receptor. Firstly, the expression of VDR mRNA by monocytes and activated B and T cells was recognized (Provvedini *et al.*, 1983). Measured by ELISA, both CD4^+ and CD8^+ T cells expressed VDR protein, which was up-regulated upon activation and exposition to $1,25(\text{OH})_2\text{D}$ (Veldman *et al.*, 2000). In resting naïve T cells, VDR protein levels were absent, but up-regulated on stimulation with anti-CD3 and anti-CD28 via the p38 TCR-signaling pathway (Von Essen *et al.*, 2010). In purified resting CD4^+ T cells, VDR mRNA expression was almost absent, but was up-regulated upon activation with phytohaemagglutinin (PHA) or anti-CD3 (Correale *et al.*, 2009). Addition of $1,25(\text{OH})_2\text{D}$ further up-regulated VDR mRNA expression. Resting mature myeloid and plasmacytoid DC's, as well as immature myeloid DC's and monocytes, expressed similarly high levels of VDR mRNA, which were not further enhanced by addition of $1,25(\text{OH})_2\text{D}$ (Penna *et al.*, 2007). Others observed low, but detectable levels of VDR expression in monocytes, which were instantly up-regulated by a DC differentiation cocktail comprising granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4 (Széles *et al.*, 2009). Resting B cells express undetectable amounts of VDR mRNA, but after activation or exposure to $1,25(\text{OH})_2\text{D}$, expression of the VDR is up-regulated (Chen *et al.*, 2007).

It can be concluded that APC express the receptor for $1,25(\text{OH})_2\text{D}$, irrespective of activation or $1,25(\text{OH})_2\text{D}$ exposition, while resting lymphocytes express very low amounts of VDR, which is up-regulated on activation or exposition to $1,25(\text{OH})_2\text{D}$. Therefore, immune cells appear to be a target for $1,25(\text{OH})_2\text{D}$. Additionally, lymphocytes appear to become more susceptible for the actions of $1,25(\text{OH})_2\text{D}$ when activated.

5.2 Metabolism of vitamin D by immune cells

The largest proportion of the vitamin D metabolites present in the circulation, lymphoid organs, and tissues, comprises $25(\text{OH})\text{D}$. Only a small part (0.1%) consists of

the biologically active metabolite 1,25(OH)₂D. Numerous immune cell types, however, have the ability to express 25(OH)D-1 α -hydroxylase (CYP27B1) and actively form 1,25(OH)₂D. Activated T cells up-regulate the expression of CYP27B1, either on activation with anti-CD3 and anti-CD28 in a T cell/DC co-culture (Sigmundsdottir *et al.*, 2007), or on activation with PHA in a CD4⁺ T cell monoculture (Correale *et al.*, 2009). *In vitro*, this up-regulation also results in the formation of 1,25(OH)₂D out of 25(OH)D (Sigmundsdottir *et al.*, 2007; Correale *et al.*, 2009). Several APC, including macrophages and DC, also express high levels of CYP27B1. Resting blood DC's synthesize limited amounts of 1,25(OH)₂D, which is dramatically up-regulated by activation with LPS, accompanied by an up-regulation of CYP27B1 expression (Fritsche *et al.*, 2003). The presence of 1,25(OH)₂D inhibits the expression of CYP27B1. In addition, human resting monocytes do not express CYP27B1, whereas *in vitro* differentiated macrophages and DC do express CYP27B1 and subsequently form 1,25(OH)₂D out of 25(OH)D (Gottfried *et al.*, 2006; Széles *et al.*, 2009). B-cells are also able to form 1,25(OH)₂D out of 25(OH)D upon activation with several activation cocktails (Chen *et al.*, 2007; Heine *et al.*, 2008). Additionally, B cells express 1,25(OH)₂D-24-hydroxylase (CYP24A1) on exposure to 1,25(OH)₂D when in an activated state (anti-IgG/anti-CD40/ IL-21) (Chen *et al.*, 2007; Heine *et al.*, 2008). Also, monocytes, macrophages and DC (Gottfried *et al.*, 2006; Penna *et al.*, 2007), as well as T cells (Correale *et al.*, 2009) express CYP24A1 upon exposure to 1,25(OH)₂D. To evoke catabolism of 1,25(OH)₂D, activation of these cells is not mandatory.

In summary, activation of an immune response is accompanied by a local induction of 1,25(OH)₂D synthesis out of the locally available 25(OH)D (**Figure 3**). When 1,25(OH)₂D is abundantly present, both activated and resting immune cells catabolize this metabolite. The observations that almost all immune cells carry the VDR and metabolize its ligand upon activation, suggest that vitamin D might have an important autocrine function for immune regulation. Interestingly, CYP27A1, the enzyme which catalyzes the vitamin D-25-hydroxylation, is also expressed by macrophages and DC's upon activation, resulting in a local formation of 25(OH)D (Gottfried *et al.*, 2006; Sigmundsdottir *et al.*, 2007). The relevance of this mechanism in the periphery, where 25(OH)D is abundantly available, is probably limited. However, in the skin, where vitamin D₃ itself is mostly present, this mechanism is important to induce effective 1,25(OH)₂D synthesis.

5.3 *In vitro* effects of 1,25(OH)₂D on the immune response

Since activated immune cells synthesize 1,25(OH)₂D and express the VDR, 1,25(OH)₂D is likely to affect the immune response. Several studies investigated the effect of 1,25(OH)₂D exposition on the immune response *in vitro*. Studies on lymphocytes can be divided in studies which assessed the effect of 1,25(OH)₂D on proliferation, cytokine production, and the phenotype of cells. Unless stated otherwise, all data concern studies with human cells. Results are summarized in **table 1**.

Table 1 Direct in vitro effects of 1,25(OH)₂D on proliferation and cytokine production of immune cells

Cell population	Stimulus	Proliferation	Pro-inflammatory cytokines/ chemokines	Anti-inflammatory cytokines/ chemokines	Phenotypic markers	Reference
PBMC	PWM, PHA, DO, or SAC	Inhibited	↓IFN- γ , ↓GM-CSF, ↓IL-2, ↓IgM, ↓ IgG, ↓ IgA	-	-	Rigby <i>et al.</i> , 1984, Lemire <i>et al.</i> , 1984, Bhalla <i>et al.</i> , 1986, Reichel <i>et al.</i> , 1987, Tobler <i>et al.</i> , 1987, Iho <i>et al.</i> , 1986, Chen <i>et al.</i> , 1987, Shiozawa <i>et al.</i> , 1987, Müller <i>et al.</i> , 1992b
	Mixed-lymphocyte reaction	Inhibited	↓IFN- γ	-	-	Fritsche <i>et al.</i> , 2003, Penna and Adorini 2000
	Tetanus toxoid in vaccinated subjects	Inhibited	↓IL-2	-	-	Bhalla <i>et al.</i> , 1986
	Epstein Bar Virus	Unaffected	= IgM, = IgG, = IgA	-	-	Müller <i>et al.</i> , 1992b
T cells (purified or enriched populations)	Anti-CD3 coated with(out) anti-CD28 monoculture	Unaffected	= IFN- γ	-	-	Penna and Adorini 2000
	PHA	Inhibited	↓IFN- γ , ↓GM-CSF	-	-	Reichel <i>et al.</i> , 1987, Tobler <i>et al.</i> , 1987
CD4+ T cells	PHA monoculture	Inhibited	[↓IL-6, ↓IL-17] ^o	[↑IL-10] ^o	-	Correale <i>et al.</i> , 2009
	Cognate antigen with irradiated PBMC	Inhibited	[↓IL-6, ↓IL-17] ^o	[↑IL-10] ^o	-	Correale <i>et al.</i> , 2009
	Anti-CD3, anti-CD28 monoculture	Unaffected	↓IFN- γ , ↓IL-2, ↓IL-17, ↓IL-21	↑IL-4, ↑IL-5, ↑IL-10	↑FoxP3, ↑CTLA-4, ↑CD25 ⁺ FoxP3 ⁺	Barrat <i>et al.</i> , 2002, Jeffery <i>et al.</i> , 2009, Correale <i>et al.</i> , 2009
	Anti-CD3 coated monoculture	-	↓IL-6, ↓IL-17	↑IL-10	-	Correale <i>et al.</i> , 2009

Cell population	Stimulus	Proliferation	Pro-inflammatory cytokines/ chemokines	Anti-inflammatory cytokines/ chemokines	Phenotypic markers	Reference
CD8 ⁺ T cells	Anti-CD3 with autologous monocytes	Slightly inhibited	↓IFN- γ , ↓IL-2, ↓IL-17, ↓IL-21	↑IL-10	↑CTLA-4, = FoxP3	Jeffery <i>et al.</i> , 2009
	OVA + APC	-	↓IFN- γ	↑IL-4, ↑IL-5, ↑IL-10	-	Barrat <i>et al.</i> , 2002, Boonstra <i>et al.</i> , 2001
	Con-A	Slightly promoted	-	-	-	Veldman <i>et al.</i> , 2000
Monocytes ^b	PHA	-	↓IL-2, ↓IFN- γ	↑/↓IL-4, ↑IL-13	-	Thien <i>et al.</i> , 2005, Pichler <i>et al.</i> , 2002
	IL-4, GM-CSF (exposed to 1,25(OH) ₂ D during 6–7 days differentiation towards M-DC)	-	-	-	↓CD1a, ↑CD14, ↑CD32, =/CD40, =CD54, =/↓CD86, ↑MR, ↑MHC-I, =/↓MHC-II	Penna and Adorini 2000, van Halteren <i>et al.</i> , 2001, Piemonti <i>et al.</i> , 2000
	LPS, CD40L (exposed to 1,25(OH) ₂ D during 7 days differentiation towards M-IDC on GM-CSF + IL-4)	-	↓IL-12 p70	=IL-10	↓CD1a, ↑CD14, ↑CD32, =/↓CD40, =CD54, =/↓CD80, ↓CD83, ↓CD86, ↑MR, ↓MHC-I, ↓MHC-II	Piemonti <i>et al.</i> , 2000
Immature M-DC (derived from isolated monocytes)	IL-4, GM-CSF (exposed to 1,25(OH) ₂ D after 7 days differentiation towards M-DC on GM-CSF + IL-4)	-	-	-	↓CD1a, ↑CD14, ↓/=CD40, =CD80, ↓CD86, =MHC-I, ↓MHC-II	Piemonti <i>et al.</i> , 2000

Cell population	Stimulus	Proliferation	Pro-inflammatory cytokines/ chemokines	Anti-inflammatory cytokines/ chemokines	Phenotypic markers	Reference
	LPS, CD40L (exposed to 1,25(OH) ₂ D, GM-CSF and IL-4 after 7 days differentiation towards M-DC on GM-CSF + IL-4).	-	-	-	↓CD40, ↓CD80, ↓CD83, ↓CD86, ↓MHC-I, ↓MHC-II	Piemonti <i>et al.</i> , 2000
	LPS, CD40L (exposed to 1,25(OH) ₂ D during maturation)	-	↓IL-12p70, ↓IL-12 p75	↑IL-10	=CD1a, ↑CD14, =/↓CD40, =CD54, ↓CD58, =/↓CD80, =/↓CD83, =/↓CD86, ↑MR, =/↓MHC-II	Penna and Adorini 2000, van Halteren 2002
Blood derived M-DC	Unstimulated and CD40L	-	↓CCL17, ↓IL-12 p75	↑CCL22	↓CD40, ↓CD80, ↓CD86, ↓MHC-II, ↑ILT3	Penna <i>et al.</i> , 2007
B cells	Anti-IgM, anti-CD40, IL-21	Inhibited	↓ IgG,	-	↑CD38, ↓IgG, ↑IgM	Chen <i>et al.</i> , 2007
	B cell crosslinking, anti-CD40, IL-4	Unaffected	IgG unaffected	↑ IL-10	↑CD38	Heine <i>et al.</i> , 2009

Direct effects of 1,25(OH)₂D on immune cells as observed in *in vitro* cultures. The effect of 1,25(OH)₂D on proliferation, cytokine/ chemokine/ antibody production and phenotype of the given cell populations when added to cultures with the given stimuli are designated as ↑ : increased; ↓ : decreased; = : not significantly different. Abbreviations used: CCL: chemokine (C-C motive) ligand; Con-A: Concanavalin A; DO: dermatophyton O; ILT3: immunoglobulin-like transcript 3; IL: interleukin; M-DC: myeloid dendritic cell; PBMC: peripheral blood mononuclear cell; PHA: Phytohemagglutinin; PWM: Pokeweed Mitogen; SAC: Staphylococcus Aureus Cowan I. ^aThese results were not shown, but described in the paper by Corneale *et al.* 2009. ^bOlder studies on monocytes are not included in the present table, since phenotyping differs from the current studies. These studies comprise: Ohta 1985, Bhalla *et al.*, 1986, Rigby *et al.*, 1990, d'Ambrosio *et al.*, 1998 and Müller *et al.*, 1992b.

Addition of 1,25(OH)₂D to cell cultures directly inhibits proliferation of peripheral blood mononuclear cells (PBMC), T cells and CD4⁺ T cells on several mitogenic (Rigby *et al.*, 1984; Lemire *et al.*, 1984, Correale *et al.*, 2009, Fritsche *et al.*, 2003) and antigen-specific stimuli (Correale *et al.*, 2009). Purified CD45RO⁺ T cells were more responsive to 1,25(OH)₂D suppression than CD45RA⁺ T cells, suggesting a predominant suppression of memory T cells (Müller and Bendtzen, 1992a). Interestingly, monocultures of T cells stimulated with anti-CD3 and anti-CD28 could not be suppressed directly by 1,25(OH)₂D (Penna and Adorini, 2000; Jeffery *et al.*, 2009). Additionally, proliferation of purified naïve T cells of chronic haemodialysis patients on anti-CD3 and anti-CD28 has even been reported to be promoted by 1,25(OH)₂D (Von Essen *et al.*, 2010). It can be concluded that 1,25(OH)₂D can directly suppress proliferation of PBMC and CD4⁺ T cells unless the stimulus is too strong (anti-CD3 and anti-CD28), and rather suppresses proliferation of memory than naïve T cells. Addition of the precursor 25(OH)D also suppresses proliferation of PBMC in mixed-lymphocyte reactions (Fritsche *et al.*, 2003), and PHA-driven proliferation of CD4⁺ T cells in monoculture (Correale *et al.*, 2009). The metabolism of 25(OH)D by CYP27B1 upon activation therefore seems to have autocrine effects, modulating the functional behavior of the lymphocytes. Since 25(OH)D is also the most abundantly available vitamin D metabolite *in vivo*, the latter experimental model might also be the most relevant for studying vitamin D and lymphocyte interactions. Catabolites of vitamin D, 24,25(OH)₂D and 25,26(OH)₂D do not induce suppression of proliferation (Correale *et al.*, 2009).

Much attention has been paid to the effects of 1,25(OH)₂D exposition on the cytokine production by activated T cells *in vitro*. Addition of 1,25(OH)₂D suppressed pro-inflammatory cytokine production in cultures of PBMC, T cells or CD4⁺ T cells stimulated with mitogens (Rigby *et al.*, 1984, Reichel *et al.*, 1987; Tobler *et al.*, 1987; Müller *et al.*, 1991), with T cell receptor agonists (Correale *et al.*, 2009), or specific antigens (Boonstra *et al.*, 2001; Jeffery *et al.*, 2009). Cytokines suppressed by 1,25(OH)₂D included IFN- γ , IL-2, IL-6, IL-17, IL-21, and GM-CSF. In addition to the suppression of pro-inflammatory cytokines, the production of anti-inflammatory cytokines seemed to be promoted. Addition of 1,25(OH)₂D to T cell monocultures or DC co-cultures, stimulated with T cell receptor agonists (Boonstra *et al.*, 2001; Barrat *et al.*, 2002, Correale *et al.*, 2009) or specific antigens (Boonstra *et al.*, 2001; Barrat *et al.*, 2002) resulted in increased production of IL-4, IL-5, and IL-10. These studies show that exposition of activated T cells to 1,25(OH)₂D results in a skewing of pro-inflammatory towards anti-inflammatory cytokine production. The effects seen in monocultures reveal that there is a direct interaction between vitamin D and T cells.

The shift in proliferation and cytokine production of CD4⁺ T cell, has also been reflected in phenotypic changes. Culture of PBMC in the presence of 1,25(OH)₂D resulted in a dramatically increased proportion of functionally active Tregs

(CD25⁺FoxP3⁺ CD4⁺ T cells) (Correale *et al.*, 2009). In addition, in an anti-CD3 and anti-CD28-driven monoculture of CD4⁺ T cells, 1,25(OH)₂D treatment induced FoxP3 and CTLA-4 expression (Jeffery *et al.*, 2009), again suggesting the induction of Tregs. Interestingly, the up-regulation of FoxP3 was completely abrogated by the stimulation with autologous monocytes and anti-CD3, which was again restored by addition of IL-2 (Jeffery *et al.*, 2009). Altogether, 1,25(OH)₂D appears to induce a regulatory T cell phenotype in activated CD4⁺ T cells. The combination of proliferation, cytokine production and phenotypic analysis shows that vitamin D induces directly a more tolerogenic CD4⁺ T cell compartment (**Figure 3**).

Figure 3 Vitamin D and the adaptive immune response

At sites of immune activation, expression of CYP27B1 and subsequent formation of 1,25(OH)₂D out of 25(OH)D is induced in activated immune cells. This results in a micro environment of high local levels of 1,25(OH)₂D. Binding of 1,25(OH)₂D to the VDR, which is present in almost every immune cell, results in a functional modulation of APC/DC and activated B and T cells. Vitamin D exerts its effects on lymphocytes both directly and via modulation of APC/DC. When exposed to 1,25(OH)₂D, lymphocytes and DC's express CYP24A1, catalyzing the inactivation of 1,25(OH)₂D. Altogether, activation of the adaptive immune response is accompanied by local activation of 25(OH)D, subsequently establishing increased levels of homeostasis.

Direct effects of 1,25(OH)₂D on CD8⁺ T cells are uncertain. CD8⁺ T cells do express the VDR protein and, in contrast to CD4⁺ T cells, exposition to 1,25(OH)₂D modestly improved proliferation rate (Veldman *et al.*, 2000). However, the cytotoxicity of CD8⁺ T cells, as measured in a ⁵¹Cr release assay, was inhibited by 1,25(OH)₂D (Meehan *et al.*, 1992). The effects on cytokine production match these of the CD4⁺ T cells, with an inhibition of IL-2 and IFN-γ and promotion of IL-4 and IL-13 (Thien *et al.*,

2005). In cord blood samples cultured under the same conditions, however, IL-4 production by CD8⁺ T cells was suppressed by 1,25(OH)₂D (Pichler *et al.*, 2002). It can be concluded that the effect of 1,25(OH)₂D on CD8⁺ T cells has not been very consistent so far, probably because CD8⁺ T cells have received only little attention yet.

Besides the direct effects on the T cell compartment, 1,25(OH)₂D also affects the maturation and cytokine production of APC. In older studies, monocytes cultured with pro-inflammatory stimuli and 1,25(OH)₂D display a VDR-dependent loss of maturation markers (Rigby *et al.*, 1990; Griffin *et al.*, 2000) and a loss of IL-6, IL-12, and tumor necrosis factor α (TNF- α) production (d'Ambrosio *et al.*, 1998; Müller *et al.*, 1992b). Treatment with 25(OH)D gives the same effects, albeit to a lesser extent. Alternatively, however, treatment of monocytes and macrophages with only 1,25(OH)₂D has been reported to increase the production of the pro-inflammatory cytokine IL-1 and cathelicidin antimicrobial peptide (Bhalla *et al.*, 1986; Gombart *et al.*, 2005). A promotion of monocyte proliferation has also been reported (Ohta *et al.*, 1985). Therefore, regarding monocytes and macrophages, the effects of vitamin D seem to depend on the way these cells are challenged. However, when monocytes are cultured supporting DC differentiation, the effect of 1,25(OH)₂D becomes more consistent. Addition of 1,25(OH)₂D to monocyte cultures hinders their differentiation to immature myeloid DC (Penna and Adorini 2000; van Halteren *et al.*, 2002; Piemonti *et al.*, 2000). Additionally, fully differentiated immature myeloid DC can also lose their DC phenotype after exposition to 1,25(OH)₂D, reflected by a loss of CD1a and upregulation of CD14 (Piemonti *et al.*, 2000). Different maturation protocols of immature myeloid DC to mature DC are also inhibited by 1,25(OH)₂D, as reflected by surface markers of maturation (Griffin *et al.*, 2001; Penna and Adorini 2000; Piemonti 2000; van Halteren *et al.*, 2002). Interestingly, functional consequences have also been described. As suggested by the preservation of the mannose receptor and CD32, the antigen uptake capacity of the 1,25(OH)₂D-matured myeloid DC is improved (Piemonti *et al.*, 2000). DC which reach a mature stage under 1,25(OH)₂D, produce almost no IL-12 and more IL-10 (Penna and Adorini 2000; van Halteren *et al.*, 2002). 1,25(OH)₂D treatment also induces apoptosis in mature myeloid DC (Penna and Adorini 2000). It can be concluded that 1,25(OH)₂D dramatically hampers maturation and affects function of myeloid DC. However, the phenotype and functionality of blood isolated plasmacytoid DC is not affected by 1,25(OH)₂D (Penna *et al.*, 2007).

Therefore, besides direct effects of 1,25(OH)₂D on the T cell compartment, vitamin D might also modulate the T cell response via the APC compartment. Indeed, pre-treatment of monocytes with 1,25(OH)₂D inhibited proliferation of T cells on tetanoid toxin in a co-culture (Rigby *et al.*, 1990). Interestingly, 25(OH)D pre-treatment also induced inhibition of proliferation, albeit to a lesser extent. Pre-treatment of P-DC with 1,25(OH)₂D did not affect T cell function in a subsequent co-

culture (Penna *et al.*, 2007). Maturation of myeloid DC in the presence of $1,25(\text{OH})_2\text{D}$ reduced the level of allogenic T cell activation by mature DC in a mixed lymphocyte reaction (Griffin *et al.*, 2000; Piemonti *et al.*, 2000; Penna and Adorini 2000). Pre-treatment of antigen-loaded immature myeloid DC with $1,25(\text{OH})_2\text{D}$ completely blocked the subsequent production of cytokines by auto-reactive T cell clones, although proliferation was not affected (Van Halteren *et al.*, 2002). Culture of mature $1,25(\text{OH})_2\text{D}$ -treated myeloid DC with T cell clones and their specific antigen reduced both T cell cytokine production and proliferation, and stimulated apoptosis of T cells, when compared to culture with untreated DC (van Halteren 2002). When T cells were, after co-culture with $1,25(\text{OH})_2\text{D}$ -treated myeloid DC, re-exposed to mature myeloid DC, a large proportion appeared to have become hypo-responsive (Piemonti *et al.*, 2000; Penna and Adorini, 2000). In conclusion, the effects of $1,25(\text{OH})_2\text{D}$ on myeloid DC also affect their capacity to evoke an (autoreactive) immune response. Besides direct effects on the CD4^+ T cell compartment, $1,25(\text{OH})_2\text{D}$ also modulates the T cell response via APC (**Figure 3**).

Direct exposure of B cells to $1,25(\text{OH})_2\text{D}$ has also been a topic of interest. Proliferation of PBMC and B cells on different combinations of B cell specific and aspecific stimuli is inhibited by $1,25(\text{OH})_2\text{D}$ (Chen *et al.*, 2007; Lemire *et al.*, 1984; Iho *et al.*, 1986). However, $1,25(\text{OH})_2\text{D}$ exposition also induced apoptosis of proliferating B cells (Chen *et al.*, 2007). Furthermore, phenotypic changes were noticed (Chen *et al.*, 2007; Heine *et al.*, 2008; Chen *et al.*, 1987) and differentiation to plasma cells was inhibited (Chen *et al.*, 2007). The subsequent synthesis of IgM and IgG by PBMC and B cells on different stimuli was inhibited (Chen *et al.*, 1987; Chen et al 2007; Iho *et al.*, 1986; Lemire *et al.*, 1984). When isolated CD19^+ B cells were stimulated by B cell receptor cross-linking, anti-CD40 and IL-4, $1,25(\text{OH})_2\text{D}$ induced the secretion of IL-10 (Heine *et al.*, 2008). Interestingly, the cells cultured by this protocol excreted no IgG, and their proliferation was unaffected by $1,25(\text{OH})_2\text{D}$. Although $1,25(\text{OH})_2\text{D}$ has direct effects on the B cells, several authors argued that indirect modulation of the B cell response might be more likely in physiological conditions. Much lower doses of $1,25(\text{OH})_2\text{D}$ were needed to inhibit PHA driven T cell proliferation compared to pokeweed mitogen driven B cell proliferation (Shiozawa *et al.*, 1987). In addition, PBMC depletion of either monocytes/macrophages (Chen *et al.*, 1987, Müller *et al.*, 1991) or T cells (Müller *et al.*, 1991), dramatically abrogated the reduction of IgG synthesis by $1,25(\text{OH})_2\text{D}$. Direct stimulation of antibody production by Epstein Bar Virus was also not affected by $1,25(\text{OH})_2\text{D}$ (Müller *et al.*, 1991). Nevertheless, the B cell appears to be a potential direct target of $1,25(\text{OH})_2\text{D}$ when exposed to a number of stimuli. The role of $1,25(\text{OH})_2\text{D}$ in the induction of IL-10 producing regulatory B-cells in vivo adds an exciting new area to the field of vitamin D and autoimmune diseases.

It can be concluded that, both directly and indirectly, $1,25(\text{OH})_2\text{D}$ skews the CD4^+ T cell compartment to a less inflammatory response. In vitro assays showed

that this skewing can be effectuated directly or via modulation of APC (**Figure 3**). The interaction between CD8⁺ T cells and vitamin D is at present not fully understood and requires further attention. In T cell mediated auto-immune diseases, the inhibition of IFN- γ producing Th1 and IL-17 producing Th17 cells in combination with promotion of Tregs, can be of benefit for relieving disease activity.

6. Vitamin D and experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is a useful animal model for MS. Although still a matter of debate, Th1/ Th17 cells are considered to be the most important effector cells. Since Th1/Th17 response is inhibited by 1,25(OH)₂D *in vitro*, a beneficial effect of 1,25(OH)₂D on a Th1/Th17 mediated disease like EAE seems logical. Indeed, whole body ultraviolet radiation before immunisation, associated with a rise in vitamin D₃ photosynthesis, prevented the development of clinical signs of EAE (Hauser *et al.*, 1984).

In several studies, oral or intraperitoneal administration of 1,25(OH)₂D before EAE-induction with myelin proteins completely prevented the appearance of any symptoms (Cantorna *et al.*, 1996; Lemire and Archer 1991). This was also established by a low calcium diet before immunisation, possibly by raising the plasma 1,25(OH)₂D level physiologically (Cantorna *et al.*, 1999). After immunization, but before the appearance of clinical symptoms, 1,25(OH)₂D was still able to prevent disease (Muthian *et al.*, 2006; van Etten *et al.*, 2003). In the animals in which EAE was not prevented, the disease was milder and survival longer (Branisteanu *et al.*, 1995; Branisteanu *et al.*, 1997; Lemire and Archer 1991; Muthian *et al.*, 2006; Nashold *et al.*, 2001; van Etten *et al.*, 2003). In animals with clinical active disease, 1,25(OH)₂D halted disease progression and even reduced disability scores (Cantorna *et al.*, 1996; Nashold *et al.*, 2000; Nataf *et al.*, 1996). The effects observed were dose-dependent (Lemire and Archer 1991; Lemire *et al.*, 1994). Typically, one study reported that none of the treated animals relapsed despite cessation of therapy as early as 5 days post-immunisation (Lemire and Archer, 1991). In another study relapses occurred within 10 days after discontinuation of therapy (Cantorna *et al.*, 1996). Whether these differences can be explained by differences in mouse strains, i.e. SJL/J mice versus B10.PL mice, or by distinct antigens, rat versus guinea pig spinal cord, remains to be determined. In Vitamin D Receptor (VDR)-knockout mice EAE also developed, but 1,25(OH)₂D was not able to prevent the development of EAE, indicating that the VDR is necessary for the beneficial effect of 1,25(OH)₂D (Meehan and DeLuca, 2002b). Exposure to vitamin D₃, a precursor of 1,25(OH)₂D₃, significantly inhibited EAE only in female mice with intact ovaries (Spach and Hayes, 2005). This suggests a gender-related difference in vitamin D₃ metabolism which

might, if the same in humans, contribute to the difference in MS prevalence between males and females.

As expected, in the animals where EAE symptoms were prevented or resolved, the extent and the intensity of histopathological inflammation of the CNS was respectively limited or reduced (Cantorna *et al.*, 1998; Lemire and Archer 1991; Nashold *et al.*, 2000). In semi-quantitative immunohistochemical analysis, a relative decline of OX-42 (monocytes, macrophages and microglia), OX-6 (MHC-class II) and NOSII (monocytes, macrophages, microglia and astrocytes) positive cells was observed in inflammatory CNS lesions of 1,25(OH)₂D-treated mice compared to vehicle-treated animals (Garcion *et al.*, 1997; Garcion *et al.*, 2003; Nataf *et al.*, 1996). A decline of CD4⁺ cells (T lymphocytes, activated microglia and some macrophages) was also observed (Garcion *et al.*, 2003; Nataf *et al.*, 1996).

Not only the clinical and histopathological effects, but also the effect of 1,25(OH)₂D on the immune response in EAE itself has been investigated. In the sera of 1,25(OH)₂D-treated animals, a decline in antibody production against Myelin Basic Protein (MBP) was observed (Branisteanu *et al.*, 1995; Lemire and Archer 1991), displaying either a direct or indirect modulation of the B cell response. Interestingly, treatment with 1,25(OH)₂D ameliorated symptoms of EAE in CD8 knockout mice, (Meehan and DeLuca 2002a). This observation draws attention further away from CD8⁺ T cells towards CD4⁺ T cells as main target of vitamin D's action. However, conflicting evidence exists about CD4⁺ T cell cytokine response to 1,25(OH)₂D treatment. Spleen cells from EAE-animals produced significantly less IFN- γ after *in vitro* 1,25(OH)₂D exposure (Muthian *et al.*, 2006). However, in MBP-specific Th1 lymphocytes this effect was not observed (Nashold *et al.*, 2001). *In vivo*, 1,25(OH)₂D did not affect IFN- γ transcription in peripheral lymph nodes (Cantorna *et al.*, 1998; Nashold *et al.*, 2001). In contrast, in CNS samples both no significant change in and a complete absence of IFN- γ transcription have been reported (Cantorna *et al.*, 1998; Nashold *et al.*, 2001). Transcription of TNF- α , also a Th1 cytokine, was not influenced by 1,25(OH)₂D (Nashold *et al.*, 2001). In lymph nodes and CNS samples of clinically improved 1,25(OH)₂D-treated mice, both a significant increase as well as no significant change in IL-4 transcription were found (Cantorna *et al.*, 1998; Nashold *et al.*, 2001). However, 1,25(OH)₂D had a less favourable effect on clinical EAE in IL-4 knockout mice (Cantorna *et al.*, 2000). These conflicting observations with regard to cytokine profiles provide no additional insights in the effects of 1,25(OH)₂D on the balance between Th1 and Th2 response *in vivo* in EAE.

An interesting observation was made in a MBP-specific TCR-transgenic mouse-strain. In Rag-1^{+/-} mice 1,25(OH)₂D was able to ameliorate disease, but in Rag-1-knockout mice this effect was not observed (Nashold *et al.*, 2001). This observation excludes APC's as the exclusive modulator of 1,25(OH)₂D's action on EAE. Another interesting observation was made in an IL-10 knockout mouse strain. In these mice, both vitamin D and 1,25(OH)₂D failed to prevent symptoms of EAE (Spach *et al.*,

2006). This was also the case in IL-10R β -knockout mice. Therefore, the IL-10/IL-10R pathway is involved in the ameliorating effect of 1,25(OH) $_2$ D and vitamin D. These observations support the view that an IL-10 producing Rag-1-dependent cell plays an important role. This might be the regulatory T cell. Indeed, dexamethason and 1,25(OH) $_2$ D-cultured Treg-cells ameliorated or completely blocked EAE when transferred to mice (Barrat *et al.*, 2002). A beneficial effect of Treg cells on EAE has previously been reported (Nicholson *et al.*, 1997). A raised TGF- β 1 transcription was found after 1,25(OH) $_2$ D treatment in both peripheral lymph nodes and in CNS samples of EAE animals (Cantorna *et al.*, 1998). However, this anti-inflammatory cytokine is not exclusively produced by Treg cells. Interestingly, increased plasma TGF- β 1 was also observed after vitamin D supplementation in a human MS-population (Mahon *et al.*, 2003). To assess whether the results of the experiments in the MBP-specific TCR transgenic mice could be attributable to an actual effect on the Treg cell, Foxp3 transcription, a marker of a certain subset of Treg cells, was quantified in the spinal cord samples of 1,25(OH) $_2$ D and placebo-treated animals 4 weeks post-immunization. No enhancement of expression was found (Spach *et al.*, 2006). This indicates that no accumulation of Foxp3-expressing Treg cells occurred in the spinal cord samples, but does not rule out the priming and activation of Treg lymphocytes elsewhere, for instance in the lymphoid tissue. Another explanation is that the 1,25(OH) $_2$ D-stimulated Treg lymphocytes responsible for the ameliorating effects of the hormone do not express Foxp3.

In summary, the *in vivo* modulation of the immune response in EAE by 1,25(OH) $_2$ D results in a change in Th1 and Th2 response, but study results are conflicting. Nevertheless, the role of APC's and Th1-Th2 shift is probably limited and Treg-cells appear to be important target cells for 1,25(OH) $_2$ D action (Damoiseaux, 2006). The modulation leads to an inhibition of the inflammatory immune response in the CNS and is accompanied by clinical and histopathological improvement of disease.

7. Vitamin D and MS

Strong evidence that directly supports the hypothesis that vitamin D is able to modulate disease in MS is lacking. However, a lot of clinical observations were made in MS-populations that support a role for vitamin D in MS. These include associations with serum 25(OH)D levels, the geographic distribution of MS-prevalence, the low bone mass density found in MS patients, the seasonal fluctuation of serum 25(OH)D levels, MS disease parameters and MS births, the clinical remission of disease during pregnancy and the associations of various genetic polymorphisms of the VDR with MS.

7.1 Serum 25(OH)D level

Low circulating 25(OH)D levels in adolescence are associated with a high incidence of MS in a white American population; a 41% decrease of incidence for every 50 nmol/L increase in 25(OH)D has been calculated (Munger *et al.*, 2006). Interestingly, increased outdoor activities in childhood and adolescence, hypothetically associated with raised 25(OH)D levels, are also associated with a decreased risk of developing MS in a Norwegian and Tasmanian population and a North-American twin population (Islam *et al.*, 2007; Kampman *et al.*, 2007; van der Mei *et al.*, 2003).

After the first onset of symptoms of MS, circulating 25(OH)D levels dropped significantly in the American population (Munger *et al.*, 2006). In a Scandinavian cohort of newly-diagnosed MS patients, summer serum 25(OH)D levels were significantly lower than in control subjects (Soliu-Hänninen *et al.*, 2005). A mean serum 25(OH)D level lower than 50 nmol/L has been described in 48–71% of all patients in several MS-populations (Mahon *et al.*, 2003; Nieves *et al.*, 1994; Ozgocmen *et al.*, 2005; Soliu-Hänninen *et al.* 2007).

Several efforts have been made to correlate clinical outcome measures of MS disease severity with serum 25(OH)D levels. A significant correlation of plasma 25(OH)D with IgG index and gadolinium enhancing T1 or T2-weighted MRI lesions could not be found (Soliu-Hänninen *et al.*, 2005; Soliu-Hänninen *et al.*, 2007). In Tasmanian patients with a longer disease history, decreased serum 25(OH)D levels were strongly correlated with an increased EDSS-score (van der Mei *et al.*, 2007). Questionnaires revealed reduced sun-exposure because of disability as being an important mechanism for this association. Lower 25(OH)D levels have also been reported during relapses in relapsing remitting MS patients (Soliu-Hänninen *et al.*, 2005; Soliu-Hänninen *et al.*, 2007).

Another clinical observation is the positive association between lighter skin types and lower disability scores in female patients with MS for longer than 10 years (Woolmore *et al.* 2007). Although 25(OH)D levels were not measured in the latter study, a positive association between skin type in terms of melanin density and 25(OH)D levels has been described in MS patients (van der Mei *et al.*, 2007).

7.2 Geographical distribution

MS has a typical geographical distribution: it has a low prevalence in equatorial regions and an increasing prevalence with increasing latitudes in both hemispheres. For example, in the northern regions of Europe and the USA an increased MS prevalence is shown in comparison with the southern regions (Kurtzke 2000). The opposite is true for Australia, where a decreased prevalence is seen in the southern coast in comparison with the sub-tropical northern coast (Hammond *et al.*, 1988).

A significant inverse correlation between MS-prevalence and the average December daily solar radiation, the average annual hours of sunlight and the average annual UVR-level has been observed in several studies (Acheson *et al.*, 1960; van

der Mei *et al.*, 2001). Therefore, the geographical differences in these parameters are considered to be a contributing factor to the typical distribution of MS. Not only latitude, but also altitude influences MS prevalence. The reported lower MS prevalence at high altitudes (>1000m) compared with low altitudes (<1000m) in Switzerland corresponded with the more optimal solar radiation at greater heights (Kurtzke 1967). Although a significant confounding effect of ethnicity and genetics has also been observed, migrating populations seem to acquire the MS risk of the area they move to (Hammond *et al.*, 2000; Pugliatti *et al.*, 2002). Limited sunlight exposure in MS patients is also shown by the lower incidence of actinic damage and non-melanoma skin cancer in this cohort, both associated with high levels of sun-exposure (Freedman *et al.*, 2000; Goldacre *et al.*, 2004; van der Mei *et al.*, 2003). Whether MS is a consequence of or rather a reason for this is uncertain.

Photosynthesis of vitamin D₃ is positively correlated with solar radiation. The mechanism by which the sun influences the prevalence of multiple sclerosis might be the promotion of vitamin D synthesis.

7.3 Seasonal variation

The seasonal variation in serum 25(OH)D levels was first described in 1974 by Stamp and McLaughlin. Since then, worldwide studies have underlined this phenomenon and geographical variations were noticed. Oral intake of vitamin D does not effect this fluctuation because it only contributes a small amount of the total 25(OH)D level (Maxwell 1994). In MS populations, fluctuations of 25(OH)D levels similar to those in healthy control-populations have been found (Soliu-Hänninen *et al.*, 2007).

In relapsing remitting MS, a seasonal variation in the exacerbation rate was first described in the 1970's. This fluctuation differs per geographical location. The peak exacerbation rate was observed in the warmer months in Arizona, in the warmest and coldest months in Japan and in the winter and spring in Switzerland (Bamford *et al.*, 1983; Ogawa *et al.*, 2004; Wuthrich and Rieder 1970). A comparable fluctuation was also found in plasma IFN- γ and TNF- α levels, hallmarks of Th1 lymphocyte activity (Balashov *et al.*, 1998; Killestein *et al.*, 2002). An inverse fluctuation of the Treg cytokine IL-10 was found (Stewart *et al.*, 2007). Unfortunately, these observations were not directly related to plasma levels of 25(OH)D. A seasonal variation has also been reported in the number of active lesions on MRI scan. In a small German study, more gadolinium enhancing lesions were found in spring and early summer (April) and less in autumn (October) (Auer *et al.*, 2000). A strikingly similar inverse fluctuation was also found in the plasma 25(OH)D levels of a similar but healthy population in the same geographical location (Embry *et al.*, 2000). In other larger studies in different geographical areas, these observations could not be reproduced with significant results (Killestein *et al.*, 2002; Koziol and Feng, 2004; Rovaris *et al.*, 2001).

7.4 Season of birth

Another seasonal fluctuation is seen in the month of birth of MS patients. In Denmark, Sweden, Great Britain and Canada, many MS patients are born in May and relatively few in November (Sadovnick and Yee, 1994; Tremlett and Devonshire 2006; Willer *et al.*, 2005). In a recent large Canadian study, this only differed significantly from the normal population in the relapsing remitting and not in the primary progressive population (Sadovnick *et al.*, 2007). Other observations of the clinical consequences of season of birth are scarce. Being born in January has been reported to be marginally associated with a slower disease progression and in another small study summer babies had a later age of MS onset (Sotgiu *et al.*, 2006; Tremlett and Devonshire 2006).

The reduced expression of several proteins associated with MS has been observed in the offspring of severely vitamin D depleted female rats (Almeras *et al.*, 2007). This underlines the important role of vitamin D in pregnancy, but it remains uncertain whether the excess of MS patients born in winter can be attributable to maternal 25(OH)D levels. Other factors during pregnancy and early youth, such as virus infections, low birth weight and maternal folate deficiency, might interfere.

7.5 Pregnancy

In MS patients, clinical remission of the disease is often observed during pregnancy with a higher relapse rate post-partum (Korn-Lubetzki *et al.*, 1984). Pregnancy has been reported to protect against the development of MS in a healthy population and against disease progression in MS patients (Runmarker and Andersen 1995). It is also known that during pregnancy, 1,25(OH)₂D levels increase physiologically and peak during the third trimester with an abrupt decline post-partum (Verhaeghe and Bouillon 1992). It has been suggested that this rise in 1,25(OH)₂D might be an explanation for the clinical remission of the disease (Schwartz 1993). Indeed, in pregnant MS patients a shift in cytokine production from a Th1 to a Th2/ Treg-profile with a shift towards a Th1 cytokine profile postpartum has been observed (Al-Shammri *et al.*, 2004; Gilmore *et al.*, 2004). This is compatible with the *in vitro* effects of 1,25(OH)₂D on T lymphocytes (**Figure 3**).

In EAE, a mouse model of MS, a positive correlation between oestrogen production and immune suppressing activity of 1,25(OH)₂D has been described (Spach and Hayes 2005). This might also illustrate, if the mechanism in humans is the same, an interaction between hormonal changes and the ameliorating effects of vitamin D on disease. However, it is not yet clear whether clinical remission in MS patients can be contributed to a change in the level of 1,25(OH)₂D or is largely confounded by numerous other physiological immune suppressing changes in the pregnant woman.

7.6 Bone Mineral Density

MS patients are known to have a lower Bone Mineral Density (BMD) and to be more at risk for fractures after minor injury (Schwid *et al.*, 1996). An increased fracture rate in the absence of major trauma has been reported (Cosman *et al.*, 1998). This was historically considered to be explained by immobilisation and glucocorticoid use. Indeed, BMD in the femoral neck is significantly inversely correlated with disability and EDSS-score (Ozgocmen *et al.*, 2005; Schwid *et al.*, 1996). However, there is conflicting evidence regarding the direct effects of the intensity of glucocorticoid use on bone density in MS populations (Formica *et al.*, 1997; Ozgocmen *et al.*, 2005; Schwid *et al.*, 1996). Besides these findings, the serum 25(OH)D-level is also significantly correlated with a low BMD (Nieves *et al.*, 1994). Thus, vitamin D deficiency, together with immobilisation and glucocorticoid therapy, also contributes to low bone mass in MS patients.

7.7 Vitamin D receptor gene polymorphisms

A role for genetic factors in the development of MS is well known (Hauser and Oksenberg 2006). Genetic polymorphisms of several vitamin D related proteins have been investigated. Neither a difference in frequency and transmission of the vitamin D Binding Protein gene, nor of transmission of the genotype of CYP27B1 has been found (Niino *et al.*, 2002; Steckley *et al.*, 2000).

In the VDR-gene (VDRG) on chromosome 12q, numerous polymorphisms have been described (Uitterlinden *et al.*, 2004). A significant association with MS was found for the VDRG polymorphisms Bsm-I in a Japanese, Apa I in a Japanese and Australian and Taq-I in an Australian population (Fukazawa *et al.*, 1999; Niino *et al.*, 2000; Tajouri *et al.*, 2005). A trend for the polymorphism Fok-I has been described in an UK population (Partridge *et al.*, 2004). These associations are not universal and geographical differences are noticeable. For example, no preferential transmission of Apa-I and Taq-1 from MS-affected parents to affected offspring was found in a Canadian study and the Taq-I polymorphism was not associated with MS in an UK population (Partridge *et al.*, 2004; Steckley *et al.*, 2000).

However, the associations point to an increased vulnerability of developing MS in individuals with these polymorphisms in certain populations. Nevertheless, only the Fok I polymorphism is located in an exon of the VDRG and is known to have a functional impact on the immune system (van Etten *et al.*, 2007). The other polymorphisms are restriction length polymorphisms in an intron of the gene and their biological effects are uncertain (Uitterlinden *et al.*, 2004). Linkage with a not yet revealed truly functional allele might be a mechanism of action. Further studies assessing vitamin D and MS parameters or prospective trials with oral vitamin D supplementation will have to define the clinical relevance of those polymorphisms in MS.

8. Vitamin D therapy in MS

Potential effects of oral vitamin D intake on MS have been observed. In a recent Norwegian case control study a protective effect of regular fish consumption and cod liver oil was found (Kampman *et al.*, 2007). The lower MS mortality and disability rates in the coastal regions compared to the inlands was earlier hypothesised to be attributable to a difference in dietary fish intake (Westlund 1970). In a large observational study in the United States, the use of supplemental vitamin D, mostly in the form of multi-vitamins, by healthy female nurses seemed to lower their risk on MS with 40% (Munger *et al.*, 2004).

In two prospective but very small patient series, supplementation of vitamin D [10 µg/d (400 IU/d) and 125 µg/d (5000 IU/d)] in combination with other nutriment seemed to have an effect on exacerbation rate and EDSS, but these studies were neither blinded, nor placebo controlled (Goldberg *et al.*, 1986; Nordvik *et al.*, 2000). In 39 MS patients, 6-month supplementation of 25 µg/d (1.000 IU/d) vitamin D₃ led to an increase of serum 25(OH)D levels, a significant increase of the cytokine TGF-β and a decline in IL-2 mRNA levels; but the correlation with clinical parameters was not investigated (Mahon *et al.*, 2003). A recent safety study in 12 patients with supplementation of up to 7000 µg/week [280.000 IU/week) vitamin D₃ for 28 weeks showed a decline in the number of gadolinium enhancing lesion on MRI (Kimball *et al.*, 2007). Supplementation with vitamin D metabolites has been investigated, but also more in relation to safety and toxicity than to clinical effectiveness. In small series of 5 and 13 patients, supplementation with 1.5 µg/d 1α(OH)D for 6 months and 2.5 µg/d 1,25(OH)₂D for 12 months respectively, only led to mild hypercalcaemia when compliant to dietary advice (Achiron *et al.*, 2003; Wingerchuk *et al.*, 2005).

Therefore little evidence of a beneficial effect of oral vitamin D supplementation on clinical disease in MS can be provided. Only a few small patient series have been conducted so far. This might be attributable to some concerns about the safety of vitamin D supplementation. The major side effect of 1,25(OH)₂D supplementation seen in EAE is the physiological increase in plasma calcium, ultimately leading to a dose-dependent hypercalcaemia (Cantorna *et al.*, 1996; Lemire and Archer 1991). This can lead to reduction of bone mineral density and potential life-threatening complications such as renal and heart failure. In human populations, people with sarcoidosis or Mycobacterium infections and people treated with thiazide diuretics are believed to be sensitive to hypercalcaemia and hypervitaminosis D (Hathcock *et al.*, 2007). Therefore, a cautious attitude towards vitamin D supplementation is often found among clinicians.

In human osteoporosis and bone metabolism research, numerous trials have been conducted with supplementation of vitamin D. In a recent extensive review, a no adverse effects limit (NOAEL) of 250 µg (10.000 IU) has been proposed for sup-

plementation of vitamin D to healthy adults (Hathcock *et al.*, 2007). This daily intake leads to a plasma 25(OH)D level of 220 nmol/L, whereas much higher levels would be needed to evoke a hypercalcaemia. Supplementation of up to 1000 µg/ day for 28 weeks in 12 MS patients led to a mean serum 25(OH)D concentration of 386 nmol/L without causing hypercalcaemia or hypercalciuria (Kimball *et al.*, 2007). Therefore it seems relatively safe to treat vitamin D deficient patients with substantial doses of vitamin D.

In EAE research, experiments have been conducted with hypocalcaemic analogues of 1,25(OH)₂D with sustained immune regulating properties (Lemire *et al.*, 1994; van Etten *et al.*, 2003). More than 270 analogues of 1,25(OH)₂D have been developed by adding a benzene-ring to or changing side-chains of the molecule (Bouillon *et al.*, 1995). To our knowledge, no human studies with hypocalcaemic analogues of vitamin D in MS patients have yet been published.

9. Conclusion

There is evidence to support the role of vitamin D as an immune modulating agent. An inhibitory effect on Th1 cell function and a promoting effect on Th2 and Treg cells have been described *in vitro*. In EAE, a beneficial effect of 1,25(OH)₂D on the clinical and histological features of disease has also been described. The most important target cell in the action of 1,25(OH)₂D on the immune response in EAE is probably the Treg lymphocyte.

Vitamin D is an immune modulator, but if it can be considered as a disease modulator in MS remains uncertain. Various observations in MS populations show an association between various vitamin D parameters and MS and are suggestive of an immune and disease modulating effect of vitamin D, but cannot be considered as solid evidence. Nevertheless, these observations provide a good reason for further research in this direction. Before conclusions can be drawn about the potential of vitamin D as an immune modulator in MS, further studies investigating the immune modulating mechanism as well as double-blind placebo-controlled trials are necessary.

Association of vitamin D metabolite levels with relapse rate and disability in patients with multiple sclerosis

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Abstract

Background: Multiple Sclerosis (MS) has been associated with low serum concentrations of 25-hydroxyvitamin D (25(OH)D). The purpose of this cross-sectional study was to investigate the association of the serum concentrations of 25(OH)D and 1,25-dihydroxyvitamin D (1,25(OH)₂D), the biologically active metabolite of vitamin D, with clinical MS severity as expressed by EDSS-score and relapse rate.

Patients and Methods: Serum samples from 267 MS patients were collected for measurement of serum 1,25(OH)₂D and 25(OH)D concentrations. Clinical MS parameters at the date of serum sampling were determined.

Results: Both metabolite levels were significantly lower in the progressive MS phenotypes when compared to the relapsing remitting (RR)MS phenotype. In RRMS patients (disease course ≤ 5 years), high 25(OH)D levels were associated with a high chance of remaining relapse-free. Low 25(OH)D levels were associated with high EDSS-scores. The serum level of 1,25(OH)₂D was not directly associated with relapse rate or EDSS-score, and was dependent of age and 25(OH)D level.

Conclusion: Circulating 25(OH)D is associated with both relapse rate and disability in MS patients. These results are suggestive for a disease modulating effect of the serum concentrations of 25(OH)D on MS. The low circulating 1,25(OH)₂D levels in progressive MS are due to older age and lower 25(OH)D levels. The potential consequences of vitamin D supplementation in MS will be discussed.

1. Introduction

A combination of as yet poorly defined genetic and environmental factors is needed to develop Multiple Sclerosis (MS) (Compston and Coles 2005). Recent studies suggest that a poor vitamin D status is an important environmental risk factor (Smolders *et al.*, 2008a, **chapter 2**). Vitamin D is predominantly photosynthesised in the skin by sunlight. In the human circulation, it is mainly found as two metabolites: the principal circulating metabolite 25-hydroxyvitamin D (25(OH)D), and the biologically active metabolite 1,25-dihydroxyvitamin D (1,25(OH)₂D). The circulating concentration 1,25(OH)₂D is mainly the result of hydroxylation of 25(OH)D in the kidney (Jongen *et al.*, 1984). *In vitro*, 1,25(OH)₂D is a potent immune modulator, which inhibits pro-inflammatory and promotes anti-inflammatory cells and cytokines (Smolders *et al.*, 2008a, **chapter 2**). In the murine model experimental autoimmune encephalomyelitis (EAE), supplementation of both vitamin D metabolites either prevents the disease or guides the disease into remission. Raising the circulating levels of 1,25(OH)₂D by supplementing 1,25(OH)₂D effectively prevented or ameliorated EAE (Cantorna *et al.*, 1996; Lemire *et al.*, 1991). Supplementation of vitamin D before EAE-induction caused a rise in the serum concentration of 25(OH)D, but not of 1,25(OH)₂D (Spach *et al.*, 2005). In the animals in which remission was achieved, elevated levels of 1,25(OH)₂D were found in the CNS, considered as locally hydroxylised 25(OH)D by for instance macrophages or dendritic cells.

Low circulating levels of 25(OH)D in adolescence correlate with a high risk of developing MS in humans (Munger *et al.*, 2006). Lower 25(OH)D levels have been reported in MS compared with healthy control populations (van der Mei *et al.*, 2007). In MS-populations, Expanded Disability Status Scale (EDSS) scores correlate negatively with circulating levels of 25(OH)D and these levels are lower during relapses than remission in relapsing remitting MS (Soliu-Hänninen 2005; Soliu-Hänninen 2008; van der Mei 2007). However, their causality is dubious: either disability by MS influences sun-exposure and thus 25(OH)D levels (van der Mei *et al.*, 2007), or 25(OH)D influences MS disability, or maybe even both. Recent studies revealed an association between sun-exposure, disability and 25(OH)D levels, but supplementation of small amounts of vitamin D also changed the cytokines in the blood of MS patients to a anti-inflammatory profile (Mahon *et al.*, 2003). An association of 25(OH)D levels with relapse rate, MRI-parameters or cerebrospinal fluid IgG-indices has not been found (Soliu-Hänninen 2005; Soliu-Hänninen 2008).

The role of circulating 1,25(OH)₂D in MS is even less well defined. In an MS population, the serum concentration of 1,25(OH)₂D correlated positively with serum 25(OH)D levels (Barnes *et al.*, 2007). *In vitro* and in EAE-research, it seems that higher serum levels of 1,25(OH)₂D alter the immune system, causing it to be less pro-inflammatory (Smolders *et al.*, 2008a, **chapter 2**). We hypothesised that interference with serum 1,25(OH)₂D could be an effector pathway by which vitamin D

modulates MS. No large studies have yet addressed the association of clinical MS severity with circulating 1,25(OH)₂D levels. Two small pilot studies investigated supplementation of 1,25(OH)₂D for 48 weeks in 15 patients and 1 α (OH)D for 6 months in 5 MS patients (Achiron *et al.*, 2003; Wingerchuk *et al.*, 2005). However, these studies were primarily concerned with the safety of supplementation rather than the clinical outcome.

The main purpose of our study was to investigate if a low serum concentration of the vitamin D metabolites 25(OH)D and 1,25(OH)₂D is associated with severe clinical MS as expressed by a high EDSS-score or a high relapse rate. We assessed relapse rate in the total relapsing remitting (RR)MS cohort and in a subgroup with a short disease course (≤ 5 years). In the latter group, the predominantly inflammatory rather than neurodegenerative disease process most closely resembles neuroinflammation as seen in the EAE model. Therefore, we hypothesised that a clinical manifestation of the anti-inflammatory effect of vitamin D, as observed both in vitro and in EAE, might be most prominent in this group.

2. Patients and Methods

267 MS patients with clinically and MRI-confirmed MS (McDonald *et al.*, 2005) and a disease duration of ≥ 0.5 years since the first symptoms of the disease, were recruited from consecutive patients admitted to our University Hospital outpatient clinic in the period 2005–2007. They all lived in the southern area of the Netherlands (latitude 50° N – 51° N). The characteristics of this population are shown in **Table 1**.

Table 1 Population characteristics.

		N/ Mean	%/ SD
Sex	Male	66	24.7%
	Female	201	75.3%
MS phenotype	RRMS	126	47.2%
	SPMS	85	31.8%
	PPMS	48	18.0%
	Uncertain	8	3.0%
Age (years)		47.0	± 11.6
Age at disease onset (years)		34.1	± 10.1
Disease duration (years)		12.9	± 9.4
EDSS-score (years)		4.2	± 2.3

Serum samples were shielded from direct light after collection and stored at -20°C . Both the metabolite levels were measured by routine analysis in our clinical chemical laboratory. Forced by closure of the first manufacturer, the 25(OH)D assay switched in June 2006 from a chemiluminescence immunoassay (Nichols Institute Diagnostics, California, USA) to a radioimmunoassay (Immunodiagnostic Systems, Boldon, UK). Forty two samples were tested with both assays and showed a good correlation ($R^2 = 0.92$) without significant differences ($P = 0.495$). 1,25(OH) $_2$ D levels were determined with a commercially available radioimmunoassay kit (Immunodiagnostic Systems, Boldon, UK). In an international consensus meeting, a 25(OH)D concentration of 70–80 nmol/L has been argued to be adequate for a normal calcium metabolism (Dawson-Hughes *et al.*, 2005). A recent paper concluded that 25(OH)D levels should exceed at least 70 nmol/L to prevent osteoporosis (Vieth 2005). Therefore, we considered 25(OH)D levels below 70 nmol/L as indicative for a suboptimal vitamin D status.

Clinical MS characteristics were retrieved from our patient database (iMed system). We collected EDSS-scores at serum sampling and counted the number of relapses in a 2 year period before serum sampling. The one year relapse rate was calculated from this count, also in patients with a disease course of < 2 years ($N=15$). Furthermore, we determined other clinical variables as age, sex, MS phenotype (RRMS, secondary progressive (SPMS), primary progressive (PPMS), unknown), disease course since first symptom, and the occurrence of a relapse within ± 6 weeks after blood collection.

SPSS software (SPSS Inc., version 12.0, Chicago, USA) was used for statistical analysis. Mean values were compared using T-tests, or in small sample sizes with the Mann-Whitney U test. Mean values are provided with standard deviation (SD; $\pm$), mean differences (MD) with standard error (SE; $\pm$). Relationships were evaluated using regression analysis. The relationship of the vitamin D metabolites with EDSS-score was analysed by a linear regression model, the relationship with relapse (yes/no) and relapse rate (N/year) by respectively a logistic and a Poisson regression model. All potential confounders were included simultaneously in the models, in order to adjust the analysis. Both the crude and adjusted odds ratios (OR) or regression coefficients (β) with corresponding 95% confidence intervals (95% CI) are provided. Potential confounders were: sex (male/ female), age (years), disease duration (years). A 2-sided P-value < 0.05 was considered statistically significant. Since all stratifications were hypothesis-driven, no correction for multiple testing was applied.

3. Results

3.1 Vitamin D metabolite levels

The mean 25(OH)D and 1,25(OH)₂D concentrations stratified for clinical MS phenotype are shown in **Table 2**. Compared with the RRMS group, the circulating levels of both metabolites were significantly lower in the progressive MS groups (respectively $P < 0.001$ and $P = 0.002$). In the RRMS group with a disease course of ≤ 5 years, the 25(OH)D and 1,25(OH)₂D values did not differ significantly from the total RRMS group (respectively $P = 0.191$; $P = 0.173$). The percentage of patients with a suboptimal vitamin D status was substantial even in the RRMS-cohort with a disease course of ≤ 5 years (34.9%), and exceeding 81.2% in the SP-cohort. There was no difference in 25(OH)D and 1,25(OH)₂D levels between the sexes, and both metabolites did not display a clear significant seasonal fluctuation (data not shown).

Table 2 Vitamin D metabolite concentrations in the MS-cohort stratified by clinical phenotype.

	Total MS (N = 267)	RRMS Duration ≤ 5 yrs. (N = 43)	RRMS (N = 126)	SPMS (N = 85)	PPMS (N = 48)
25(OH)D (nmol/L)*	62.53 (± 32.4)	79.47 (± 32.2)	72.06 (± 31.9)	51.27 (± 28.8)	57.40 (± 31.7)
1,25(OH) ₂ D (pmol/l)*	107.65 (± 43.2)	127.88 (± 43.4)	116.92 (± 45.4)	99.48 (± 41.8)	100.79 (± 36.5)
Suboptimal vitamin D status**	170 (63.7%)	15 (34.9%)	61 (48.8%)	69 (81.2%)	33 (68.8%)

RRMS: relapsing remitting MS; SPMS: secondary progressive MS; PPMS: primary progressive MS. *Mean ($\pm$ SD). **N(%) with serum 25(OH)D levels < 70 nmol/L.

3.2 Relapse rate

In the whole RRMS cohort ($N = 126$), the mean relapse rate was $0.69 (\pm 1.02)/$ year. We analysed both the raw, and the in the regression model adjusted vitamin D data. There was no association of both the raw and adjusted vitamin D metabolite data with relapse rate (**Table 3**).

In the RRMS cohort with a disease course of ≤ 5 years, the mean relapse rate was $1.17 (\pm 1.09)/$ year and the mean EDSS-score $2.68 (\pm 1.74)$. There was no significant association of the vitamin D metabolites with relapse rate (**Table 3**). However, when the patients with and without relapses were compared, the relative risk of remaining relapse free prior to serum sampling increased by 51% for each 10 nmol/L increase of 25(OH)D ($P = 0.017$). The association with the other metabolite, 1,25(OH)₂D, was not statistically significant ($P = 0.201$). The mean EDSS-score did not differ significantly between the groups with or without relapses (MD: $0.63 (\pm 0.69)$; $P = 0.385$).

Table 3 The raw and adjusted regression coefficients and odds ratios of vitamin D metabolites and clinical parameters of disease severity.

	25(OH)D				1,25(OH) ₂ D			
	Crude		Adjusted**		Crude		Adjusted**	
	β / OR	(95% CI)	β / OR	(95% CI)	β / OR	(95% CI)	β / OR	(95% CI)
All MS (N=267)								
EDSS-score (β)	-0.023*	(-0.031 – -0.015)	-0.014*	(-0.022 – -0.006)	-0.006	(-0.013 – 0.000)	0.000	(-0.006 – 0.006)
RRMS (N=126)								
Relapsefree (OR)	1.000	(0.989 – 1.012)	0.993	(0.979 – 1.007)	1.006	(0.998 – 1.014)	1.003	(0.933 – 1.013)
Relapse Rate (β)			-0.005	(-0.013 – 0.003)			0.000	(-0.005 – 0.005)
RRMS; course ≤ 5 yrs. (N=43)								
Relapsefree (OR)	0.975	(0.949 – 1.002)	0.949*	(0.909 – 0.991)	0.984	(0.966 – 1.003)	0.985	(0.964 – 1.007)
Relapse Rate (β)			-0.007	(-0.016 – 0.003)			0.000	(-0.008 – 0.007)
RRMS; course ≤ 5 yrs; EDSS ≤ 3.5 (N=31)								
Relapsefree (OR)	0.964*	(0.932 – 0.998)	0.963*	(0.889 – 0.998)	0.966*	(0.936 – 0.997)	0.964*	(0.931 – 0.999)
Relapse Rate (β)			-0.009	(-0.019 – 0.001)			-0.006	(-0.014 – 0.002)

RRMS: relapsing remitting MS; EDSS: Expanded Disability Status Scale; β: regression coefficient; OR: Odds Ratio. Dependent variables: EDSS-score (linear regression model), Relapse free (yes/no; logistic regression model), Relapse Rate (N/year; Poisson regression model). *P < 0.050. **Predictive value of serum 25(OH)D and 1,25(OH)₂D adjusted for age, sex, disease duration.

In this cohort, we conducted a subgroup-analysis of patients who were fully ambulatory at the time of serum sampling (EDSS-score ≤ 3.5). This subgroup analysis was performed in order to assess the association in a presumably more homogenous cohort regarding sun-exposure. Both 25(OH)D and 1,25(OH)₂D levels were lower in patients with 1 or more relapses compared to patients with no relapses prior to serum sampling (**Figure 1**). EDSS-scores did not differ significantly between the groups (MD: 0.60 (±0.48); P = 0.226). Both high 25(OH)D (P = 0.043) and 1,25(OH)₂D levels (P = 0.046) were associated with a high chance of remaining relapse free (**Table 3**). The association of 25(OH)D level with relapse rate reached a significance level of P = 0.087.

Figure 1 Serum vitamin D metabolite levels in fully ambulatory RRMS patients with and without relapses prior to serum sampling. (N = 31; disease duration ≤ 5 years; EDSS score < 3.5 . Grey boxes: 25(OH)D, White boxes: 1,25(OH)₂D. Test for significance: Mann-Whitney U test

3.3 EDSS-score

In the whole MS population, the raw 25(OH)D levels correlated negatively with EDSS-score (**Table 3**). There was no significant association between EDSS-score and raw 1,25(OH)₂D levels ($P = 0.065$). To address the elusive causality between disability and vitamin D, both metabolites were tested as predictors as well as dependents of EDSS-score in a multiple linear regression model. Only the adjusted 25(OH)D level was an independent predictor of EDSS-score (**Table 3**). When the vitamin D metabolites were considered as dependents of disability, only 25(OH)D was predicted by EDSS-score ($\beta = -3.155$; 95%CI $-4.936 - -1.374$). The 1,25(OH)₂D level was dependent upon 25(OH)D level ($\beta = 0.550$; 95%CI $0.395 - 0.705$) and age ($\beta = -0.810$; 95% CI $-1.310 - -0.310$), but not upon EDSS-score.

4. Discussion

This is the first study to assess the serum levels of both 25(OH)D and 1,25(OH)₂D in a large MS-population, including correlation of 1,25(OH)₂D with clinical disease severity. We showed that low circulating levels of both 25(OH)D and 1,25(OH)₂D are associated with the progressive MS phenotypes. Only low 25(OH)D levels are associated with an increase in clinical MS severity as measured by EDSS-score and the occurrence of relapses. Circulating 1,25(OH)₂D is not independently associated with these clinical parameters, and is dependent of age and 25(OH)D level.

Although the association with relapse rate did not reach statistical significance, we did find significantly higher 25(OH)D levels in RRMS patients without relapses compared to patients with relapses, despite comparable EDSS-scores at serum sam-

pling. This finding supports the observations of Souliu-Hänninen *et al.*, who also investigated 2 year relapse rate before baseline 25(OH)D measurement (Souliu-Hänninen *et al.*, 2008). They observed a trend towards lower 25(OH)D levels in patients with a high relapse rate. Their small sample-size ($N = 26$) probably explains this lack of significance, but also the inclusion of patients with a disease course of up to 15 years might contribute. We did only observe an association in patients with a disease course of ≤ 5 years, and not in our total RRMS cohort. As we hypothesised, the specific inflammatory disease process at RRMS-onset might explain this phenomenon, but we can speculate about other explanations. The lower vitamin D metabolite levels in the secondary progressive group might be indicative of an association between low 25(OH)D levels and neurodegeneration, i.e. disease progression.

Our findings show that the serum concentration of 1,25(OH)₂D does not play a key role in the interaction between vitamin D and clinical MS severity. The lower 1,25(OH)₂D levels that we found in the fully ambulatory patients with relapses and in the patients with high EDSS-scores are most likely attributable to the correlation between the vitamin D metabolites (Barnes *et al.*, 2007). The 1,25(OH)₂D levels present in the serum, hydroxylized in the kidneys, are of vital significance for calcium metabolism, but are not likely to be involved in a disease modulating mechanism in MS. In the previously described EAE models, a beneficial reaction of disability on serum 1,25(OH)₂D elevation has been observed (Cantorna *et al.*, 1996; Lemire *et al.*, 1991). However, this is most likely attributable to the use of supra-physiological levels of the hormone, leading to serious side-effects like hypercalcaemia. Apart from 25(OH)D, age is an important negative predictor of serum 1,25(OH)₂D. In progressive MS patients, impaired renal function with low glomerular filtration rates is often found (Calabresi *et al.*, 2002). Chronic damage to the kidney could lead to a impaired 1 α -hydroxylation of 25(OH)D over the decades and thereby contribute to lower serum 1,25(OH)₂D levels in older MS patients. Therefore, besides the MS modulating potential, vitamin D supplementation might certainly be very important in helping to maintain proper serum 1,25(OH)₂D levels and a healthy calcium metabolism in this population, made vulnerable for loss of bone mineral density (Nieves *et al.*, 1994).

The causality of the interaction between 25(OH)D levels and MS is dubious; the low 25(OH)D levels during relapses or in patients with high EDSS-scores can be interpreted in various ways. However, the association that we found between relapses and circulating 25(OH)D levels in RRMS patients with a disease course ≤ 5 years, more strongly suggests a modulating effect of vitamin D on MS pathobiology. The mechanism underlying this modulation is still elusive, but some suggestions have been made in experimental research (Smolders *et al.*, 2008a, **chapter 2**). The potential therapeutic consequences of vitamin D also remain to be determined. There is more evidence appearing that supplementation of substantial doses of

vitamin D in MS patients is an arguable and relatively safe option (Kimball *et al.*, 2007). Therefore, we suggest that it is time for double-blind placebo-controlled trials to assess the role of vitamin D in MS treatment.

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Vitamin D status is positively correlated
with regulatory T cell function in patients
with relapsing remitting multiple sclerosis

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Abstract

Background: In several autoimmune diseases, including multiple sclerosis (MS), a compromised regulatory T cell (Treg) function is believed to be critically involved in the disease process. *In vitro*, the biologically active metabolite of vitamin D has been shown to promote Treg development. A poor vitamin D status has been linked with MS incidence and MS disease activity. In the present study, we assess a potential *in vivo* correlation between vitamin D status and Treg function in relapsing remitting MS (RRMS) patients.

Methodology/ Principal Findings: Serum levels of 25-hydroxyvitamin D (25(OH)D) were measured in 29 RRMS patients. The number of circulating Tregs was assessed by flow-cytometry, and their functionality was tested *in vitro* in a CFSE-based proliferation suppression assay. Additionally, the intracellular cytokine profile of T helper cells was determined directly ex-vivo by flow-cytometry. Serum levels of 25(OH)D correlated positively with the ability of Tregs to suppress T cell proliferation ($R = 0.590$, $P = 0.002$). No correlation between 25(OH)D levels and the number of Tregs was found. The IFN- γ /IL-4 ratio (Th1/Th2-balance) was more directed towards IL-4 in patients with favourable 25(OH)D levels ($R = -0.435$, $P = 0.023$).

Conclusions/ Significance: These results show an association of high 25(OH)D levels with an improved Treg function, and with skewing of the Th1/Th2 balance towards Th2. These findings suggest that vitamin D is an important promoter of T cell regulation *in vivo* in MS patients. It is tempting to speculate that our results may not only hold for MS, but also for other autoimmune diseases. Future intervention studies will show whether modulation of vitamin D status results in modulation of the T cell response and subsequent amelioration of disease activity.

1. Introduction

Multiple Sclerosis (MS) is a disabling, chronic inflammatory disease of the central nervous system (CNS). Although some patients experience progression of disability from the first symptoms onwards, the disease is at its onset mostly characterised by repetitive sub-acute deteriorations of symptoms with remissions afterwards, i.e. relapsing remitting MS (RRMS). After several years, sustained and progressive disability often occurs and the disease becomes secondary progressive (Compston and Coles, 2005).

Although the pathophysiological process in MS is not yet fully elucidated, T cell mediated inflammation within the CNS plays an important role. Auto-reactive T cells move through the blood brain barrier, and contribute to local inflammation of the CNS (Bar-Or 2008). Historically, these auto-reactive T cells were believed to comprise predominantly interferon gamma (IFN- γ) producing T helper 1 (Th1) cells. More recently interleukin-17 (IL-17) producing Th17 cells have been argued to be important pathogenic T cells in MS (Kebir *et al.*, 2007; Steinman 2007). In a healthy T cell compartment, regulatory T cells (Treg) regulate the quantity and quality of the immune response, and prevent auto-reactive T cell responses, and hereby autoimmune diseases (Sakaguchi *et al.*, 2008). Although the number of Tregs is not decreased, their capability to suppress polyclonal or antigen-specific proliferation of T cells *in vitro* has been shown to be compromised in MS patients (Haas *et al.*, 2005; Venken *et al.*, 2006; Viglietta *et al.*, 2004).

Experimental studies showed that the biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH) $_2$ D), is able to skew the T cell compartment into a more anti-inflammatory and regulated state, with inhibition of Th1 and Th17 cells and promotion of Th2 and Treg cells (Correale *et al.*, 2009; Smolders *et al.*, 2008a, **chapter 2**). Interestingly, in the experimental autoimmune encephalomyelitis (EAE) animal model of MS, treatment with vitamin D prevented, and even cured, the disease (Cantorna *et al.*, 1996). These findings suggest a potential role for vitamin D in MS, as has been investigated in several epidemiological studies. Near the equator, where vitamin D photosynthesis is optimal, MS incidence is low (Kurtzke 1967). Furthermore, increased sun exposition and a good vitamin D status in childhood and adolescence, reflected by the serum values of 25-dihydroxyvitamin D (25(OH)D) (Hollis 1996), have been associated with a decreased risk for developing MS (Islam *et al.*, 2007; Kampman *et al.*, 2007; Munger *et al.*, 2006). An increased activity and disability of MS has also been associated with a poor vitamin D status (Kragt *et al.*, 2009; Smolders *et al.*, 2008b, **chapter 3**; van der Mei *et al.*, 2007). Since vitamin D modulates the immune response *in vitro* and in animal models, it is highly interesting to assess whether this modulation can also be found *in vivo* in MS patients. This could be, beside regulation of the transcription of MS-associated alleles (Ramapolagan *et al.*, 2009), an important driver of the associations between vitamin

D status and MS. At present, however, no published studies assessed the potential relationship between serum levels of 25(OH)D and the T cell compartment in MS.

In the present study, we assess whether vitamin D status is correlated with the *in vivo* composition and function of the T cell compartment in MS patients. Since in RRMS patients with a short disease duration disease pathophysiology has been argued to be predominantly inflammatory (Compston and Coles 2005) and Treg function has been shown to be particularly compromised (Venken *et al.*, 2006), we assess a potential anti-inflammatory and in particular Treg-promoting effect of vitamin D, as being related to vitamin D status, in these patients.

2. Materials and methods

2.1 Subjects

We included in this study 29 Caucasian subjects, by approaching consecutive patients matching the inclusion criteria, who visited the outpatient clinic of our MS centre. All subjects had RRMS according to the revised McDonald criteria (Polman *et al.*, 2005), with a disease duration of <5 years since the first to MS attributable symptoms. Both patients treated with interferon beta 1a or 1b, and patients untreated with immune modulation were included. Exclusion criteria were: no clinically definite phenotype, use of other immune modulation than interferon beta 1a or 1b, occurrence of a relapse <5 weeks before blood collection, recent start (<¹/₂ year) of vitamin D supplementation. The characteristics of the patients are described in **Table 1**.

Table 1 Patients characteristics.

	Median / N (%)	Range [min – max]
Male / Female (N)	10 (34 %) / 19 (66%)	
Age (years)	39.23	[22.61 – 58.63]
Duration MS (years)	3.48	[0.69 – 4.89]
Relapse Rate (N/year)	0.00	[0.00 – 3.00]
Relapse free (years)	1.04	[0.10 – 3.91]
EDSS* score (points)	2.0	[0.00 – 6.00]
Interferon beta treated (N)	25 (86 %)	

*Expanded disability status scale

2.2 Ethical statement

Written informed consent was acquired from all subjects participating in this study, according to the declaration of Helsinki. The study was reviewed and approved by both the regional ethical committee on human research 'Atrium MC, Orbis MC,

Zuyd', and the institutional ethical committee 'Local Advisory Board on Scientific Research'.

2.3 Vitamin D assays

Blood withdrawal was performed in the period from September 2008 until February 2009. The cells for the cellular assays and the serum for vitamin D measurement were retrieved at the same time, and cellular assays were performed on the day of blood collection. The collected serum was immediately shielded from direct light and stored at -20°C. At the end of the study, all samples were analysed simultaneously. The serum values of 25(OH)D were measured with a commercially available radioimmuno assay (Immunodiagnostic Systems, Boldon, UK) (Smolders *et al.*, 2008b, **chapter 3**).

2.4 Cell culture reagents

Cells were cultured in RPMI glutamax medium (Gibco Invitrogen, Breda, The Netherlands) supplemented with 10% Foetal Calf Serum (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), 1% non-essential amino acids (Gibco Invitrogen), 1% sodium pyruvate (Gibco Invitrogen) and 2% penicillin-streptomycin (Gibco Invitrogen) in U-bottom 96-wells plates.

2.5 Cell purification

Cell isolation was performed as described before, with minor modifications (Venken *et al.*, 2007). PBMC were isolated by Ficoll gradient centrifugation (Histopaque; Sigma Aldrich, Zwijndrecht, The Netherlands). CD4⁺ T cells were selectively isolated with RosetteSep (Stem Cell Technologies, Grenoble, France). Approximately 20–30 x 10⁶ CD4⁺ T cells were incubated at 4°C for 30 minutes with anti-CD4-APC (BD Biosciences, Breda, The Netherlands), anti-CD25-PE (BD Biosciences) and anti-CD127-FITC (BD Biosciences). Human CD4⁺CD25⁺CD127⁻ Tregs (Liu *et al.*, 2006; Seddiki *et al.*, 2006) and CD4⁺CD25⁻ responder T cells (Tresps) were sorted on a FacsAriaTM (BD Biosciences) cell sorter (**Figure 1A**). The mean purity of the sorted populations was 97.51% (SD 0.81) for the Tregs and 97.45% (SD 1.65) for the Tresps. Accessory cells were obtained by irradiating autologous PBMC with 66.2 gray.

Figure 1 Results proliferation suppression assay.

(A) Representative dot-plot of the sorting protocol. $CD25^+CD127^-$ cells within the $CD4^+$ lymphogate were sorted as Tregs, $CD25^+$ cells were sorted as Tresp. (B) The percentages of suppression which were achieved for the respective Treg/Tresp ratios of the individual patients are shown. The Treg/Tresp ratio at which 50% inhibition of proliferation was achieved is defined as ED50 of suppression. The distribution of ED50 is shown in the rightmost column. The lines show the median values. The closed dots represent the Beta Interferon-treated patients, the open dots the untreated patients.

2.6 Proliferation assay

The CFSE-based proliferation suppression assay was adapted from Venken et al. (Venken *et al.*, 2007). In short, the freshly isolated $CD4^+CD25^-$ Tresp were labelled with carboxy fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes Invitrogen, Breda, The Netherlands). In a U-bottom 96-wells plate, 2×10^5 Tresp were stimulated with soluble anti-CD3 (2.0 pg/mL, WT32 IgG2a monoclonal antibody, kindly provided by dr. W Tax, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) in the presence of 2×10^5 irradiated accessory cells. Tresp were co-cultured for 5 days with varying amounts of Tregs (Treg/Tresp ratios 0/1, 0.25/1, 0.5/1, 1/1 or 2/1). All conditions were performed in triplo in a final volume of 200 μ L. After culture, the cells were stained with anti-CD4-APC and 7AAD (BD Biosciences), and the CFSE signal of cells in the $CD4^+7AAD^-$ lymphogate was analysed by Flow Cytometry on a FacsCaliburTM flowcytometer (BD Biosciences). Data were analysed with CellQuest software (BD Biosciences). The amount of proliferating

eration achieved in the 0/1 ratio was set at 0% suppression. The mean relative amount of suppression in the other ratios was calculated. By linear interpolation, the Treg/Tresp ratio at which 50% suppression of proliferation was achieved (ED50) was calculated.

2.7 T cell phenotyping

Tregs were defined as CD25⁺FoxP3⁺ CD4⁺ T cells (Fontenot *et al.*, 2003; Hori *et al.*, 2003) and as CD25⁺CD127⁻ CD4⁺T cells (Liu *et al.*, 2006; Seddiki *et al.*, 2006). For determining the number of Tregs, PBMC were stained with anti-CD3-PerCP (BD Biosciences), anti-CD4-APC, anti-CD4-FITC (BD Biosciences), anti-CD25-PE, anti-CD127-FITC or anti-FoxP3-APC (E-Bioscience, San Diego, USA), and analysed on a FACS Calibur™ flowcytometer. The absolute number of lymphocytes was determined with a haematological cell counter (Beckman Coulter, Woerden, The Netherlands).

T helper cell subsets were determined by assessing the intracellular cytokine pattern of CD3⁺CD8⁻ lymphocytes, which are further referred to as CD4⁺ T cells, by flowcytometry (Ordonez *et al.*, 2009). PBMC were stimulated for 5 hours with calcium ionomycin (Sigma Aldrich), phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) and monensin (BD Biosciences). Cells were stained intra-cellularly with anti-IL-4-PE (BD Biosciences), anti-IFN-γ-FITC (BD Biosciences), anti-IL-17-PE (E-Bioscience) and anti-IL-10-PE (E-Bioscience), and extra-cellularly with anti-CD3-PerCP and anti-CD8-APC.

2.8 Statistics

Statistical analysis was conducted with Statistical Package for Social Sciences version 15.0 software (SPSS inc., Chicago IL, USA) and figures were constructed with GraphPad Prism 5 software (GraphPad Software inc., La Jolla CA, USA). Of continuous variables, the median and corresponding range (min – max) are provided. When normally distributed (Shapiro-Wilk test $P > 0.05$), a linear relationship between two continuous variables was tested with the Pearson correlation coefficient (Pearson R). Abnormally distributed variables were Log-transformed. In case of persistent abnormal distribution, the Spearman correlation coefficient (Spearman R) was determined. A P value < 0.05 was considered statistically significant.

3. Results

3.1 Serum 25(OH)D levels.

We determined the serum levels of 25(OH)D, the vitamin D metabolite which is accepted to reflect vitamin D status best (Hollis *et al.*, 1996). The median serum 25(OH)D level was 54 nmol/L.

3.2 Suppressive capacity of Tregs correlates positively with 25(OH)D levels.

To evaluate Treg suppressive capacity, we conducted an in vitro CFSE-based proliferation suppression assay (**Figure 1B**). Of 4 patients, not enough cells were retrieved to start the assay. In all other individual patients, Tregs suppressed proliferation of T responder cells in a dose-dependent manner. There was no apparent difference in Treg suppression between Beta Interferon treated and untreated patients (**Figure 1B**). The median Treg/Tresp ratio at 50% suppression of proliferation (ED50) was 0.82 (0.16 – 1.96). Serum 25(OH)D levels correlated negatively with ED50 (Spearman $R = -0.590$, $P = 0.002$) (**Figure 2A**). This correlation was reflected by the positive correlation between vitamin D status and the amount of suppression in the individual Treg/Tresp ratios (**Figure 2B–E**).

3.3 Number of circulating Tregs does not correlate with 25(OH)D levels.

Next, we evaluated the absolute and relative number of Tregs in the circulation (**Figure 3**). Regulatory cells within the $CD4^+$ T cell compartment were defined as $CD25^+CD127^-$ and $CD25^+FoxP3^+$ cells. The median percentage of $CD25^+FoxP3^+$ Tregs in the $CD4^+$ T cell compartment was 5.63% (2.67 – 15.81) (absolute number 9.61×10^7 c/L (4.65 – 25.41)). The median percentage of $CD25^+CD127^-$ Tregs was 6.56% (2.93 – 12.32) (absolute 10.32×10^7 c/L (4.44 – 24.14)). A strong positive correlation was observed between the numbers of $CD25^+CD27^-$ and $CD25^+FoxP3^+ CD4^+$ Tregs ($R = 0.826$, $P < 0.001$). Serum levels of 25(OH)D were not correlated with the relative and absolute number of Tregs (**Figure 4**).

3.4 The Th1/Th2 balance correlates negatively with 25(OH)D levels.

$CD4^+$ T cells were classified as Th1, Th2 or Th17 by assessing their intracellular cytokine profile (IFN- γ , IL-4 and IL-17, resp.) (**Figure 5A**). We also assessed the percentage of $CD4^+$ T cells producing the regulatory cytokine IL-10 (Correale *et al.*, 2009). The median percentage of IFN- γ^+ cells within the $CD4^+$ T cell compartment was 12.11% (2.60 – 26.18), of IL-4 $^+$ cells 2.07% (0.90–4.93), of IL-17 $^+$ cells 0.92% (0.31 – 2.35), and of IL-10 $^+$ cells 0.83% (0.43 – 1.45). No significant correlations between serum 25(OH)D levels and the individual percentages of T helper cell subsets were found, although there was a trend towards a negative correlation with the percentage of IFN- γ^+CD4^+ T cells (Pearson $R = -0.336$ $P = 0.060$). The balance between Th1 and Th2 is used to indicate a more pro- or anti-inflammatory composition of the T cell compartment (Steinman 2007). This balance is described by the ratio of IFN- γ^+ and IL-4 $^+CD4^+$ T cells. The IFN- γ /IL-4 ratio correlated negatively with serum 25(OH)D levels (Pearson $R = -0.435$ $P = 0.023$) (**Figure 5B**).

Figure 2 Correlation between Treg suppressive function and serum 25(OH)D levels. (A) The correlation between ED50 and serum 25(OH)D levels. (B–E) Correlation between inhibition of suppression and 25(OH)D levels for the individual Treg/Tresp ratios. The Spearman correlation coefficient is shown. The regression line illustrates the direction of the association. The closed dots represent the Beta Interferon-treated patients, the open dots the untreated patients.

Figure 3 Relative and absolute number of circulating Tregs. Within the CD3⁺CD4⁺ lymphogate, Tregs were defined as either being (A) CD25⁺FoxP3⁺ or being (B) CD25⁺CD127⁻. (C) The percentages of Tregs within the CD4⁺ T cell compartment. (D) Absolute number of circulating Tregs. The lines indicate the median values.

Figure 4 Correlation of the number of circulating Tregs with serum 25(OH)D levels. (A-D) Correlation of relative and absolute number of Tregs with 25(OH)D levels. The Pearson correlation coefficient and corresponding regression line are shown.

Figure 5 Correlation of T helper cell subsets with serum 25(OH)D levels. (A) The percentages of IFN- γ ⁺, IL-4⁺, IL-17⁺ and IL-10⁺ CD4⁺ T cells. The lines show the median values. (B) The correlation between IFN- γ /IL-4 ratio and serum 25(OH)D levels. The Pearson correlation coefficient and corresponding regression line are shown.

3.5 No association between MS disease activity and vitamin D status or T cell parameters.

Neither vitamin D status, nor T cell parameters as Treg function or T cell phenotype did correlate with measures of MS disease activity as described in **Table 1** (data not shown).

4. Discussion

Vitamin D status has been linked with MS either in large epidemiological studies or in experimental *in vitro* and animal studies. This is the first study to investigate the correlation between vitamin D status and the composition of the T cell compartment and in particular Treg function *in vivo* in MS patients. Our data shows that serum 25(OH)D levels correlate positively with the ability of CD4⁺CD25⁺CD127⁻ Tregs to suppress proliferation of Tresp cells. The absolute and relative Treg numbers in the circulation were not correlated with vitamin D status. Although we did not observe a significant association between 25(OH)D levels and the individual percentages of CD4⁺ T helper cell subsets, the decreasing IFN- γ /IL-4 ratio reflected a skewing of the Th1/Th2-balance towards a more Th2 phenotype in patients with higher serum 25(OH)D levels. Altogether, this data indicates that high serum 25(OH)D levels are associated with a less inflammatory and better regulated T cell compartment in MS.

In our data, we found no association between vitamin D status and disease severity and activity of MS. This is in contrast with earlier observations (Kragt *et al.*, 2009; Smolders *et al.*, 2008b; **Chapter 3**; van der Mei *et al.*, 2007). However, since we measured patients in the period from September until February, the seasonal fluctuation of vitamin D status is likely to confound these associations (Kimlin *et al.*, 2008). In contrast to the MS activity variables, this seems not critical for the correlation with the T cell tests: the data obtained reflects the status of the T cell com-

partment at the moment of blood collection. Additionally, several studies have shown a seasonal fluctuation of immunological parameters in MS patients comparable to the fluctuation of serum 25(OH)D levels (Balashov *et al.*, 1998; Stewart *et al.*, 2007).

In a previous study, CD4⁺CD25^{high} Tregs of RRMS patients achieved only about 40% suppression in a 1:1 Treg/Tresp ratio as measured in a ³H-Thymidine assay (Viglietta *et al.*, 2004). A later study confirmed a mean suppression of 40% in untreated RRMS patients, and of 50% in Beta interferon users (Venken *et al.*, 2006). Our RRMS patients reached in the 1:1 ratio a mean suppression of 60% (20–90). Beta Interferon treatment has been shown to promote Treg function (Venken *et al.*, 2008). Therefore, the fact that most of our patients were treated with Beta Interferon might have contributed to this substantially more favourable suppression. Additionally, we included the α -chain of the IL-7 receptor (CD127), which has been shown to be specifically absent on CD4⁺CD25⁺FoxP3⁺ Tregs, in our sorting protocol to define Tregs (Liu *et al.*, 2006; Seddiki *et al.*, 2006). Therefore, the sorted population of CD4⁺CD25⁺CD127⁻ Tregs may comprise a more pure Treg fraction, compared to CD4⁺CD25^{high} sorted T cells alone (Michel *et al.*, 2008).

Our data shows that in RRMS patients with a short disease duration, in which Treg function has been shown to be particularly compromised (Venken *et al.*, 2006), Treg suppressor function correlates positively with serum 25(OH)D levels. The relationship between Treg function and 25(OH)D levels is likely to contribute to the inter-individual variation of Treg suppressive function in MS cohorts in other studies (Haas *et al.*, 2005; Venken *et al.*, 2006; Viglietta *et al.*, 2004). However, we could not find an association of serum vitamin D values with the number of CD4⁺CD25⁺FoxP3⁺ or CD4⁺CD25⁺CD127⁻ Tregs in the circulation. This discrepancy between the impaired number and function of Tregs has been shown in other studies on Tregs in MS and other autoimmune diseases (Constantino *et al.*, 2008). However, the underlying mechanism is not known. Interestingly, a recent publication showed a negative correlation between the percentage of FoxP3⁺ Tregs within the T cell compartment of MS patients and their vitamin D status (Royal III *et al.*, 2009). However, the heterogeneous study population considering race and disease duration, and the use of linear regression models to analyse not-equally distributed parameters in a limited sample size, require careful interpretation of these results. The discrepancy with our data not showing a comparable correlation, might be attributable to these methodological differences.

Additionally, we found a negative correlation between serum 25(OH)D levels and the IFN- γ /IL-4 ratio. This ratio is commonly used to describe the balance in the immune system between pro-inflammatory IFN- γ ⁺ Th1 cells and anti-inflammatory IL-4⁺ Th2 cells (Steinman 2007). Our data suggests that, in patients with relatively high 25(OH)D-levels, the balance between Th1 and Th2 is more skewed in the direction of Th2. Interestingly, *in vitro* research also suggested that vitamin D skews the T

cell compartment from a Th1 towards a Th2 phenotype (Correale *et al.*, 2009; Smolders *et al.*, 2008a; **Chapter 3**). Therefore, high 25(OH)D levels appear to be associated with a less-pro-inflammatory T-cell compartment in MS patients.

In conclusion, we have shown that serum 25(OH)D levels are associated with the suppressive function of Tregs and the composition of the T cell compartment in MS patients. These findings further support the hypothesis that vitamin D is an important promoter of T cell regulation in MS. Since in RRMS patients the Treg compartment has been shown to be compromised, our findings provide additional arguments to maintain a healthy vitamin D status in these patients. Also other autoimmune diseases have been associated with both low vitamin D levels, and a reduced regulatory T cell function (Arnson *et al.*, 2008; Constantino *et al.*, 2008). Whether a poor vitamin D status correlates in healthy populations with a reduced Treg function and potentially a subsequently increased risk for developing autoimmune diseases, remains to be established. Altogether, it is tempting to speculate about the potential therapeutic effect of vitamin D supplementation in autoimmune diseases. Previously, it was demonstrated that therapy with Beta Interferon in MS results in a promotion of regulatory T cell function (Venken *et al.*, 2008) and a decrease of relapse rate (IFNB MS study group, 1995). Since we observed an additional effect of 25(OH)D levels in Beta Interferon users, this might provide a rationale for add-on therapy with vitamin D during Beta Interferon therapy in MS. Randomized clinical trials should assess an additional role of vitamin D treatment on the T cell compartment and more importantly on disease activity of MS.

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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 has been proposed as a potential 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 (Bar Or 2008). In experimental studies, vitamin D inhibits Th1 and Th17 cells, and promotes Th2 and regulatory T cells (Treg) (Correale *et al.*, 2009; Smolders *et al.*, 2008a, **chapter 2**). Recently, we showed that vitamin D status, as reflected by serum levels of 25-hydroxyvitamin D, correlated positively with Treg functionality, and with a more towards Th2 directed Th1/Th2 balance in patients with relapsing remitting MS (RRMS) (Smolders *et al.*, 2009d, **chapter 4**). Association studies showed that vitamin D status was poorer in subjects developing MS in later life (Munger *et al.*, 2006), and correlated negatively with disease activity (Smolders *et al.*, 2008b, **chapter 3**). Although 25(OH)D is the most abundant metabolite, the biologically active metabolite 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 hydroxylation of 25(OH)D in the kidneys upon signals from calcium metabolism, e.g. calcium and parathyroid hormone (PTH) (Jongen *et al.*, 1984). An increase in serum 1,25(OH)₂D results in increasing serum calcium levels, by promoting calcium resorption 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 (Smolders *et al.*, 2009d, **chapter 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 yrs. (23–59), the median disease duration 3.5 yrs (0.7–4.9), the median relapse rate 0/yr (0–3), and the median expanded disability status scale (EDSS) score 2 (0–6). Twenty-five (86%) patients were treated with beta interferon, 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 in the period September 2008 – February 2009. Serum values of 25(OH)D and 1,25(OH)₂D were determined with commercially available radioimmuno assays (Immunodiagnostic Systems, Boldon, UK). Serum total

calcium levels were determined with the Immunolite 2000TM (Siemens Healthcare Diagnostics, Deerfield IL, USA), and intact PTH levels were determined with the Synchron LXTM (Beckman Coulter, Woerden, The Netherlands). Reference values of the clinical chemical laboratory are provided.

T cell variables were determined as described extensively before (Smolders *et al.*, 2009d, **chapter 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 hour 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 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 FACS AriaTM 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 FACS CaliburTM flow-cytometer and analyzed with CellQuestTM software (both BD Biosciences).

Statistical analysis was performed with 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, serum 25(OH)D values correlated positively with Treg suppressive function (**Figure 1A**). Serum 1,25(OH)₂D, PTH and calcium did not correlate significantly with Treg functionality (**Figure 1 B-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), of CD25⁺FoxP3⁺ Tregs 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, serum 25(OH)D correlated negatively with the Th1/Th2-ratio (**Figure 1E**). The other calcium metabolism parameters did not correlate significantly with Th1/Th2 ratio (**Figure 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 (**Figure 1 I-L**).

Figure 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 a 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 ionomycin, and (I-L) the proportion of IL-17⁺ CD4⁺ T cells in a PBMC culture stimulated with PMA and calcium ionomycin. 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.

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, 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 ani-

mal-model of MS, dietary calcium intake and the subsequent elevation of serum calcium concentration were critical factors in the clinical and immunological effects of $1,25(\text{OH})_2\text{D}$ therapy (Cantorna *et al.*, 1999). Calcitonin, a protein released during hypercalcaemia, enhanced the clinical effectiveness of $1,25(\text{OH})_2\text{D}$ in EAE (Becklund *et al.*, 2009a), but was not mandatory (Becklund *et al.*, 2009b). 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, Soliu-Hänninen *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 $25(\text{OH})\text{D}$ levels (Soliu-Hänninen *et al.*, 2008). Additionally, intact PTH and $25(\text{OH})\text{D}$ levels were, respectively, higher and lower during relapse when compared to remission.

Contrastingly, our data suggest that serum calcium, PTH, and $1,25(\text{OH})_2\text{D}$ are not critically involved in the interaction between vitamin D status and T cell regulation in MS patients. The trend towards correlation of $1,25(\text{OH})_2\text{D}$ with Treg suppressive function is likely to be secondary to the strong relationship between $25(\text{OH})\text{D}$ and $1,25(\text{OH})_2\text{D}$. Likewise, the apparent negative correlation between disability and $1,25(\text{OH})_2\text{D}$ levels in MS patients that we earlier reported, appeared to be dependent on the correlation of EDSS with serum $25(\text{OH})\text{D}$ in a multiple regression analysis (Smolders *et al.*, 2008b, **chapter 3**). A patient control study also suggested that the contribution of serum $1,25(\text{OH})_2\text{D}$ levels to the risk for developing MS is limited (Kragt *et al.*, 2009). 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 $1,25(\text{OH})_2\text{D}$ out of $25(\text{OH})\text{D}$ at sites of immune activation (Correale *et al.*, 2009). These local levels of $1,25(\text{OH})_2\text{D}$ 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 (Torkildsen *et al.*, 2008). Additionally to a deficiency of serum $1,25(\text{OH})_2\text{D}$ levels in these patients, the defect in the $25(\text{OH})\text{D}$ - 1α -hydroxylase gene also abolishes $1,25(\text{OH})_2\text{D}$ formation by activated immune cells. Although speculative, our data suggest the latter mechanism to be most important in the reported association. Royal III *et al.*, reported that the ratio between $1,25(\text{OH})_2\text{D}$ and $25(\text{OH})\text{D}$ correlated with the proportion of Tregs in the CD4^+ T cell compartment (Royal 3rd *et al.*, 2009). We could not reproduce these results in our patient cohort.

The positive correlation of $1,25(\text{OH})_2\text{D}$ with the percentage of Th17 cells seems contradictory. Th17 cells have been proposed to be the most important pathogenic cells in MS (Bar Or 2008). Therefore, a positive correlation of serum $1,25(\text{OH})_2\text{D}$ levels and these cells suggests a negative effect of vitamin D on MS, which has, however, not been observed in clinical studies (Kragt *et al.*, 2009; Smolders *et al.*,

2008b, **chapter 3**). 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 (Zhou *et al.*, 2009). Alternatively, several autoimmune disease, including MS, have been associated with a loss of bone mineral density (BMD) (Nieves *et al.*, 1994). Th17 cells have been proposed to promote osteoclast function via IL-17 production, and to establish hereby loss of BMD (Nakashima *et al.*, 2009). 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, 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.

Safety and T cell modulating effects of high dose vitamin D₃ supplementation in relapsing remitting multiple sclerosis

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Abstract

Background: A poor vitamin D status has been associated with a high disease activity of multiple sclerosis (MS). Recently, we described associations between vitamin D status and peripheral T cell characteristics in relapsing remitting MS (RRMS) patients. In the present study, we studied the effects of high dose vitamin D₃ supplementation on safety and T cell related outcome measures.

Methodology/ Principal Findings: Fifteen RRMS patients were supplemented with 20 000 IU/d vitamin D₃ for 12 weeks. Vitamin D and calcium metabolism were carefully monitored, and T cell characteristics were studied by flowcytometry. All patients finished the protocol without side-effects, hypercalcaemia, or hypercalciuria. The median vitamin D status increased from 50 nmol/L (31–175) at week 0 to 380 nmol/L (151–535) at week 12 ($P < 0.001$). During the study, 1 patient experienced an exacerbation of MS and was censored from the T cell analysis. The proportions of (naïve and memory) CD4⁺ Tregs remained unaffected. Treg suppressive function improved not significantly. An increased proportion of IL-10⁺ CD4⁺ T cells was found after supplementation. Additionally, a decrease of the ratio between IFN- γ ⁺ and IL-4⁺ CD4⁺ T cells was observed.

Conclusion/ Significance: Twelve week supplementation of high dose vitamin D₃ in RRMS patients was well tolerated and did not induce decompensation of calcium metabolism. The skewing towards an anti-inflammatory cytokine profile supports the evidence on vitamin D as an immune-modulator, and may be used as outcome measure for upcoming randomized placebo-controlled trials.

Registration: This study was registered at www.clinicaltrials.gov as NCT00940719.

1. Introduction

Multiple Sclerosis (MS) is an inflammatory disease of the central nervous system, probably of autoimmune origin. It is presumed to arise from a combination of genetic and environmental factors (Compston and Coles, 2008). One of the environmental factors which gained much attention during the last decades, is vitamin D. A poor exposure to either vitamin D or sunlight, its most important inducer, has been associated with a high risk of developing MS (Ascherio *et al.*, 2010). The underlying mechanism has not been unravelled yet, but a central role for the actions of vitamin D on immune regulation has been proposed (Hayes *et al.*, 1997; Smolders *et al.*, 2008a, **chapter 2**). The biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$), has potent immune modulating properties, both *in vitro* and in the experimental animal model of MS, Experimental Autoimmune Encephalomyelitis (EAE) (Correale *et al.*, 2009; Cantorna *et al.*, 1996). *In vitro*, exposure to $1,25(\text{OH})_2\text{D}$ inhibits CD4^+ T cell proliferation and pro-inflammatory cytokine production (IFN- γ and IL-17), and promotes anti-inflammatory cytokine production (IL-4 and IL-10) and acquisition of regulatory T cell (Treg) phenotype (Smolders *et al.*, 2008a, **chapter 2**).

Not only incidence, but also disease activity of MS has been associated with vitamin D status. Disability of MS is inversely correlated with vitamin D status [Smolders *et al.*, 2008b, **chapter 3**; van der Mei *et al.*, 2007], but the causality of this association is uncertain (Ascherio and Munger, 2008). Additionally, a poor vitamin D status has been associated with an increased risk on relapses (Simpson *et al.*, 2010; Smolders *et al.*, 2008b, **chapter 3**; Soliu-Hänninen *et al.*, 2005). A recent study revealed an increased proportion of relapse free relapsing remitting MS (RRMS) patients in a cohort supplemented with 14 000 IU vitamin D_3/d for 52 weeks (Burton *et al.*, 2010). The immune modulating properties of vitamin D have been proposed to underlie these associations. Supplementation of 1 000 IU vitamin D_3/d for 6 months induced elevated circulating TGF- β levels in MS patients (Mahon *et al.*, 2003). We studied the correlation of vitamin D status with T cell regulatory status in a cross-sectional design (Smolders *et al.*, 2009d, **chapter 4**). Although no correlations with the circulating numbers of CD4^+ Treg were found, vitamin D status correlated positively with the suppressive capacity of Treg. Additionally, the ratio between pro-inflammatory IFN- γ^+ and anti-inflammatory IL-4 $^+$ CD4^+ T cells was higher in RRMS patients with a poor vitamin D status. These associations were independent from the effects of vitamin D on calcium homeostasis (Smolders *et al.*, 2010, **chapter 5**).

These clinical observations and experimental data on vitamin D and MS warrant the development of well designed clinical trials to assess the disease and immune modulating effects of vitamin D in MS. The aim of the present pilot study was to explore the safety and the *in vivo* effects on the peripheral T cell compartment of high dose vitamin D_3 supplementation in MS.

2. Materials and methods

2.1 Subjects

Between October – December 2009, 15 RRMS patients were enrolled (Polman *et al.*, 2005). Inclusion criteria were age >18 years, disease duration <6 years, use of Beta Interferon (IFN- β) 1a or 1b, and being relapse free ≥ 6 weeks prior to enrolment. Exclusion criteria were current use of drugs associated with an increased susceptibility to hypercalcaemia, treatment with immune modulating/ suppressive drugs other than IFN- β ≤ 6 weeks prior to enrolment, pregnancy, hypercalcaemia, serum creatinine >100 $\mu\text{mol/L}$, and a history of primary hyperparathyroidism, hypercalcaemia, renal dysfunction, cardiac disease, malignancy, or granulomatous disease. Holidays to sunny locations and the use of solariums were not allowed. Continuation of multivitamins containing vitamin D ≤ 600 IU $\mu\text{g/day}$ was allowed. Written informed consent was obtained from each subject. The regional ethical committee 'Atrium-Orbis-Zuyd' and the institutional 'Local Advisory Board on Scientific Research' approved this study. This study is registered at www.clinicaltrials.gov as NCT00940719.

2.2 Vitamin D₃ supplementation

To achieve serum 25-hydroxyvitamin D (25(OH)D) levels comparable to the study by Burton *et al.* within 12 weeks (Burton *et al.*, 2010), participants were supplemented with 20 000 IU vitamin D₃/d. An oil-based vitamin D₃ solution in a concentration of 20 000 IU/mL was obtained (Vigantol Oil; Merck Serono, Darmstad, Germany) and patients were instructed to ingest 1 mL each morning (Maalouf *et al.*, 2008). No dose-escalation was incorporated, no dietary restrictions were imposed, and no additional calcium supplementation was provided. Vitamin D₃ solution was provided twice for 6 weeks. At the end of week 6 and 12, residual volume of the vitamin D₃ solution was assessed. Additionally, patients registered their vitamin D₃ intake daily on a calendar. As a safety follow-up, additional visits were performed at week 16 and 24.

2.3 Biochemical analysis

At each study visit, calcium, albumin, and 25(OH)D were measured. Additionally, we periodically measured parathyroid hormone (PTH), creatinine, urea, electrolytes and liver function enzymes [alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP)]. A urine sample was obtained the morning of the study visits to determine the ratio between the molar concentrations of calcium and creatinine. We measured PTH on the Immunolite 2500 analyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Other serum and urine biochemical analytes were measured on the Beckman DXC880i analyzer (Beckman Coulter, Woerden, The Netherlands). A radioimmunoassay kit (Immunodiagnostic Systems,

Boldon, UK) was used to measure serum 25(OH)D and 1,25(OH)₂D concentrations. Toxicity of vitamin D₃ manifests as hypercalcemia (serum total calcium $\geq$ 2.6 mmol/L) or hypercalciuria (urinary calcium to creatinine ratio $>$ 1.0 mmol/L x (mmol/L)⁻¹) (Kimball *et al.*, 2007).

2.4 Cell isolation and purification

Cell isolation was performed as described before (Smolders *et al.*, 2009d, **chapter 4**). PBMC were isolated by Ficoll gradient centrifugation (Histopaque; Sigma Aldrich, Zwijndrecht, The Netherlands). CD4⁺ T cells were isolated by negative selection with RosetteSep (Stem Cell Technologies, Grenoble, France). CD4⁺ T cells were incubated at 4°C for 30 minutes with anti-CD4-APC (Biolegend, Uithoorn, The Netherlands, #300514), anti-CD25-PE (BD Biosciences, Breda, The Netherlands, #555432) and anti-CD127-FITC (E-Bioscience, Hatfield, UK, #11-1278). Human CD4⁺CD25⁺CD127⁻ Treg (Liu *et al.*, 2006), and CD4⁺CD25⁻ responder T cells (Tresp) were sorted on a FACS Aria (BD Biosciences) cell sorter. After the sort, the median proportion CD4⁺CD25⁺CD127⁻ cells of all events was 98.9% (97.6 – 99.5) in the Treg tube, and the median proportion CD4⁺CD25⁻ cells was 99.1% (91.0 – 99.6) in the Tresp tube. Accessory cells were obtained by irradiating autologous PBMC with 66.2 gray.

2.5 Proliferation suppression assay

The CFSE-based proliferation suppression assay was performed as described before (Smolders *et al.*, 2009d, **chapter 4**). In short, the freshly isolated Tresp were labelled with CFSE (Molecular Probes Invitrogen, Breda, The Netherlands). In a U-bottom 96-wells plate, 2×10^4 Tresp were stimulated with soluble anti-CD3 (2.0 pg/mL, WT32 IgG2a monoclonal antibody, kindly provided by dr. W Tax, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) in the presence of 1×10^5 irradiated accessory cells. Tresp were co-cultured for 5 days with varying amounts of Treg (Treg/Tresp ratios 0/1, 0.25/1, 0.5/1, 1/1 or 2/1). All conditions were performed in triplo in a final volume of 200 μ L. Control conditions were incorporated to validate the assay, including monocultures of un-stimulated Tresp, and of stimulated accessory cells, Treg, and surpluses of Tresp. After culture, cells were stained with anti-CD4-APC and 7AAD (BD Biosciences, #559925), and the CFSE signal of cells in the CD4⁺7AAD⁻ lymphogate was analysed by flow cytometry on a FACS Canto II flowcytometer (BD Biosciences). Data were analysed with FACS Diva software version 6.1.2. (BD Biosciences). The amount of proliferation achieved in the 0/1 ratio was set at 0% suppression. The median relative amount of suppression in the Treg/Tresp co-cultures was calculated. By linear interpolation, the inhibitor ratio (Treg/Tresp) ratio at which 40% suppression of proliferation was achieved (IR40) was calculated.

2.6 T cell phenotyping and cytokine analysis

Treg were defined as CD25⁺FoxP3⁺ CD4⁺ T cells (Fontenot *et al.*, 2003) and as CD25⁺CD127⁻ CD4⁺ T cells (Liu *et al.*, 2006). Furthermore, CD45RA expression was used to stratify CD25⁺CD127⁻ CD4⁺ T cells for memory (CD45RA⁻) and naïve (CD45RA⁺) Treg (Seddiki *et al.*, 2006). For phenotyping, PBMC were stained with different combinations of anti-CD3-horizon 450 (BD biosciences, #560365), anti-CD3- PerCP (BD biosciences, #27355), anti-CD4-APC, anti-CD4-PerCP (Biolegend, #300528), anti-CD25-PECy7 (BD Biosciences, #54679), anti-CD45RA-PE (BD Biosciences, #07804), anti-CD127-FITC or anti-FoxP3-PE (E-Bioscience, #12-4776), and analysed on a FACS Canto II flowcytometer. The absolute number of lymphocytes was determined with a haematological cell counter (Beckman Coulter).

The cytokine profile of CD4⁺ T cells was determined by assessing the intracellular cytokine pattern of CD3⁺CD8⁻ lymphocytes, which are further referred to as CD4⁺ T cells, by flowcytometry (Smolders *et al.*, 2009d, **chapter 4**). PBMC were stimulated for 5 hours with calcium ionomycin (Sigma Aldrich), PMA (Sigma Aldrich) and cytokine excretion was blocked with monensin (BD Biosciences). CD69 expression was included to assess the activation status of the cells. Cells were stained intracellularly with anti-IL-4-PE (Biolegend, #500704), anti-IFN- γ -FITC (BD Biosciences, #340449), anti-IL-17A-PerCP-CY5.5 (Biolegend, #512314), anti-IL-10-APC (Biolegend, #501410) and anti-CD69-PE-CY7 (Biolegend, #310912), and extracellularly with anti-CD3-horizon 450 and anti-CD8-APC-H7 (BD Biosciences, #641400). Samples were analysed on a FACS Canto II flowcytometer.

2.7 Statistical analysis

Statistical analysis was conducted with SPSS version 15.0 software (SPSS inc., Chicago IL, USA) and figures were constructed with GraphPad Prism 5 software (GraphPad Software inc., La Jolla CA, USA). The median and corresponding range (min – max) are provided for continuous variables. Differences between two related samples (week 0 and 12) were assessed with the Wilcoxon signed-ranks test, the Man Whitney U test was used for unpaired testing. Paired testing was applied unless indicated otherwise. A p-value <0.05 was considered statistically significant.

3. Results

3.1 Adverse events and patient compliance

The characteristics of the 15 participants are shown in **Table 1**. All patients completed the 12 week supplementation protocol without side effects other than increased flatulence (N = 1). Patients reported some beneficial changes, including decreased fatigue (N = 8), decreased headache (N = 2), decrease in eczematous eruptions (N = 1). During the 12 weeks of supplementation, 1 patient developed a

relapse of MS, at week 7 of the protocol, which was confirmed by the treating neurologist. This patient was treated with pulsed prednisolone therapy. During the follow-up period, 1 additional patient suffered from an MS relapse (week 19), resulting in a mean annualized relapse rate of 0.27 (SD 0.70) over 24 weeks in this cohort, compared to 0.80 (SD 0.77) in the year prior to this study (Mann Whitney U test $P = 0.014$). No further (serious) adverse events were registered. The median total number of missed days of vitamin D₃ intake was 0/ 84 days (0 – 5), according to the patient's diaries. Measurement of residue in the returned vitamin D bottles revealed a median difference of -0.2/ 42 mL (-8.0 – 8.0) at week 6, and of +0.4/ 42 mL (-7.5 – 5.0) at week 12.

Table 1 Patient characteristics.

	Median/ Category	Min-Max range/ N (%)
Sex	Female	N = 8 (53%)
	Male	N = 7 (47%)
Age	35.15 year	25.47 – 49.36
MS duration since first symptoms	3.47 year	0.63 – 5.58
Time since last relapse	0.96 year	0.21 – 3.35
Number of relapses last 12 months	1.0	0.0 – 3.0
EDSS-score*	2.0	0.5 – 4.0
IFN- β type	Avonex	N = 3 (20%)
	Betaferon	N = 3 (20%)
	Rebif	N = 9 (60%)
Race	Caucasian	N = 14 (93%)
	Hispanic	N = 1 (7%)

*Expanded Disability Status Scale (Kurtzke 1983)

3.2 Biochemical assessment

Serum levels of 25(OH)D increased gradually during the study in all patients (**Figure 1A**). The median level was 50 nmol/L (31 - 175) at week 0, and the serum level 25(OH)D of 2 patients exceeded 100 nmol/L (109 and 175 nmol/L, respectively). Remarkably, the patient with the highest serum 25(OH)D level used no vitamin supplements. At week 12, the median 25(OH)D levels was 380 nmol/L (151 - 535) ($P < 0.001$). Additionally, a rise in serum 1,25(OH)₂D levels was observed, which stabilized after 4 weeks (**Figure 1B**). After 6 weeks of supplementation, a decrease in serum PTH was observed (Table 2). Serum uncorrected and albumin corrected calcium levels were not significantly elevated, and remained below 2.6 mmol/L in all subjects (**Figure 1C**). Additionally, no hypercalciuria (urinary calcium to creatinine ratio > 1.0) was observed (**Figure 1D**). Liver enzyme tests remained unaffected. However, serum creatinine levels gradually increased during vitamin D₃ supplementation, but remained stable during the follow-up visits (**Table 2**).

Table 2 Biochemical follow-up

Parameter	Supplementation of vitamin D ₃				Follow-up after cessation			
	W0	W6	W12	W16	W24	W36	W48	W60
	Median	(Min–Max)	Median	(Min–Max)	Median	(Min–Max)	Median	(Min–Max)
25(OH)D (nmol/L)	50	(31 – 175)	285**	(122 – 401)	0.001	(151 – 535)	0.001	(151 – 535)
1,25(OH) ₂ D (pmol/L)	146	(58 – 272)	208**	(99 – 434)	0.002	(90 – 400)	0.003	(90 – 400)
Total calcium (mmol/L)	2.3	(2.2 – 2.5)	2.4	(2.2 – 2.5)	0.133	(2.2 – 2.5)	0.208	(2.2 – 2.5)
Albumin (g/L)	39	(32 – 47)	39	(31 – 44)	0.134	(31 – 46)	0.606	(31 – 46)
Corrected calcium (mmol/L) ^A	2.36	(2.14 – 2.52)	2.38*	(2.32 – 2.56)	0.048	(2.26 – 2.50)	0.082	(2.24 – 2.58)
Phosphorus (mmol/L)	1.0	(0.7 – 1.1)	1.1	(0.8 – 1.4)	0.057	(0.9 – 1.5)	0.008	(0.8 – 1.4)
PTH (pmol/L)	4.6	(1.9 – 10.5)	2.8**	(1.7 – 6.7)	0.003	(0.9 – 9.6)	0.003	(0.9 – 9.6)
Sodium (mmol/L)	139	(136 – 142)	139	(138 – 143)	0.150	(138 – 143)	0.005	(137 – 142)
Potassium (mmol/L)	4.1	(3.4 – 4.6)	4.0	(3.5 – 4.6)	0.414	(3.6 – 4.7)	0.008	(3.6 – 4.7)
Urea (mmol/L)	3.5	(2.3 – 6.0)	3.5	(2.4 – 6.9)	0.776	(2.6 – 6.4)	0.221	(2.7 – 5.8)
Creatinine (μmol/L)	65	(47 – 84)	69	(49 – 99)	0.060	(49 – 109)	0.018	(49 – 97)
AST (U/L)	31	(21 – 42)	32	(21 – 40)	0.820	(21 – 40)	-	(20 – 52)
ALT (U/L)	34	(19 – 95)	33	(22 – 80)	0.975	(22 – 80)	-	(19 – 120)
ALP (U/L)	67	(42 – 93)	62	(45 – 85)	0.529	(45 – 85)	-	(43 – 85)

^ACalculated with the formula: $\text{Calciumcorr (mmol/L)} = \text{total Calcium (mmol/L)} + [(40 - \text{Albumin (g/L)}) \times 0.02]$. ^BDifference when compared to baseline levels was tested with the Wilcoxon Signed-Ranks test. * $P < 0.050$; ** $P < 0.010$. Abbreviations: 25(OH)D: 25-hydroxyvitamin D; 1,25(OH)₂D: 1,25-dihydroxyvitamin D; PTH: parathyroid hormone; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase.

Figure 1 Vitamin D and calcium metabolism during vitamin D₃ supplementation. A-B) The serum levels of 25(OH)D (A) and 1,25(OH)₂D (B) are shown for the study visits at week 0, 2, 4, 6, and 12. The thick line indicates the median level, the grey zone the interquartile range. The thin lines show the minimum and maximum values. C) Total serum calcium levels at all study visits (week 0, 2, 4, 6, 12, 16, and 24). Hypercalcaemia is designated as a serum total calcium level ≥ 2.6 mmol/L. The grey lines indicate the median values. D) Urine levels of total calcium and creatinine levels and their ratio at week 12 and 24. Hypercalciuria is designated as a urine total calcium/creatinine ratio > 1.0 mmol/L*(mmol/L)⁻¹. The lines indicate the median values.

3.3 Regulatory T cell numbers are not affected by vitamin D supplementation

The difference in composition and function of the T cell compartment between week 0 and week 12 was assessed in the 14 patients that remained relapse-free during the first 12 weeks. No difference was observed in the absolute amount of T cells in the circulation [1.37×10^6 (0.91 – 2.39) vs. 1.32×10^6 (0.90 – 2.24) cells/mL; $P = 0.761$]. PBMC were stained directly *ex vivo* to determine the proportions of circulating CD4⁺ Treg cells (**Figure 2A**). Week 0 and week 12 proportions of CD25⁺FoxP3⁺ and CD25⁺CD127⁻CD4⁺ Treg did not differ significantly (**Figure 2B–C**). Proportions of these two Treg phenotypes correlated well (Pearson $R = 0.891$, $P < 0.001$; ICC = 0.872, $P < 0.001$), and 74.03% (52.09 – 89.75) of CD25⁺CD127⁻ Treg was FoxP3⁺. The expression of CD45RA was used to stratify Treg for CD45RA⁺ naïve and CD45RA⁻ memory Treg (**Figure 2A**). There was no difference in the circulating proportions of both naïve and memory Treg between week 0 and week 12 (**Figure 2D**).

Figure 2 Phenotypic analysis of circulating regulatory T cells (Treg). A) Isolated PBMC were analyzed directly ex-vivo by flow cytometry. In the lymphogate, CD3⁺CD4⁺ cells were assessed for the proportions of CD25⁺FoxP3⁺ and CD25⁺CD127⁻ Treg cells. Expression of CD45RA was analyzed to phenotype naïve (CD45RA⁺) and memory (CD45RA⁻) Treg. B-D) The proportions of circulating CD25⁺FoxP3⁺ (B) and CD25⁺CD127⁻ (C)Treg, and of naïve Treg (D) before and after vitamin D₃ supplementation (week 0 and 12). Significance was assessed with the Wilcoxon signed ranks comparison test.

3.4 No significant improvement of regulatory T cell suppressive function

The suppressive capacity of Treg was assessed ex-vivo in a proliferation suppression assay. Of 1 patient, Tresp cells were anergic to anti-CD3 stimulation at week 0. Of the remaining 13 patients, a proliferation suppression assay was acquired at week 0 and week 12. No difference was observed in the amount of Tresp cell proliferation in the culture system [82.8% (53.9 – 93.3) vs. 83.4% (64.0 – 96.3); $P = 0.848$]. At both time points, an increasing suppression of Tresp proliferation was seen with increasing Treg/Tresp ratio (**Figure 3**). At week 0, the median IR40 was at a Treg/Tresp ratio of 0.79 (0.37 – 1.31). At week 12, the median IR40 was at 0.48 (0.29 – 1.27) (Mann Whitney U test $P = 0.090$). However, there was no statistically significant improvement of Treg suppressive function ($P = 0.143$). Baseline 25(OH)D levels did not differ significantly between improving and non-improving subjects (49 nmol/L (39 – 109) vs. 74 nmol/L (48 – 104), respectively; $P = 0.260$). However, non-improving subjects tended to have better suppression at week 0 when compared to

improving subjects (IR40 0.51 (0.37 – 0.99) vs. 0.86 (0.49 – 1.31), respectively; $P = 0.076$).

Figure 3 Regulatory T cell suppressive function.

A) A representative example of the CFSE signal in the $CD4^+7AAD^-$ lymphogate. The upper panel (0.00) shows proliferation of Tresp cells without Treg. Co-culture with an increasing proportion of Treg results in an inhibition of Tresp cell proliferation (downward panels, ratio 0.25:1, 0.5:1, 1:1 and 2:1, respectively). An increasing proportion of CFSE⁺ Treg can be observed, and a subsequent decrease of proliferating CFSE⁺ Tresp cells. Between the Treg/Tresp ratio 0.25 and 2.0, all patients achieved at both time points (week 0 and 12) 40% suppression of proliferation (IR40). B) The box and whisker plots show the suppression at each Treg/ Tresp ratio before (week 0, open boxes) and after supplementation (week 12, dashed boxes). The boxes show the interquartile range, the whiskers the minimum and maximum value. C) IR40 before and after vitamin D₃ supplementation (week 0 and 12).

3.5 $CD4^+$ T cell cytokine profile shifts from pro- to anti-inflammatory

The cytokine profile of the $CD4^+$ T cells was assessed with an intracellular FACS-staining (**Figure 4A**). Cytokines of interest were IFN- γ (Th1 cytokine), IL-17 (Th17 cytokine), IL-4 (Th2 cytokine) and IL-10 (regulatory cytokine). The efficacy of stimulation, as evaluated by the expression level of the activation marker CD69, was comparable between week 0 and 12 (median MFI = 91 030 (45 880 – 169 520) and 88 580 (56 550 – 123 710), respectively). When week 0 and 12 were compared, no difference was observed in the proportions of $CD4^+$ T cells positive for IFN- γ [7.82% (3.45 – 12.74) vs. 6.51% (1.07 – 14.66)], IL-17 [0.90% (0.50 – 2.09) vs. 0.85% (0.16 – 2.19)], or IL-4 (2.04% (0.80 – 3.62) vs. 2.37% (0.88 – 4.21)]. However, the proportion of IL-10⁺ $CD4^+$ T cells was significantly increased [0.36% (0.20 – 0.95) vs. 0.69% (0.13 – 1.14); $P = 0.021$] (**Figure 4B**). When the Th1/ Th2 balance was assessed, we found

that the $\text{IFN-}\gamma^+/\text{IL-4}^+$ balance was decreased after supplementation [3.68 (1.86 – 10.76) vs. 2.98 (0.71 – 5.55); $P = 0.035$] (**Figure 4C**).

Figure 4 T helper cell cytokine profiles. A) One million PBMC were stimulated for 5 hours with PMA and calcium ionomycin in the presence of monensin, and subsequently stained for flow cytometric analysis. In the lymphogate, $\text{CD3}^+\text{CD8}^-$ cells were assessed for the proportions of $\text{IFN-}\gamma^+$, IL-4^+ , IL-17^+ and IL-10^+ cells. CD69 was included to evaluate activation. B) The proportions of $\text{IL-10}^+\text{CD4}^+$ T cells before and after supplementation. C) The ratio between Th1 ($\text{IFN-}\gamma^+$) and Th2 (IL-4^+) cells before and after supplementation. Significance was assessed with the Wilcoxon signed ranks comparison test.

4. Discussion

In this study, the effects of high dose vitamin D_3 supplementation on safety and T cell parameters in patients with RRMS were assessed. We observed that supplementation of 20 000 IU/d vitamin D_3 for 12 weeks was without negative side-effects and without decompensation of calcium metabolism (i.e. hypercalcaemia and hypercalciuria). The number of Treg remained unaffected, as well as the numbers of Treg memory and naïve subtypes. Results regarding Treg suppressive capacity were inconclusive. A shift was observed in the cytokine profiles of CD4^+ T cells, with a

relative decline in pro-inflammatory ($\text{IFN-}\gamma^+$) and a relative increase in anti-inflammatory (IL-4^+ and IL-10^+) CD4^+ T cells. These results further add to the notion that vitamin D is a physiological immune modulator *in vivo*. Monitoring the cytokine profile of CD4^+ T cells could be a valuable tool to assess the immunological efficacy of vitamin D_3 in randomized controlled clinical trials on vitamin D supplementation in patients with MS.

This study confirmed that supplementation of high doses vitamin D_3 does not induce a decompensation of calcium metabolism in patients with MS (Burton *et al.*, 2008; Kimball *et al.*, 2007). Without a dose-escalation supplementation scheme, we did not detect hypercalcaemia or hypercalciuria. The slight non-significant increase in serum calcium and phosphorus most likely reflects a correction of vitamin D insufficiency in this cohort. A gradual increase in serum creatinine levels was observed during vitamin D_3 supplementation, which however remained below $110 \mu\text{mol/L}$ in all patients. The dramatic complication of a hypercalcaemia associated decrease of glomerular filtration rate (GFR) is unlikely, since neither hypercalcaemia nor hypercalciuria was detected. Earlier dose-escalation trials supplementing up to 280 000 IU/w for 28 weeks and up to 40 000 IU for 52 weeks did not report either significant elevations of serum creatinine levels, or calcifications in the kidneys (Burton *et al.*, 2008; Kimball *et al.*, 2007). Interestingly, however, earlier reports described a rise in serum creatinine which was not accompanied by a decreased GFR in patients treated with $1,25(\text{OH})_2\text{D}$ or $1\alpha(\text{OH})\text{D}$ (Andreev *et al.*, 1992). Physiologically, $25(\text{OH})\text{D}$ is filtrated in complex with vitamin D binding protein (DBP) by the glomeruli and re-absorbed by proximal tubular epithelial cells (Doorenbos *et al.*, 2009). Interestingly, creatinine is being secreted in the proximal renal tubuli as well. We postulate that a massive increase of $25(\text{OH})\text{D}$ -DBP complex reabsorption competes with tubular creatinine excretion and hereby increases serum creatinine levels. Although we believe that the increased serum creatinine levels do not reflect a decreased GFR, future studies on massive dose vitamin D supplementation should be cautious towards patients with an impaired kidney function. On the whole, biochemical data show the absence of vitamin D_3 toxicity in participants of this study.

We found no effect of vitamin D supplementation on the number of Treg in the circulation. In MS, the function rather than the number of Treg has been reported to be impaired (Venken *et al.*, 2006; Viglietta *et al.*, 2004), and to relate with serum $25(\text{OH})\text{D}$ levels (Smolders *et al.*, 2009d, **chapter 4**). Additionally, other immune modulating therapies also have been shown to improve Treg function rather than Treg numbers (Haas *et al.*, 2009; Korporeal *et al.*, 2008). An expansion of naïve Treg has been described in Glatiramer Acetate treatment (Haas *et al.*, 2009), and a decrease of memory Treg cells in $\text{IFN-}\beta$ treatment (Korporeal *et al.*, 2008). Therefore, we also assessed these Treg subsets, but found no effect of vitamin D_3 supplementation on the proportions of naïve and memory Treg.

Previously, we observed a correlation of Treg suppressive capacity with serum 25(OH)D levels (Smolders *et al.*, 2009d, **chapter 4**). Although Treg suppressive function was improved in a proportion of patients after 12 weeks of vitamin D₃ supplementation, this improvement was not statistically significant. The size of the cohort assessed is most likely too small to detect a difference in this complex assay. Alternatively, one could speculate from our previous work that only in the patients with the poorest vitamin D statuses, an improved suppression can be expected (Smolders *et al.*, 2009d, **chapter 4**). Although vitamin D statuses at week 0 were lower in the 9 improving patients when compared to the 4 non-improving patients, the difference was not significant. Additionally, Treg suppressive function is not impaired in all RRMS patients, especially when treated with IFN- β (Haas *et al.*, 2009; Korporeal *et al.*, 2008; Michel *et al.*, 2008; Venken *et al.*, 2006). Therefore, it can be speculated that an adequate Treg function cannot be improved further on vitamin D₃ supplementation. Indeed, the patients of whom Treg suppressive function improved tended to have a poorer suppression at week 0. Altogether, the data collected in this study regarding Treg suppressive function remain inconclusive.

A significant effect on the composition of the T helper cell compartment was detected. The ratio of IFN- γ ⁺/IL-4⁺ CD4⁺ T cells represents the balance between pro-inflammatory Th1 and anti-inflammatory Th2 cells, and is regarded an important variable in autoimmune diseases (Abbas *et al.*, 1996). During supplementation, this ratio changed towards a less pro-inflammatory profile. This is in accordance with previous cross-sectional findings (Smolders *et al.*, 2009d, **chapter 4**). Interestingly, we detected also more IL-10⁺ CD4⁺ T cells after supplementation. The source of IL10⁺ cells in the CD4⁺ T cell compartment may be the T regulatory cell type 1 (Tr1) (Moore *et al.*, 2001). Alternatively, IL-10 has also been proposed to be a typical Th2 cytokine. However, we measured no double producers of IL-10 and the (other) typical Th2 cytokine IL-4 (data not shown). Therefore, we speculate that the rise in IL-10⁺ CD4⁺ T cells might reflect expansion of inducible regulatory Tr1 cells. The finding of a decreased ratio between pro- and anti-inflammatory CD4⁺ T cells conforms to observations in experimental studies (Correale *et al.*, 2009) and further supports the assessment of vitamin D₃ as a natural immune modulator in MS (Hayes *et al.*, 1997).

Although the present study did not comprise a placebo-group, it delivered valuable new information. We earlier performed a cross-sectional study, in which serum 25(OH)D levels correlated with peripheral T cell homeostasis (Smolders *et al.*, 2009d, **chapter 4**). However, the cross-sectional design did not allow statements on causality, since physical exercise and UV exposure per se could be the real underlying mediators (Becklund *et al.*, 2010). Our current study revealed 25(OH)D as a good causal candidate. Ultimately, however, placebo controlled studies should demonstrate whether vitamin D₃ is an immune modulator *in vivo*. Regarding these upcoming trials, it is also of interest that we included patients treated with immune modulating drugs (IFN- β). Interestingly, therapy with 1,25(OH)₂D showed synergistic ef-

fects with IFN- β in an EAE model of MS (van Etten *et al.*, 2007). Upcoming clinical trials will most likely assess add-on effects of vitamin D₃ on current immune modulating drugs. The present study suggests that an add-on immune modulating effect of vitamin D₃ on top of IFN- β is a feasible outcome measure.

In conclusion, we showed in a cohort of RRMS patients that supplementation of high doses vitamin D₃ did not result in a decompensated calcium metabolism. Additionally, vitamin D₃ appeared to skew the CD4⁺ T cell compartment to a more pronounced anti-inflammatory state. Herewith, we confirmed that the peripheral CD4⁺ T cell compartment is a potentially important outcome measure in upcoming randomized controlled clinical trials. Ultimately, these trials should demonstrate whether vitamin D₃ is an immune and disease modulating compound in MS.

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Fok-I vitamin D receptor gene polymorphism (rs10735810) and vitamin D metabolism in multiple sclerosis

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Abstract

Multiple sclerosis (MS) has been associated with low serum levels of 25-hydroxy-vitamin D (25(OH)D). Several genetic polymorphisms of the vitamin D receptor gene (VDRG), of whom Fok-I (rs10735810) has functional consequences for receptor protein structure, have been studied in relation to MS with variable results. The purpose of our study was to assess an association of the Fok-I VDRG polymorphism with MS, and to further unravel the interaction of this polymorphism with vitamin D metabolism. Therefore, we genotyped 212 MS patients and 289 healthy controls for the Fok-I polymorphism and determined levels of the vitamin D metabolites 25(OH)D and 1,25(OH)₂D. No association of the Fok-I VDRG polymorphism with MS was found. The F-allele was associated with lower winter and summer serum 25(OH)D levels in our MS patients, and with lower 25(OH)D levels in healthy controls. Remarkably, the F-allele corresponded with higher 1,25(OH)₂D levels in MS patients. In all groups, carriers of the F-allele had higher 1,25(OH)₂D/ 25(OH)D-ratios compared to their f-allele counterparts. In conclusion, we demonstrated the functional consequences of the Fok-I VDRG polymorphism for vitamin D metabolism. This should be taken into account in association and ultimately intervention studies on vitamin D and MS.

1. Introduction

Limited vitamin D exposure is considered to be an environmental risk factor for developing multiple sclerosis (MS) (Smolders *et al.*, 2008a, **chapter 2**). Twenty-five-hydroxyvitamin D (25(OH)D) is the principal vitamin D metabolite in serum, and is widely used to determine the vitamin D status of an individual (Hollis 1996). Low serum levels of 25(OH)D in adolescence are associated with an increased risk of developing MS in a white American population (Munger *et al.*, 2006). Limited sunlight exposure, the principal source of vitamin D synthesis, is associated with a high risk for MS in American, Tasmanian and Norwegian populations (Islam *et al.*, 2007; Kampman *et al.*, 2007; van der Mei *et al.*, 2003). One of the genetic factors contributing to this risk, might be the genetic constitution of the vitamin D receptor (VDR), the receptor for the biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)₂D).

The vitamin D receptor gene (VDRG) is located on chromosome 12q 12–14. Various single nucleotide polymorphisms (SNP's) in the VDRG have been described. The most frequently studied in MS are usually referred to by the names of the digestion enzymes used for genotyping. The polymorphisms Apa-I (rs7975232), Bsm-I (rs1544410) and Taq-I (rs731236) are located in an intron near the 3' end, and the polymorphism Fok-I (rs10735810) in an exon near the 5' end of the VDRG. The Fok-I polymorphism leads to a 3 amino-acids longer VDR protein by directly introducing a start codon. A functional impact of this polymorphism on the immune response has been demonstrated (Colin *et al.*, 2000; van Etten *et al.*, 2007). The biological consequences of the other polymorphisms are less certain.

Several studies have addressed the association of these VDRG polymorphisms with MS, with variable results (Fukazawa *et al.*, 1999; Niino *et al.*, 2000; Partridge *et al.*, 2004; Steckley *et al.*, 2000; Tajouri *et al.*, 2005; Yeo *et al.*, 2004). Since the studies in which an association was reported were performed in a subtropical area with much sunlight (Tajouri *et al.*, 2005), and an area where the diet contains large amounts of fat fish (Fukazawa *et al.*, 1999; Niino *et al.*, 2000), these populations were presumably exposed to relatively large environmental supplies of vitamin D. We hypothesized that an association of a VDRG polymorphism with MS might only be penetrant in a population with an adequate vitamin D exposition. We determined the VDRG polymorphism and 25(OH)D levels in patients and controls, in order to assess a possible association with selective inclusion of patients with an adequate vitamin D status. However, upon validation of this approach, there appeared a direct interaction of the polymorphism with vitamin D metabolism.

This study had several purposes. First of all, a possible association of the Fok-I VDRG polymorphism with MS was assessed in our population. Secondly, we investigated an association of the Fok-I polymorphism with 25(OH)D levels in a large population. In order to further evaluate the consequences of the polymorphism for vita-

min D metabolism, we also assessed an association of the Fok-I VDRG polymorphism with serum levels of the biologically active metabolite of vitamin D, 1,25(OH)₂D. To limit the effect of seasonal fluctuation of vitamin D photosynthesis, these associations were assessed both in summer and in winter period.

2. Subjects and Methods

2.1 Population

Two-hundred and twelve patients with clinically and MRI-confirmed MS (McDonald *et al.*, 2001) and 289 healthy community controls were included in this study, after informed consent was acquired. The characteristics these two cohorts are shown in **Table 1**. Patients and controls all lived in the southern part of the Netherlands (latitude 50° N – 51° N). This project was approved by the local medical ethics committee.

Table 1 Population characteristics of the MS and control population.

		Patients (n = 212)		Healthy Controls (n = 289)	
		N/Mean	%/SD	N/Mean	%/SD
Sex	M	62	29.2%	138	50.2%
	F	150	70.8%	137	49.8%
MS phenotype	RRMS	97	45.8%		
	SPMS	72	34.0%		
	PPMS	38	17.9%		
	Unknown	5	2.4%		
Age (years)		46.7	±11.9	34.92	±14.3
MS duration (years)		12.7	±9.92		
EDSS-score		4.24	±2.28		

Values are presented either as absolute number with corresponding percentage (%), or as mean value with corresponding standard deviation (±). EDSS: Expanded Disability Status Scale; RRMS: relapsing remitting MS; SPMS: secondary progressive MS; PPMS: primary progressive MS.

2.2 Clinical and biochemical parameters.

Blood samples of our MS patients were collected in summer (from May to October) and winter (from November to April). Plasma samples were available only of a limited amount of healthy controls (N = 133) and the exact dates of blood collection were not known. The serum 25(OH)D and 1,25(OH)₂D levels were determined as previously described (Smolders *et al.*, 2008b, **chapter 3**). We described the relationship between these metabolites as ratios (1000 x 1,25(OH)₂D / 25(OH)D). Parameters such as sex, age, clinical MS-phenotype (RR = relapsing remitting, SP = secondary progressive, PP = primary progressive, unknown), disease course and Expanded

Disability Status Scale (EDSS)-score at the time of serum sampling were collected from our patient-database.

2.3 Genotyping

DNA was extracted from peripheral blood leukocytes with a High Pure PCR Preparation Kit (Roche, Woerden, The Netherlands) following the manufacturer's instructions, and stored at -20°C . The Fok-I polymorphism was determined by a previously described method (Tajouri *et al.*, 2005). In brief, the regions were amplified by a polymerase chain reaction with normal unlabeled nucleotides. The amplicon was digested with the Fok-I restriction enzyme (Roche, Woerden, The Netherlands) and electrophoresed on a gelstar-stained 2% agarose gel. The presence of a restriction site was represented by the lowercase ('f', nucleotide A), and its absence by the uppercase ('F', nucleotide G) allele.

2.4 Statistics

Genotype and allele frequencies were calculated from the analysed genotypes. SPSS-software (version 12.0, SPSS Inc., California, USA) was used for analysis. Deviation from Hardy-Weinberg equilibrium (HWE; $P < 0.05$) was tested with the χ^2 test. The relationship between VDR genotype or allele frequency and MS was tested in a logistic regression model, adjusted for age (years) and sex (male/ female) by introducing them simultaneously in the model. Results are presented as odds ratios (OR) with 95% confidence intervals (95% CI). The relationship between Fok-I VDR genotype and vitamin D metabolite levels was tested in a linear regression model. Unequally distributed metabolite values (Lilliefors corrected Kolmogorov-Smirnov test $P < 0.05$) were log-transformed. We adjusted this relationship for confounders, by introducing them simultaneously in the model. Confounders of the relationship between 25(OH)D and Fok-I VDR genotype were age, sex and EDSS-score (Smolders *et al.*, 2008b, **chapter 3**). The relationship between 1,25(OH)₂D and Fok-I VDR genotype was adjusted for age, sex and vitamin D status (25(OH)D-levels) (Smolders *et al.*, 2008b, **chapter 3**). Results are presented as regression coefficients (C) with 95% CI. Variation in the 1,25(OH)₂D/ 25(OH)D-ratios was assessed with an analysis of variance (ANOVA). A two-sided P-value < 0.050 was considered significant.

3. Results

3.1 No association of Fok-I VDR genotype with MS

The genotype and allele-frequencies of the Fok-I VDRG polymorphism in the whole MS and control populations are described in **Table 2**. Both cohorts were in Hardy-Weinberg equilibrium. There was no difference in the distribution of genotypes or alleles between patients and controls.

Table 2 Distribution of the Fok-I vitamin D receptor gene polymorphism in MS patients and in healthy controls.

	Patients (n = 212)		Healthy controls (n = 289)		OR	(95% CI)
	N	%	N	%		
F/F	79	37.3%	113	39.1%	1.015	(0.649–1.588)
F/f	103	48.6%	134	46.4%	1.002	(0.647–1.553)
f/f	30	24.1%	42	14.5%	0.966	(0.516–1.811)
F	261	61.6%	360	62.3%	1.027	(0.552–1.406)
f	163	38.4%	218	37.7%	0.974	(0.711–1.336)
HWE			HWE			
	P = 0.77		P = 0.90			

HWE: Hardy-Weinberg equilibrium; OR: age- and sex adjusted odds-ratios with corresponding 95% confidence intervals

Next, we intended to identify a possible association between the Fok-I VDRG polymorphism and MS by selective inclusion of patients with an adequate vitamin D status. However, since a possible association between 25(OH)D levels and the Fok-I VDRG polymorphism could be a confounder, we further explored the association of the polymorphism with the two vitamin D metabolites.

3.2 Association of Fok-I VDR genotype with 25(OH)D levels in MS patients.

In the MS cohort, carriers of two F-alleles had both lower summer and winter serum 25(OH)D levels than carriers of two f-alleles (**Figure 1**). The Fok-I genotype was a significant predictor of summer 25(OH)D level ($C = 7.109$, 95% CI 0.935–13.283, $P = 0.024$). The association with log-transformed winter 25(OH)D level did not reach significance ($C = 0.038$, 95% CI -0.006–0.081, $P = 0.088$). Since 25(OH)D status is associated with the level of disability in MS patients, we adjusted our analysis for EDSS-score, and found that both summer ($C = 8.338$, 95% CI 2.547–14.129, $P = 0.005$) and log-transformed winter 25(OH)D levels ($C = 0.043$, 95% CI 0.001 – 0.085, $P = 0.043$) were significantly associated with Fok-I VDR genotype.

3.3 Association of Fok-I VDR genotype with 25(OH)D levels in healthy controls.

In the healthy controls, carriers of an F-allele had also lower 25(OH)D levels (**Figure 1**), and the Fok-I VDR genotype was found to be a significant predictor of 25(OH)D levels ($C = 6.388$, 95% CI 0.841–11.936, $P = 0.024$).

3.4 Association of Fok-I VDR genotype with 1,25(OH)₂D levels in MS patients.

In contrast to the 25(OH)D levels, we observed slightly higher serum 1,25(OH)₂D levels in carriers of two F-alleles compared to carriers of two f-alleles (**Figure 1**). This is surprising, since we found a positive correlation between the two metabolites

(summer: $R = 0.367$, $P < 0.001$ and winter: $R = 0.354$, $P < 0.001$). The $1,25(\text{OH})_2\text{D}/25(\text{OH})\text{D}$ -ratios show that carriers of an F allele have relatively higher $1,25(\text{OH})_2\text{D}$ levels related to $25(\text{OH})\text{D}$ levels in winter ($P = 0.034$), and a trend towards this association in summer ($P = 0.079$).

Figure 1 Serum levels of $25(\text{OH})\text{D}$ and $1,25(\text{OH})_2\text{D}$ and the $1,25(\text{OH})_2\text{D}/25(\text{OH})\text{D}$ -ratio of the MS population in summer and winter, and of the healthy control population, stratified for Fok-I VDR genotype.

The dots show the mean value with corresponding 95% CI. A line was inserted to illustrate the direction of the association.

When analysed uncorrected for possible confounders, both log-transformed summer and winter $1,25(\text{OH})_2\text{D}$ levels were not predicted by Fok-I VDR genotype (data not shown). In the adjusted regression model, Fok-I VDR genotype predicted log-transformed summer $1,25(\text{OH})_2\text{D}$ levels ($C = -0.032$, 95% CI $-0.062 - -0.001$, $P = 0.045$), and showed a trend towards prediction of log-transformed winter $1,25(\text{OH})_2\text{D}$ levels ($C = -0.032$, 95% CI $-0.066 - -0.003$, $P = 0.073$).

3.5 Association of Fok-I VDR genotype with 1,25(OH)₂D levels in healthy controls.

In the healthy control cohort, there was descriptively no clear association of plasma 1,25(OH)₂D levels with the F or f-allele to be observed (**Figure 1**). Like in the MS cohort, 1,25(OH)₂D and 25(OH)D levels correlated positively ($R = 0.424$, $P < 0.001$). Interestingly, the 1,25(OH)₂D/ 25(OH)D-ratio did also show an association of the F-allele with relatively higher 1,25(OH)₂D levels compared to 25(OH)D status ($P = 0.024$). However, the Fok-I VDRG polymorphism failed to predict log-transformed plasma 1,25(OH)₂D levels in the adjusted regression model.

4. Discussion

This is the first study to assess serum levels of 25(OH)D and 1,25(OH)₂D, and the Fok-I VDRG polymorphism in two unrelated large cohorts of MS patients and healthy controls. Firstly, we did not find an association of the Fok-I VDRG polymorphism with MS in our population. Secondly, we observed an association of the F allele with low 25(OH)D levels in patients and controls. Thirdly, we extended these findings in our MS cohort by showing a reverse association of the F allele with high summer 1,25(OH)₂D levels, and a trend towards association with high winter values. Lastly, in both MS patients and healthy control carriers of an F-allele, 1,25(OH)₂D/ 25(OH)D-ratios were higher compared to their f-allele counterparts. These results show that the Fok-I VDRG polymorphism has important consequences for vitamin D metabolism in both MS and healthy control populations.

It is challenging to interpret our results. The association of the Fok-I VDRG polymorphism with 25(OH)D levels was earlier described by Orton *et al.*, in an MS twin cohort (Orton *et al.*, 2008). Since the serum level of this metabolite has been associated with the incidence of MS and several other autoimmune diseases, interference of the Fok-I VDRG polymorphism with its serum levels makes the polymorphism potentially relevant. Additionally, the association that we observed of the Fok-I polymorphism with 1,25(OH)₂D levels and 1,25(OH)₂D/ 25(OH)D-ratios might provide more insight in the drivers of the association initially described by Orton *et al.*, The serum level of 1,25(OH)₂D is mainly the result of a balance between 1 α -hydroxylation of 25(OH)D in the kidneys (Jongen *et al.*, 1984), and deactivation of 1,25(OH)₂D by 24-hydroxylation in its target tissues (reviewed in Dusso *et al.*, 2005). These processes are strictly regulated by serum calcium, phosphate and parathyroid hormone (PTH) levels. However, several papers also suggest a substantial role for self-regulation, i.e. negative feedback mechanisms, in vitamin D metabolism. Treatment of cell cultures with 1,25(OH)₂D has been shown to reduce the amount of 25(OH)D-1 α -hydroxylase mRNA (reviewed in Dusso *et al.*, 2005). Takeyama *et al.*, showed a critical role of the VDR in this process, using a VDR knockout mouse model (Takeyama *et al.*, 1997). The absence of the VDR gene revealed dramatically higher

serum 1,25(OH)₂D levels, but 25(OH)D levels were not significantly affected. Transcription analysis revealed inhibition of CYP27B1 by 1,25(OH)₂D only in mice expressing the VDR. Interestingly, the vitamin D inactivating enzyme CYP24A1 (1,25(OH)₂D-24-hydroxylase) was also only up-regulated by 1,25(OH)₂D in mice expressing the VDR. Furthermore, increasing levels of 1,25(OH)₂D also limit the formation of its precursor 25(OH)D out of vitamin D. In an *in vivo* animal model, rat hepatic CYP27A1 (vitamin D-25-hydroxylase) expression was inhibited by systemic 1,25(OH)₂D treatment (Theodoropoulos *et al.*, 2003b), and *in vitro* treatment of human fetal intestine tissue with 1,25(OH)₂D was also associated with a decreased CYP27A1 expression (Theodoropoulos *et al.*, 2003a). Altogether, these studies suggest a negative feedback loop in vitamin D metabolism which is activated upon VDR ligation, and limits the synthesis of both 1,25(OH)₂D and its precursor 25(OH)D. The results of our study suggest that the balance between 25(OH)D and 1,25(OH)₂D synthesis at serum level is affected by the Fok-I VDRG polymorphism. When reflected to our model of vitamin D metabolism, the reverse association of the Fok-I VDRG polymorphism with the two metabolites suggest that it primarily interferes with the serum levels of 1,25(OH)₂D, leading to secondary changes of 25(OH)D levels. Whether this interference is mediated by CYP27B1, CYP24A1, calcium, phosphate or PTH-levels remains to be determined. Although somewhat speculative, the two polymorphisms of CYP27B1, which have been related to 25(OH)D levels, are also most likely to primarily affect 1,25(OH)₂D levels (Orton *et al.*, 2008). Further experimental studies on polymorphisms of the VDR and the CYP-enzymes in vitamin D metabolism should provide more insight.

Although the Fok-I VDRG polymorphism affects 25(OH)D levels, and 25(OH)D levels have been associated with the risk on developing MS (Munger *et al.*, 2006), the anticipated association of the polymorphism with MS was not observed. Although others did describe a trend towards an association of the f-allele with a decreased risk for MS (Partridge *et al.*, 2004) and an association of the f-allele with a decreased severity of MS (Mamutse *et al.*, 2008), immunological *in vitro* studies (Colin *et al.*, 2000; van Etten *et al.*, 2007) and association studies (Orton *et al.*, 2008; Tajouri *et al.*, 2005) show conflicting results. As we hypothesised, vitamin D status is likely to be a confounding factor in these studies and our study. We initially intended to take this factor into account in our study by selective inclusion of MS patients with an optimal vitamin D status in our analysis. However, since the polymorphism appeared to interact intensively with vitamin D metabolism in both patients and healthy controls, this approach was no longer valid. Interestingly, a recent study in type 1 diabetes mellitus (T1DM) research, another disease which has been associated with 25(OH)D levels, also suggests the interference of vitamin D exposition in association studies on the VDRG. Ponsonby *et al.*, showed in a recent meta-regression analysis that the odds ratios for the association of the Fok-I VDRG poly-

morphism with T1DM increased with increasing regional winter UVR radiation levels (Ponsonby *et al.*, 2008).

This study has provided some new data and hypothesis on the consequences of the Fok-I VDRG polymorphism for vitamin D metabolism and for MS. In future intervention studies on vitamin D in MS, the polymorphism is likely to influence the response of vitamin D metabolism on exposure to large amounts of vitamin D. Further clinical and experimental studies on vitamin D metabolites and MS should take the Fok-I VDRG polymorphism into account, and further clarify the role of vitamin D, its metabolites and its receptor in MS.

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Design of a randomized, controlled clinical trial on add-on therapy with vitamin D₃ in Interferon Beta-treated patients with relapsing remitting multiple sclerosis

Submitted as: Joost Smolders, Raymond Hupperts, Frederik Barkhof, Luigi Grimaldi, Trygve Holmøy, Joep Killestein, Peter Rieckmann, Myriam Schluep, Reinhold Vieth, Ulrike Hostalek, Lizette Ghazi-Visser, Manolo Beelke, on behalf of the SOLAR study group. Efficacy of Vitamin D₃ as Add-on Therapy in Patients with Relapsing–Remitting Multiple Sclerosis Receiving Subcutaneous Interferon Beta-1a: A Phase II, Multicentre, Double-blind, Randomized, Placebo-Controlled Trial.

Abstract

Recent studies have demonstrated the immunomodulatory properties of vitamin D, and vitamin D deficiency may be a risk factor for the development of MS. The risk of developing MS has, in fact, been associated with rising latitudes, past exposure to sun and serum vitamin D level. Serum 25-hydroxyvitamin D [25(OH)D] levels have also been associated with relapses and disability progression. The identification of risk factors, such as vitamin D deficiency, in MS may provide an opportunity to improve current treatment strategies, through combination therapy with established MS treatments. Accordingly, vitamin D may play a role in MS therapy. Small clinical studies of vitamin D supplementation in patients with MS have reported positive immunomodulatory effects, reduced relapse rates and a reduction in the number of gadolinium-enhancing lesions. However, large randomized clinical trials of vitamin D supplementation in patients with MS are lacking.

SOLAR (Supplementation of Vigantol[®] oil versus placebo as add-on in patients with relapsing–remitting multiple sclerosis receiving rebif[®] treatment) is a 96-week, three-arm, multicentre, double-blind, randomized, placebo-controlled, Phase II trial (NCT01285401). SOLAR will evaluate the efficacy of vitamin D₃ as add-on therapy to subcutaneous interferon beta-1a in patients with RRMS. Recruitment began in February 2011 and is aimed to take place over 1 calendar year due to the potential influence of seasonal differences in 25(OH)D levels.

1. Introduction

Multiple sclerosis (MS) is a chronic, inflammatory and degenerative disease of the central nervous system, believed to be triggered by environmental factors in patients with complex genetic-risk profiles (Compston and Coles, 2008). Current disease-modifying drugs can reduce relapses and may delay disability progression in patients with relapsing–remitting MS (RRMS) (IFNB Multiple Sclerosis Study Group, 1993; Jacobs *et al.*, 1996; Johnson *et al.*, 1995; PRISMS Study Group, 1998), the most common disease course at onset (Weinshenker *et al.*, 1989).

Vitamin D deficiency is a putative risk factor for MS (Ascherio *et al.*, 2010; Smolders *et al.*, 2008a, **chapter 2**). Identifying such risk factors as potential targets of therapy may provide an opportunity to improve current MS treatment strategies. The risk of developing MS has been correlated to rising latitudes, past exposure to sun and serum 25-hydroxyvitamin D [25(OH)D] level (Pierrot-Deseilligny, 2009). Levels of vitamin D are determined by dietary intake, sun exposure and genetic make-up (Orton *et al.*, 2008). Vitamin D₃ (cholecalciferol), the naturally occurring form of vitamin D, is predominantly found in two metabolite forms in the circulation: the prehormone, 25-hydroxyvitamin D; and the thousand-times less abundant, biologically active metabolite, 1,25-dihydroxyvitamin D [1,25(OH)₂D]. Circulating concentrations of 1,25(OH)₂D are mainly the result of a 25-hydroxylation step in the kidney, which is regulated according to the needs of calcium homeostasis (Jongen *et al.*, 1984). This activation step into 1,25(OH)₂D can also occur within other tissues, including immune-modulating cells, where the activation is not affected by calcium homeostasis; in this case 1,25(OH)₂D is not thought to enter the circulation, but rather its role is to moderate immune function by autocrine and paracrine mechanisms, suggesting that vitamin D may have a role in human physiology beyond skeletal and calcium homeostasis (Adams and Hewison, 2008).

In several studies, a potential causal association between serum 25(OH)D concentrations and MS disease activity has been observed. A high chance of remaining relapse-free has been associated with higher serum 25(OH)D levels (Smolders *et al.*, 2008, **chapter 3**), with each 10 nmol/L increase in 25(OH)D resulting in a reduction in the risk of relapse of up to 12% (Simpson *et al.*, 2010). In addition, in paediatric-onset MS and clinically isolated syndrome, every 10 ng/mL increase in adjusted 25(OH)D level was associated with a 34% decrease in the rate of subsequent relapses (Mowry *et al.*, 2010), (equating to an approximate 14% decrease in the rate of subsequent relapses per 10 nmol/L increase). Furthermore, serum 25(OH)D and 1,25(OH)₂D levels have been reported to be lower during MS relapses than during remission (Correale *et al.*, 2009; Soliu-Hänninen *et al.*, 2005). An inverse relation between Expanded Disability Status Scale (EDSS) progression and vitamin D levels has also been reported (Smolders *et al.*, 2008, **chapter 3**).

Functional interaction of vitamin D with the main MS-linked HLA-DRB1*1501 allele (Ramagopalan *et al.*, 2009), or selective immune system regulation (Hayes *et al.*, 1997) are possible mechanisms underlying the modulating effect of vitamin D on MS pathobiology. The biologically active metabolite of vitamin D has been shown to induce suppression of IL-2, IL-1 and lymphocyte proliferation (Tsoukas *et al.*, 1984; Tsoukas *et al.*, 1989). High levels of serum 25(OH)D have been associated with an improved regulatory T cell function and with skewing of the Th1/Th2 balance towards a Th2 type response, suggesting that vitamin D may be an important regulator of peripheral T cell function in MS (Smolders *et al.*, 2009, **chapter 4**). A reduced proliferative response of CD4⁺ T cells to myelin peptides was observed in patients who received high-dose vitamin D supplementation (up to 40 000 IU daily), when compared with that in patients without supplementation and when compared with that prior to treatment (Burton *et al.*, 2010). Strong immunomodulatory properties of molecules derived from vitamin D₃ have also been demonstrated in murine experimental autoimmune encephalomyelitis (EAE), a model of MS. TX527, an analogue of 1,25(OH)₂D₃, can attenuate EAE and results in significant disease protection in combination with interferon (IFN)-beta, superior to the effects of the individual treatments (van Etten *et al.*, 2007).

Small preliminary studies of vitamin D₃ supplementation, with or without calcium, have reported positive immunomodulatory effects (Burton *et al.*, 2010), reduced relapse rates (Goldberg *et al.*, 1986) and a reduction in the number of gadolinium (Gd)-enhancing lesions (Kimball *et al.*, 2007) in patients with MS. Furthermore, vitamin D supplementation in patients with MS raised serum transforming growth factor beta-1 levels (Mahon *et al.*, 2003), which may indicate a neuroprotective effect.

In a recent pilot study, patients with RRMS receiving IFN-beta were supplemented with high-dose (20 000 IU daily) vitamin D₃, the same compound that will be used in the SOLAR study, for 12 weeks (Smolders *et al.*, 2010, **chapter 6**). A shift towards an anti-inflammatory cytokine profile, with an increased proportion of IL-10⁺ CD4⁺ T cells and a decrease in the ratio between IFN-γ⁺ and IL-4⁺ CD4⁺ T cells was observed, thus suggesting that combining IFN-beta with vitamin D₃, to further modulate the immune system of patients with MS may be beneficial.

Although there is no direct evidence demonstrating the clinical benefit of combining vitamin D₃ with IFN-beta therapy in MS, the majority of patients in studies examining vitamin D in MS were also receiving IFN-beta. To date, these studies have been limited to assessing the association between the risk of relapse and vitamin D level (Smolders *et al.*, 2008b, **chapter 3**; Simpson *et al.*, 2010; Mowry *et al.*, 2010), and the safety of high dose vitamin D (Burton *et al.*, 2010; Kimball *et al.*, 2007; Smolders *et al.*, 2010b, **chapter 6**) in MS. The recent pilot study of high-dose vitamin D₃ treatment (Smolders *et al.*, 2010, **chapter 6**), and the potential synergism for IFN-beta and vitamin D in a mouse model of MS (van Etten *et al.*, 2007), suggest that a

double-blind placebo-controlled trial is necessary to determine the potential add-on effects of vitamin D₃ to IFN-beta treatment.

The SOLAR (Supplementation of VigantOL[®] oil versus placebo as Add-on in patients with relapsing–remitting multiple sclerosis receiving Rebif[®] treatment) study is designed to assess the efficacy of vitamin D₃ as add-on therapy to subcutaneous (sc) IFN beta-1a in patients with RRMS (www.clinicaltrials.gov NCT01285401).

2. Methods

2.1 SOLAR trial design, objectives and rationale

SOLAR is a 96-week, three-arm, multicentre, double-blind, randomized, placebo-controlled, Phase II trial (**Figure 1**). The primary objective of the SOLAR study is to assess the efficacy of vitamin D₃ (cholecalciferol, Vigantol[®] oil, Merck KGaA, Darmstadt, Germany) versus placebo as add-on therapy in patients with RRMS receiving sc IFN beta-1a (Rebif[®], Merck Serono S.A. – Geneva) 44 µg three times weekly (tiw).

Figure 1 Patient recruitment and randomization.

EDSS, Expanded Disability Status Scale; IFN, interferon; sc, subcutaneous; RRMS, relapsing–remitting multiple sclerosis; tiw, three times weekly.

The secondary objectives are to: assess changes in clinical and magnetic resonance imaging (MRI) parameters; investigate the safety profile of study treatment

over 96 weeks; and explore pharmacogenetics, gene expression and circulating biomarkers, and potential correlations with vitamin D₃ treatment outcomes.

A total of 348 patients with RRMS and with serum 25(OH)D levels <150 nmol/L will be randomized (1:1) to receive vitamin D₃ or placebo as add-on therapy to IFN beta-1a, 44 µg sc tiw. Patients randomized to vitamin D₃ will receive 7000 IU (175 µg) daily for 4 weeks. If treatment with vitamin D is tolerated (as described below), patients will receive 14 000 IU (350 µg) daily for a further 92 weeks; otherwise, patients will continue to receive 7000 IU daily. In case of further intolerance, vitamin D₃ supplementation will be withdrawn; patients will be encouraged to maintain the assessment schedule until the end of the treatment period during which vitamin D₃ withdrawal occurred. The stability of the vitamin D₃ compound is guaranteed for the entire shelf life of the product. An unspecified number of patients (estimated to be 10) with supraphysiological serum 25(OH)D levels ≥150 nmol/L will receive IFN beta-1a, 44 µg sc tiw, only. For all patients, if treatment with IFN beta-1a, 44 µg sc tiw, is not well tolerated patients will receive IFN beta-1a, 22 µg sc tiw, based on the physician's decision.

Vitamin D₃ will be administered as an oral solution (500 µg/mL) each morning during breakfast, as the absorption of a fat-soluble vitamin benefits from the contemporaneous intake of fat (e.g. dropping the daily dose on a piece of buttered bread). sc IFN beta-1a will be administered before going to bed. The exception to this schedule will be during visits to the trial site where vitamin D₃, or placebo, will be administered only after the collection of blood samples.

Serum calcium (<2.6 mmol/L) levels, as well as the urinary ratio of calcium to creatinine (<1.0) will be determined before the start of treatment, week 4, week 12 and every 12 weeks thereafter. If the urinary ratio of calcium to creatinine is elevated, this measurement will be repeated as soon as possible to confirm the result before any action is taken, as urine calcium levels are highly variable. If any parameter is above the mentioned upper limit of normal for patients receiving 14 000 IU daily, the dose of vitamin D₃ may be decreased by 50% (i.e. from 14 000 IU daily to 7000 IU daily) or omitted for 1 month with a re-administration of 50% of the dose thereafter. All patients will continue to receive their dose of IFN beta-1a for the duration of the study.

2.2 Rationale for vitamin D₃ dosing in combination with IFN-beta

To be able to differentiate treatment effects between the vitamin D₃ and placebo-treated arms, patients will be supplemented with high doses of vitamin D₃, as a lower dose of vitamin D₃ within a controlled trial setting may not reveal potential benefits (Burton *et al.*, 2010). In addition, evidence for high dose vitamin D₃ effectiveness in the context of an immunomodulatory treatment is supported by results from two recent studies: where patients with RRMS, receiving IFN-beta, were supplemented with 20 000 IU daily vitamin D₃ (Smolders *et al.*, 2010, **chapter 6**); and

where patients with MS received escalating vitamin D doses up to 40 000 IU daily over 28 weeks, followed by 10 000 IU daily over 12 weeks, and further downtitrated to 0 IU daily (Burton *et al.*, 2010).

Studies investigating 25(OH)D levels have suggested that a concentration exceeding 100 nmol/L is optimal in healthy individuals (Hollis *et al.*, 2007), and may reduce the risk of MS (Munger *et al.*, 2009). In addition, studies assessing vitamin D adequacy in fracture prevention and preservation of bone mineral density suggest that concentrations of 25(OH)D should exceed 75 nmol/L (Vieth *et al.*, 2006). Moreover several cohort studies among patients with MS have shown that patients with RRMS are environmentally exposed to physiological 25(OH)D levels up to 150 nmol/L (Smolders *et al.*, 2008b, **chapter 3**; Simpson *et al.*, 2010; Mowry *et al.*, 2010).

A study designed to test the tolerability of specific 25(OH)D concentrations, reported a reduction in the number of Gd-enhancing lesions in patients with MS receiving 1200 mg elemental Ca/day alongside increasing doses of vitamin D₃, from 28 000 to 280 000 IU weekly (Kimball *et al.*, 2007). Furthermore, evidence of immunomodulatory effects was observed in patients with MS treated with high-dose oral vitamin D, which resulted in a mean serum 25(OH)D peak of 413 nmol/L (Burton *et al.*, 2010). Therefore, we selected a dose of 7000 IU daily for 4 weeks, followed by 14 000 IU daily for a further 92 weeks.

2.3 Dose safety and potential side effects

Baseline serum 25(OH)D levels of 150 nmol/L in patients treated with 14 000 IU vitamin D₃ per day, corresponding to 350 µg, is expected to increase the 25(OH)D serum level by 245 nmol/L, based on a 0.70 nmol increase for each additional 1 µg vitamin D₃ input (Heaney *et al.*, 2003). Therefore, we expect the resulting serum 25(OH)D level of 395 nmol/L in patients receiving vitamin D₃ to be below the critical threshold of 500 nmol/L that may lead to hypercalcaemia (Heaney *et al.*, 2008). In a previous study, the serum 25(OH)D levels of patients with MS receiving high doses of vitamin D₃ (increasing doses of vitamin D₃ from 28 000 to 280 000 IU weekly, for 28 weeks) was initially 78 nmol/L, rising to 386 nmol/L after treatment, meaning that patients reached twice the top of the physiologic range without eliciting hypercalcaemia or hypercalciuria (Kimball *et al.*, 2007). No significant adverse events (AEs) were observed in patients with MS treated with high doses of vitamin D₃ (up to 40 000 IU daily over 28 weeks, followed by 10 000 IU daily for 12 weeks, and further down-titrated to 0 IU daily), leading to the conclusion that approximately 10 000 IU daily was a safe regimen (Burton *et al.*, 2010). Vitamin D₃ was well tolerated and resulted in desirable vitamin D₃ levels in a study investigating long- (1 year) and short-term (8 weeks) safety in healthy adolescents at doses of 14 000 IU weekly, equivalent to 2000 IU daily (Maalouf *et al.*, 2008). In addition, 15 patients with RRMS receiving IFN-beta were recently supplemented with 20 000 IU daily of vitamin D₃ for 12 weeks without a dose-escalation scheme. The resulting median serum

25(OH)D level of 348 nmol/L did not induce AEs such as hypercalcaemia or hypercalciuria (Smolders *et al.*, 2010, **chapter 6**).

2.4 Rationale for the three-arm study design

There is currently no consensus on the optimal physiological range of vitamin D₃ in the general population, nor a recognised threshold below which individuals should be considered as vitamin D₃-deficient. Therefore, to avoid potential low vitamin D₃ level selection bias, a serum 25(OH)D level of <150 nmol/L has been defined as a cut-off value. In addition to stratifying randomized patients by serum 25(OH)D levels at baseline, patients will also be stratified (in order of importance) by body mass index (due to the fat-soluble nature of vitamin D₃), gender and number of relapses in the past 2 years, in order to assess the impact of these variables on clinical outcomes and to assure equal distribution among the treatment arms. Avoiding potential vitamin D₃ level selection bias and stratifying patients by variables is considered to be the most ethically acceptable study design, considering the potential benefits of vitamin D₃ supplementation in patients with RRMS. In addition, this approach will allow the potential synergistic mode of action (MoA) of IFN-beta with vitamin D₃ in MS to be explored, with respect to IFN-beta with placebo.

Samples from patients in the third arm, with supraphysiological serum 25(OH)D levels (≥150 nmol/L), will be used in exploratory analyses. Pharmacogenetic analyses will investigate whether common genetic polymorphisms distinguish these patients from those with serum 25(OH)D levels <150 nmol/L. In addition, it will also be of interest to determine whether these patients have a more benign course of disease than those with serum 25(OH)D levels <150 nmol/L, suggesting natural protection.

2.5 Patient criteria

Patients who meet the following criteria will be eligible for the study: aged 18–50 years; have a diagnosis of RRMS (McDonald 2005 criteria; Polman *et al.*, 2005); have a brain and/or spinal MRI with findings typical of MS; have a first clinical event occurring within 5 years prior to screening; have had at least one clinical event, or one or more Gd-enhancing or new T2 MRI lesions within the 12 months prior to screening; have an EDSS score ≤4.0 at screening; are either currently and for the first time receiving sc IFN beta-1a tiw for a minimum of 90 days and for not longer than 12 months prior to the baseline visit. All patients must be willing and able to comply with the protocol for the duration of the trial and provide written informed consent. The main exclusion criteria are: pregnancy or lactation; any disease other than MS that could better explain signs and symptoms; complete transverse myelitis or bilateral optic neuritis; a relapse within 30 days prior to study day 1 (SD1); use of corticosteroids or adrenocorticotrophic hormone within 30 days prior to SD1; abnormalities of vitamin D-related hormonal system other than due to low dietary

intake or decreased sun exposure; urine calcium/creatinine (mmol/mmol) ratio >1.0 or hypercalcaemia (11 mg/100 cc [5.5 mEq/l]); inadequate liver function (defined by alanine aminotransferase [ALT] >3 times upper limit of normal [ULN]; aspartate aminotransferase >3 times ULN; alkaline phosphatase >2.5 times ULN; or bilirubin >1.5 times ULN, if associated with any elevation of ALT or alkaline phosphatase); concomitant medications that influence vitamin D metabolism other than corticosteroids (e.g., phenytoin, barbiturates, thiazide diuretics and cardiac glycosides); currently taking >400 IU (>10 µg) of vitamin D supplement daily; or conditions with increased susceptibility to hypercalcaemia (e.g., known arrhythmia or heart disease, treatment with digitalis or hydrochlorothiazide, nephrolithiasis).

2.6 Randomization

Patients with serum 25(OH)D levels <150 nmol/L will be randomized 1:1 at SD1, by means of an interactive voice-response system, to vitamin D₃ or placebo, and will be double-blinded for all assessments. Patients with serum 25(OH)D levels ≥150 nmol/L will be automatically assigned to the sc IFN beta-1a treatment group and undergo all trial assessments without receiving either vitamin D₃ or placebo.

2.7 Primary endpoints

The primary endpoint will be a composite endpoint of MRI and clinical variables. The primary MRI endpoint will be the mean number of combined unique active (CUA) lesions at week 48. The primary clinical endpoint will be the proportion of relapse-free patients at week 96, chosen as previous studies have demonstrated correlations between vitamin D supplementation and relapse-free status (Burton *et al.*, 2010; Smolders *et al.*, 2008b, **chapter 3**; Simpson *et al.*, 2010; Mowry *et al.*, 2010). The composite endpoint will be assessed in a hierarchical manner, with the primary MRI endpoint measured at week 48 and the primary clinical endpoint measured at week 96.

2.8 Secondary endpoints

Secondary clinical endpoints will include relapses and chronic disease (i.e. disability) progression. Relapse-related endpoints will be measured by the: proportion of relapse-free patients at week 48; time to first documented relapse; annualized relapse rate at weeks 48 and 96; total number of reported relapses at all time points; and requirement for glucocorticoid treatment during the trial. Secondary disability-related endpoints will be measured by the: proportion of patients free from confirmed EDSS progression at weeks 48 and 96; time to 24 weeks' sustained disability progression on EDSS; and proportion of patients with 24 weeks' sustained disability progression on EDSS at weeks 48 and 96.

The secondary MRI endpoints (**Table 1**) will assess the anti-inflammatory effect (lesion activity, disease activity and burden of disease) and a potential neuroprotec-

tive effect (development of black holes and brain atrophy) of Vitamin D supplementation. Additional secondary efficacy endpoints will measure the proportion of patients free from disease activity and the change in cognitive function from baseline at weeks 48 and 96, measured by the Symbol Digit Modalities Test (SDMT).

2.9 Safety and immunogenicity endpoints

Safety endpoints will be evaluated up to week 96 and will include safety data from all completed trial visits, including: the occurrence of AEs, serious AEs and laboratory abnormalities; adherence to treatment; change from baseline in bone mass density at week 96 (test optional); reasons for trial termination; serum calcium level; urine calcium/creatinine ratios; serum 25(OH)D₃; and serum 1,25(OH)₂D₃ levels. Immunogenicity endpoints will include the proportion of patients, measured from baseline, who are positive for binding antibodies (BAbs) and neutralizing antibodies (NABs) to IFN beta.

2.10 Assessments

Patients will undergo 12 visits during the trial period (**Figure 2**). MRI assessments will be performed at SD1 and weeks 48 and 96. Neurological function will be assessed at screening, SD1 and every 12 weeks thereafter. Cognitive function (SDMT) will be assessed at SD1 and weeks 24, 48, 72 and 96. Relapses will be evaluated at SD1, week 4, week 12 and every 12 weeks thereafter up to week 96. BAbs and NABs to IFN beta-1a will be measured at SD1, week 12 and every 12 weeks thereafter up to week 96. Serum 25(OH)D levels will be measured at screening, SD1 and weeks 48 and 96. Gene expression profiling will be performed at SD1 and weeks 24, 48, 72 and 96, and pharmacogenetic biomarkers investigated in samples taken at SD1.

2.11 Biomarkers and pharmacogenetics

Circulating biomarkers, gene expression, and pharmacogenetic analyses will be performed on blood and plasma samples to identify potential surrogate biomarkers. Biomarker analysis will investigate potential correlations between vitamin D₃ add-on therapy and clinical- and MRI-based outcomes. In addition, biomarker analysis will explore the pharmacological response to treatment and the mechanism of action of vitamin D₃. Clinical and MRI outcomes will be evaluated in relation to single nucleotide polymorphisms of the vitamin D binding protein, vitamin D receptor, CYP27B1, CYP24A1, CYP2R1, and in MS-related CD25, CD127 and HLA-DRB1*1501. Circulating protein and gene expression profiling will be measured at relapse. It is also important to note that different or additional biomarkers may be analysed to reflect the latest advances in biomarker research.

Table 1 Additional MRI-based secondary endpoints.

Scanning sequence	Endpoint
T1 (Gd-enhancing)	Mean number of new T1 Gd-enhancing lesions per patient per scan at weeks 48 and 96 Cumulative number of T1 Gd-enhancing lesions over weeks 48 and 96 Proportion of patients free from T1 Gd-enhancing lesions at weeks 48 and 96
T1 (Gd-enhancing) and T2	Cumulative number of CUA lesions at weeks 48 and 96 Mean number of CUA lesions per patient per scan at week 96
T2	Mean change from baseline in the total volume of T2 lesions at weeks 48 and 96 (T2 BOD; mm ³)
T1 non-enhancing	Proportion of patients free from new T1 hypointense lesions (black holes) at weeks 48 and 96 Percentage of new T1 hypointense lesions (black holes) at weeks 48 and 96 within the subgroup of new or enlarging non-enhancing T2 lesions Mean change from baseline in total volume of T1 hypointense lesions at week 48 and week 96 (mm ³)
Other	Percent brain volume change at weeks 48 and 96 from baseline, and at week 96 from week 48 Proportion of T1 Gd-enhancing lesions at SD1 that transform into black holes at weeks 48 and 96

BOD, burden of disease; CUA, combined unique active; Gd, gadolinium; MRI, magnetic resonance imaging; SD1, study day 1

Figure 2 Study design.

EDSS, Expanded Disability Status Scale; MRI, magnetic resonance imaging; MSFC, multiple sclerosis functional composite; sc, subcutaneous; SD1, study day 1; SDMT, single digit modalities test; tiw, three times weekly

2.12 Sample size and statistical analysis

The study is 80% powered to detect an increase from 53% to 69% in the proportion of relapse-free patients at week 96. The study is also powered to detect futility based upon the mean number of CUA lesions at week 48 at the interim analysis. Assuming that the mean number of CUA lesions follows an approximate negative binomial distribution, with a mean (SD) for treatment group 1 (patients receiving vitamin D₃ and IFN beta-1a, 44 µg sc tiw) of 1.03 (1.61) and treatment group 2 (patients receiving placebo and IFN beta-1a, 44 µg sc tiw) of 0.48 (1.11), then 200 patients (100 per arm) provides 80% power to detect this increase in the mean number of CUA lesions over treatment group 3 (IFN beta-1a, 44 µg sc tiw only) with $p < 0.05$. The study is designed to detect at least an 18% mean decrease from baseline in the mean number of CUA lesions at week 48.

The planned sample size of 348 patients with 25(OH)D serum levels < 150 nmol/L in the two randomized arms is based upon the primary clinical endpoint.

2.13 Patient recruitment

The SOLAR study will be conducted in Europe, involving approximately 60 sites across Belgium, Denmark, Estonia, Finland, Germany, Hungary, Latvia, Lithuania, Norway, Switzerland, the Netherlands and Italy. Recruitment of patients from northern and southern Europe will allow for an investigation of the effects of latitude (sun exposure) on treatment outcomes. Recruitment is aimed to take place over 1 calendar year due to the potential influence of seasonal differences in vitamin D levels. Recruitment began in half of the countries in February/March and will begin in April/May in the other half, which should result in a close to linear recruitment curve, with an accrual rate estimate of 0.5 patient's per-month per-site. The site initiation sequence is performed only in relation to the approval of the study documents by the site IRB/EC and contract finalization, and is not dictated by any other factors (e.g. latitude of the site).

2.14 Sub-studies

The SOLAR(IUM) trial will be a parallel, immunological sub-study in the Netherlands. The trial will assess whether, in patients supplemented with vitamin D₃ for 1 year, the CD4⁺ T cell compartment displays a more pronounced anti-inflammatory state when compared with placebo-supplemented patients. The primary endpoint will be the difference in distribution of cytokine profile of peripheral CD4⁺ T lymphocytes by flow cytometry at week 48 between the two randomized treatment groups.

A second sub-study in Switzerland will aim to investigate the effect of vitamin D treatment on antigen-specific T cells in patients with MS. The secondary objective will be to assess the immunomodulatory effect of sc IFN beta-1a with or without vitamin D₃ supplementation compared with no immunomodulatory treatment, using the two randomized treatment groups. For this analysis, a fourth group of

patients will be enrolled: patients with RRMS without any treatment for the past 3 months, matched with the randomized patients in terms of EDSS score, age and sex.

3. Conclusions

Although the mechanisms underlying the modulating effect of vitamin D on MS pathobiology are not currently fully understood, current literature suggests that vitamin D is a likely environmental factor contributing to the risk of developing of MS. In addition, initial studies indicate that the dose of vitamin D₃ we plan to use in this study may be clinically beneficial to patients with MS, without inducing adverse events. SOLAR will be the first large, placebo-controlled international study to assess vitamin D₃ as add-on therapy to sc IFN beta-1a in patients with RRMS, using clinical and MRI outcomes. The study will also consider confounding factors, serum 25(OH)D levels reached, sunlight exposure and latitude. Further, analyses of specific genetic sequences already described will be performed to evaluate correlations between specific genetic preconditions and treatment response,. In addition, the SOLAR study will provide important information that will assist with the design of future trials of vitamin D supplementation in MS treatment, by identifying how different populations of patients with MS respond to vitamin D₃.

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General discussion

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1. Baseline

The vitamin D and multiple sclerosis (MS) story in Maastricht started with a review of the literature, as is described in **chapter 2**. In this review, two questions were addressed: i) is vitamin D a modulator of MS disease activity? ii) which biological processes may underlie this modulation?

Regarding the first question, evidence was limited. Only association studies and small pilot studies had been reported. These studies addressed the association of vitamin D status with both the risk on developing MS and disease activity of MS. People who developed MS in later life were exposed to lower amounts of sunlight and other correlates of vitamin D exposure in childhood and adolescence. Within MS cohorts, patients with either a relapse or high EDSS-score displayed the poorest vitamin D statuses. Although these reports could only be regarded as circumstantial evidence, they were abundant and showed a consistent picture: an increase in vitamin D exposure may reduce both the risk on developing MS, and disease activity of MS. These observations provided an excellent reason to perform further studies on vitamin D in MS. Additionally, literature revealed that not only vitamin D status, but also genetic polymorphisms of the VDR had been associated with risk on developing MS. This led to a more complicated model of the relationship between vitamin D and MS (**Figure 1**).

Figure 1 Potential interaction between vitamin D exposure, vitamin D receptor gene polymorphisms, and the risk on developing MS.

Vitamin D related genetic variation can affect the risk on MS via 3 potential mechanisms: 1.) via modulation of vitamin D status, 2.) via modulation of the quantity/ quality of the biological response to vitamin D, 3.) via a direct effect of the risk of MS, in which the vitamin D exposure is not important.

Much more experimental evidence had been published, showing that vitamin D is actively involved in the regulation of the adaptive immune response *in vitro* and in animal models of MS (discussed in **chapter 2**). This led to our hypothesis, that a lack of sufficient vitamin D exposure may result in a decompensation of the regulatory

role of vitamin D in the adaptive immune response. The subsequent disturbance of peripheral T cell homeostasis may lead to an increased risk for either developing or experiencing a more active disease course of MS. However, the translation of experimental data to epidemiological observations could be poorly made. Although low serum levels of 25(OH)D were found in MS, supplementation of EAE rodents with vitamin D was not as effective as 1,25(OH)₂D. Furthermore, the effects appeared to be restricted to female animals (Spach *et al.*, 2005). Evidence *in vivo* in MS patients on any relationship between T cell homeostasis and serum 25(OH)D levels was lacking. Therefore, it remained therefore to be seen whether vitamin D could be regarded as a peripheral immune modulator in MS.

In this thesis, we describe the work that we did on vitamin D and MS, in order to further elucidate whether vitamin D can be used as an immune modulator in MS. In the upcoming paragraphs, we will discuss the assessment of an association between vitamin D status disease activity of MS in our patient population, the assessment of vitamin D as a peripheral immune modulator in MS, the study on effects of a vitamin D receptor gene polymorphism, and the implications of these findings for the trial design of a large international trial on vitamin D₃ supplementation in MS. This chapter will end with the conclusions of this thesis, and with ideas for future research as well as for the possible application of vitamin D in clinical practice.

2. MS disease activity and vitamin D status

In **chapter 3**, we describe an association study between disease activity/ severity of MS and vitamin D status. We assessed the serum concentrations of two vitamin D metabolites: 25(OH)D, the biologically inactive yet most stable and most abundant vitamin D metabolite, and 1,25(OH)₂D, the biologically active metabolite with a short half-life. As outcome measures for disease activity/ severity, we assessed relapse activity (annualized relapse rate, relapse-free in the 2 years prior to sampling (Y/N)) and EDSS-score. We found that 25(OH)D status was negatively correlated with EDSS-score, and that patients who were relapse-free prior to serum sampling, displayed a better vitamin D status. Serum 1,25(OH)₂D levels were not correlated with EDSS-score, and were dependent on serum 25(OH)D levels and age.

Measuring disability of MS is a complicated issue. As a measure for disability we used the EDSS-score (Kurtzke 1983). Although in use for over 25 years, this scale certainly has its limitations. The lower ranks of the scale are dependent on findings in neurological examination, the higher ranks on limitation of mobility. The non-linearity of the scale makes the use of this scale in regression analysis questionable (Twork *et al.*, 2010). Novel clinimetric scales have been developed, including the Multiple Sclerosis Functional Composite (MSFC) (Cutter *et al.*, 1999). This scale has been argued to have less limitations (Polman and Rudick, 2010), but longstanding

experience with it is in many centers lacking. Therefore, in our study, we have chosen to use the EDSS-score, knowing its limitations.

Causality cannot be derived from association studies. The association between EDSS-score and vitamin D status is a good example in this perspective. EDSS-score correlates negatively with sun exposure, and sun-exposure predicts vitamin D status (van der Mei *et al.*, 2007). Alternatively, Burton *et al.* showed recently in a small phase I/II study that supplementation of high doses of vitamin D₃ increased the proportion of MS patients with a stable EDSS-score over 2 years (Burton *et al.*, 2010). In our study, we reproduced the earlier reported association between EDSS-score and vitamin D status (Nieves *et al.*, 2001, van der Mei *et al.*, 2007). Although no argument for an effect of vitamin D on MS pathophysiology, it shows that especially patients with a higher EDSS-score are at risk for having a poor vitamin D status. Therefore, these patients are logical candidates for assessment of their vitamin D status in clinical routine and eventual vitamin D supplementation to maintain a healthy calcium metabolism (Dawson-Hughes *et al.*, 2005). Other parameters are more suitable for observational and therapeutic studies in MS, than a single EDSS-score. Disability progression due to MS is believed to reflect severity of disease pathophysiology in the CNS, i.e. neuroinflammation (Poonawalla *et al.*, 2010) and maybe most importantly neurodegeneration (Minneboo *et al.*, 2009). Loss of neurons over time is a predominant feature (Ferguson *et al.*, 1997) not only in progressive MS (Furby *et al.*, 2010), but also at disease onset (Filippi *et al.*, 2003). Intervention in this pathophysiological process would be a highly relevant finding. Therefore, not disability at a certain time point per se, but rather disability progression of MS is a more suitable outcome measure (Leray *et al.*, 2010). Of the cohort described in **chapter 3**, first 5 year follow-up data on EDSS progression and vitamin D status will become available soon. This dataset will allow us to assess a contribution of vitamin D status to MS progression in a prospective longitudinal design. Additionally, the relationship of vitamin D status with other symptoms of MS can be assessed prospectively. Cognitive complaints, depression and fatigue are for instance also frequently observed in MS (Bol *et al.*, 2009). Recently, we found that vitamin D status correlated with the presence of depressive symptoms in MS (Knippenberg *et al.*, 2010). These and other parameters may also be assessed in future studies on vitamin D in MS.

The second parameter of MS disease activity which we took into account in **chapter 3**, was relapse rate of MS. Ideally, the study should have been performed prospectively, but only data on relapse-activity up to 2 years prior to serum sampling were available. With the assumption that the variance in vitamin D status found between the studied subjects would reflect the variance of vitamin D status as it was during the 2 years prior to serum sampling, we included these data in our analysis. The contribution of vitamin D status to relapse rate was assessed in a Poisson regression model (Wang *et al.*, 2009), the contribution to the odds on remaining

relapse-free in a logistic regression model. In the whole RRMS cohort, vitamin D status did not contribute significantly to either the odds on remaining relapse free, or relapse rate.

Based on our literature review, we hypothesized vitamin D to be a modulator of predominantly CD4⁺ T cell responses. When assessing an association between vitamin D status and MS, we intended to focus on the patients in which the CD4⁺ T cell-driven inflammatory response is the most predominant feature of MS pathophysiology. These have been argued to be RRMS patients with a short disease duration (Compston and Coles, 2005). Interestingly, in these patients, peripheral T cell homeostasis has been reported to be markedly disturbed (Venken *et al.*, 2007). To define this group of patients, we looked to the lower quartile of our RRMS cohort, regarding disease duration since the onset of first symptoms. This resulted in the cut-off value of 5 years, which could be argued to be an entirely artificial value. However, several studies have argued relapse activity and progression of disability in the first 5 years after MS onset to predict long term outcome (Degenhardt *et al.*, 2009). On the other hand, in a long term follow-up study, only in the first 2 years after MS onset, relapse rate predicted the time to objective EDSS endpoints (Scalfari *et al.*, 2010). Despite the questionability of this cut-off duration, the resulting cohort will have at least a more homogeneous MS pathophysiology with a larger contribution of T cell mediated inflammation, when compared to the entire RRMS cohort.

In this particular group, we found that relapse-free patients displayed higher serum 25(OH)D levels when compared to patients who relapsed within 2 years prior to serum sampling. Although multiple alternative explanations exist for this association, it was one of the first indications in the field that vitamin D might indeed influence risk on relapses of MS. Later studies with prospective designs reproduced this association, and took it as their main outcome measure (Simpson *et al.*, 2010; Mowry *et al.*, 2010). Additionally, a small phase I/II study provided an indication that supplementation of high doses vitamin D₃ might increase the odds on remaining relapse-free (Burton *et al.*, 2010). Altogether, our observation provided directions for other studies on vitamin D and MS, also in our own research line. Firstly, it provided us a defined cohort in which the assessment of an association between vitamin D status and peripheral T cell homeostasis appeared to be most relevant. Secondly, it provided us with a part of the combined primary outcome measure for the large clinical trial, and some restrictions of the patients to be included (*vide infra*).

Lastly, we touched in **chapter 3** on the issue of serum 1,25(OH)₂D versus 25(OH)D as relevant for disease modulation in MS. Although all experts agree on the idea that the serum 25(OH)D level is the most useful indicator a subjects' vitamin D status, the biologically active metabolite is also in 1000-fold lower concentrations present in the circulation. Only vitamin D dependent rickets type I and type II, and evaluation of hypercalcaemia have been proposed as diagnostic indications for measurement of serum 1,25(OH)₂D, (Hollis *et al.*, 1996). In hyper- or hypoparathy-

roidism and chronic kidney failure, serum 1,25(OH)₂D is rather a confirmatory test. In healthy individuals, these levels are tightly regulated by the serum calcium and parathyroid levels. However, we and others also found positive correlation between serum 25(OH)D and 1,25(OH)₂D levels in the serum (Barnes *et al.*, 2005; Smolders *et al.*, 2009a, **chapter 6**). Therefore, an impairment of serum 25(OH)D levels contributes to a relative impairment of serum 1,25(OH)₂D levels. This has been hypothesized to be of interest for patients with MS. In experimental studies, most frequently 1,25(OH)₂D is used to assess the effects of vitamin D (reviewed in **chapter 2**). In animal studies, 1,25(OH)₂D treatment prevented and cured symptoms of EAE to a larger extent than vitamin D (Cantorna *et al.* 1996, Spach *et al.*, 2000). Subsequently, small safety studies investigated the safety of 1,25(OH)₂D and 1 α (OH)D supplementation to patients with MS (Achiron *et al.*, 2003; Wingerchuk *et al.*, 2005). In **chapter 3**, we assessed whether serum levels of 1,25(OH)₂D contributed to EDSS-score and relapse rate of MS. In short, there was no contribution of serum 1,25(OH)₂D levels to either relapse rate or EDSS-score in our cohort. Although the raw dataset showed a difference in serum 1,25(OH)₂D between relapse-free and relapsing patients, this was not retained in the regression model after adjustment for confounders. Our results contributed to the idea that not the serum levels of 1,25(OH)₂D, but rather the serum 25(OH)D concentrations are important in MS. It needs to be said that epidemiological evidence also supports this hypothesis. No epidemiological studies reported an increased risk of MS in patients with kidney diseases and low serum levels of 1,25(OH)₂D. There has been one report on an increased risk for MS in patients with vitamin D dependent rickets, in which normal serum levels of 25(OH)D in the absence of 1,25(OH)₂D are found (Torkildsen *et al.*, 2008; Ramagopalan *et al.*, 2010). However, it is uncertain whether the serum levels of 1,25(OH)₂D, or rather the inability of (immune) cells to activate 25(OH)D locally is responsible for this association. In **chapter 5**, we further elaborate on this issue.

3. Peripheral T cell homeostasis and vitamin D status

In **chapters 4–6**, the interaction between vitamin D and the T cell compartment of MS patients is explored *in vivo*. Since vitamin D is a potent promoter of T cell homeostasis *in vitro* (Smolders *et al.*, 2008a, **chapter 2**), we hypothesized this effect to be the main driver of its associations with MS. In RRMS patients, predominantly those with a short disease duration, peripheral T cell homeostasis has been described to be disturbed (Viglietta *et al.*, 2004, Haas *et al.*, 2005, Venken *et al.*, 2006). Additionally, MS modulating drugs have been reported to restore this homeostasis (Korporal *et al.*, 2008, Haas *et al.*, 2009). Therefore, a shortage in vitamin D status may result in a more disturbed peripheral T cell homeostasis, and a more active disease course of MS. In the studies described in **chapters 4–6**, we included patients

resembling the cohort in which we found associations between vitamin D status and relapse activity in **chapter 3**. We first took a cross-sectional approach in **chapter 4**, and assessed whether there was a correlation of vitamin D status with composition and function of the CD4⁺ T cell compartment. We included hallmarks of peripheral T cell homeostasis, as have been assessed before as outcome measures in MS, including number of Treg, Treg suppressive capacity, and peripheral CD4⁺ T cell cytokine profiles.

The Treg has become a hot item in autoimmune disease research over the past decade (Damoiseaux 2006). Originally, these cells were characterized as CD25^{high} CD4⁺ T cells (Sakaguchi *et al.*, 2008). Since CD25 (IL-2R α -chain) is also an activation-marker on CD4⁺ T cells, phenotyping can be problematic. Identification of the transcription-factor Forkhead Box P3 (FoxP3) as a marker of Treg made phenotyping easier (Fontenot *et al.*, 2003; Hori *et al.*, 2003), and the lack of CD127 (IL-7R α -chain) expression further improved identification of Treg (Liu *et al.*, 2006; Seddiki *et al.*, 2006). A fascinating observation is that polymorphisms in both the IL-7 and IL-2 receptor genes surfaced in a genome wide association study as risk alleles for developing MS (International MS Genetics Consortium, 2007). However, literature is still controversial whether the CD25⁺FoxP3⁺CD127⁻ CD4⁺ T cell should be seen as 'true' Treg, or whether CD25⁺FoxP3⁺ and CD25⁺CD127⁻ cells should be seen as two distinct phenotypes with Treg properties. Although a strong correlation between the two Treg phenotypes is observed ($R = 0.826\text{--}0.891$) in our studies, only approximately 75% of CD25⁺CD127⁻ Treg is FoxP3⁺ (**chapter 4 and 6**). The number of Treg has generally been reported not to differ between healthy controls and patients with MS, regardless of their phenotype (Viglietta *et al.*, 2004, Haas *et al.*, 2005, Venken *et al.*, 2006). Beside the number of Treg, the functionality of Treg can be quantified as their ability to suppress proliferation and cytokine production of non-Treg CD4⁺ T cells (Tresp) in a proliferation-suppression assay (Venken *et al.*, 2007). In MS, the suppressive capacity of CD4⁺CD25^{high} Treg has been reported to be impaired (Viglietta *et al.*, 2004, Haas *et al.*, 2005, Venken *et al.*, 2006), but another study reported only an impairment of CD25⁺CD127⁻ Treg suppressive function during relapses of MS (Michel *et al.*, 2008).

Traditionally, the ratio of Th1 (IFN- γ ⁺) and Th2 (IL-4⁺) cells gained much attention in autoimmune research. In this model, the balance between pro- and anti-inflammatory T cells were the main drivers of autoimmune diseases (Abbas *et al.*, 1996). However, the introduction of Treg and Th17 (IL-17⁺) cells in auto-immunology complicated this picture (Steinman 2007). Nevertheless, the Th1/Th2 balance is still widely regarded as informative about the inflammatory state of the peripheral T cell compartment, and the role of Th17 cells in MS is still not consolidated. Additionally, CD4⁺ T cells can secrete the regulatory cytokine IL-10. These cells have been argued to reflect the proportion of T regulatory cell type 1 (Tr1) in the circulation (Moore *et al.*, 2001), but IL-10 production by Th2 cells and Treg has also been described. One

limitation which needs to be taken into account, is that characterization of immune cells in the circulation, does not need to reflect the state of immune cells in other compartments. Immune cells can be present in 4 relevant compartments in MS: the secondary lymphoid organs, the peripheral blood, the cerebrospinal fluid (CSF), and within the CNS. The correlation between findings in these compartments is uncertain, and the immune status within in the CSF has been argued to be more informative for MS than within the peripheral blood (Stüve *et al.*, 2006). Nevertheless, T cell characteristics in the circulation do correlate with disease status of MS. More IL-17⁺ and IFN- γ ⁺ CD4⁺ T cells were found in MS patients when compared to healthy controls, and in relapsing versus stable MS patients after stimulation with PMA and calcium ionomycin (Edwards *et al.*, 2010). One can conclude that the peripheral T cell compartment allows characterization of a more pronounced pro- or anti-inflammatory state of T cells in the periphery, but that the relevance of these parameters for the disease process of MS is only partly consolidated.

In a cohort of 29 patients, we found that the number of Treg did not correlate with vitamin D status, but that their functionality was worse in patients with a poor vitamin D status. In this assay it is, however, uncertain whether Treg are less capable to suppress CD4⁺ CD25⁻ T cell proliferation, or CD4⁺CD25⁻ Tresp are more resistant to suppression by Treg. It is remarkable that all patients with the worst Treg suppressive capacity displayed a vitamin D status lower than 50 nmol/L, which is widely accepted as the lower limit to prevent a decompensation of calcium metabolism (Dawson-Hughes *et al.*, 2005). This made us wonder whether it was not the vitamin D status per se, but rather the decompensation of calcium metabolism which was important in peripheral T cell homeostasis. Interestingly, elevation of serum calcium levels has been shown to be mandatory in the therapeutic effect of 1,25(OH)₂D in EAE (Cantorna *et al.*, 1999). We determined serum concentrations of 1,25(OH)₂D, parathyroid hormone, and total calcium, and found no correlation of Treg suppressive capacity with these parameters (described in **chapter 5**). Although more correlates of calcaemic functions of vitamin D can be proposed, our data suggest that the interaction between vitamin D and Treg suppressive capacity is not exclusively dependent on calcium homeostasis. Additionally to the Treg assay, we assessed the cytokine profile of CD4⁺ T cells in these 29 patients. We found that not the proportions of IFN- γ ⁺, IL-4⁺, IL-17⁺ and IL-10⁺ cells, but rather the Th1/Th2 (IFN- γ ⁺/IL-4⁺) ratio correlated negatively with vitamin D status. It has been argued that not the absolute proportions, but rather the ratios of pro- and anti-inflammatory cells are of interest in autoimmune diseases (Abbas *et al.*, 1996). Alternatively, however, mainly the proportion of IFN- γ ⁺ CD4⁺ T cells is decreased, suggesting that the decreased Th1/Th2 balance is mainly the result of a decreased proportion of Th1 cells. Additionally, we also showed for the Th1/Th2 ratio, that a correlation with correlates of vitamin D's calcaemic functions was absent (described in **chapter 5**). Altogether, the data presented in **chapter 4 and 5** suggest that a proportion of MS

patients with a poor vitamin D status displays a less well regulated T cell compartment.

As stated above, causality cannot be derived from association studies. Sunshine per se has been shown to prevent experimental models of MS independently of vitamin D (Becklund *et al.*, 2010), and a correlation of vitamin D status with immune regulation may be nothing more than an epiphenomena of this effect. Additionally, physical exercise has been shown to affect peripheral Treg measurements (Wilson *et al.*, 2009), and to correlate with an increased level of sun-exposure (Kampman *et al.*, 2008). In summary, only intervention studies can cast more light on the causality of the association between vitamin D status and peripheral T cell homeostasis in MS. Therefore, we wanted to assess whether the T cell parameters studied in **chapter 5 and 6** could provide useful outcome measure for intervention studies. We designed a pilot-study, in which we assessed both the safety and the effects on peripheral CD4⁺ T cell homeostasis of high-dose vitamin D₃ supplementation in MS (described in **chapter 6**).

4. Peripheral T cell homeostasis and vitamin D supplementation

In our pilot, we included 15 RRMS patients matching the inclusion criteria of **chapter 4 and 5**. Vitamin D₃ was supplemented in a dose of 20.000 IU/d (500 µg/d). The aim of this dose was to reach in a timeframe of 12 weeks circulating 25(OH)D concentrations, comparable to those reached in the study of Burton *et al.*, since in this study clinical effects of vitamin D₃ supplementation were suggested. The mean 25(OH)D level reached in our study 357 nmol/L, compared to the mean of 413 nmol/L which was reached by Burton *et al.*, (Burton *et al.*, 2010). The timeframe of 12 weeks was chosen for practical reasons: we wanted to control the level of vitamin D and sunlight that our subjects were exposed to. In **Figure 2**, the amount of vitamin D effective UV-radiation in Sittard, where our MS center is located, is shown. The first patient started with vitamin D supplementation at October 2009, and the last patient's blood was drawn for the last T cell analysis at March 2010. Within this timeframe, the amount of vitamin D synthesized in the skin was negligible, and sunshine per-se was also not likely to severely confound our results.

The first goal of the pilot, was to explore the safety of vitamin D₃ supplementation. The main side effect of a hypervitaminosis D is a decompensation of calcium metabolism (Kimball *et al.*, 2007; Hathcock *et al.*, 2007). A hypervitaminosis D could induce a hypercalcaemia, which could result in severe complications as heart failure and renal failure (Peacock 2010). However, cases of hypercalcaemia after vitamin D supplementation have only been described in subjects with additional diseases, as malignancies and granulomatous diseases (Hathcock *et al.*, 2007). Additionally, MS patient series showed that supplementation of high doses of vitamin D₃ did not

result in a decompensation of calcium metabolism (Kimball *et al.*, 2007; Burton *et al.*, 2010). Although these data provide confidence to supplement patients with high doses of vitamin D₃, carefulness is warranted. For instance, an unrecognized tuberculosis could induce a hypercalcaemia shortly after the start of supplementation, leading to complications in patients suffering from arrhythmias or renal dysfunction. Therefore, a careful inclusion of patients, and an intensive follow-up is recommended, certainly the first few months of intervention studies. Regarding the long-term safety of high dose vitamin D₃ supplementation, large datasets are lacking. Therefore, several concerns remain. One concern is the effect of a rise of total circulating calcium levels on the rate of cardiovascular events. Higher serum calcium levels have been associated with an increased carotid artery plaque thickness (Rubin *et al.*, 2007), and an increased risk of cardiovascular events in retrospective (Jorde *et al.*, 1999; Foley *et al.*, 2008) and prospective (Lind *et al.*, 1997; Tonelli *et al.*, 2005) cohort studies in healthy populations. However, intervention studies on calcium supplementation did not reported marked enhancement of the risk on cardiovascular events (Wang *et al.*, 2010). Additionally, it is uncertain whether the relatively marginal or even absent increase in total serum calcium levels as observed in our study and other studies on massive dose vitamin D₃ supplementation (Kimball *et al.*, 2007; Burton *et al.*, 2010) can be compared to the effects found between extremes in large populations. Studies on long-term vitamin D₃ with or without calcium supplementation should provide data on this matter. Another concern has been raised by a recent publication. In several (elderly) populations, supplementation of vitamin D₃ with/ without calcium reduced the risk on falls and fractures (Bischoff-Ferrari 2005; Bischoff-Ferrari 2009). Additionally, bone mass density correlated positively with vitamin D status (Bischoff-Ferrari 2004). However, in elderly women (N = 2256), once yearly supplementation of 500.000 IU (12.500 µg) of vitamin D₃ resulted in an increased fall risk (15%), and an increased fracture risk (26%), when compared to placebo (Sanders *et al.*, 2010). However, It is uncertain whether this increased risk is the result of the high doses, the lack of calcium supplementation, or the once-yearly administration. Post-hoc analysis revealed the latter mechanism to be plausible. Altogether, long-term safety of high-dose vitamin D₃ supplementation is not fully consolidated and should be included as outcome-measure in upcoming trials on vitamin D₃ supplementation in MS. Our study showed that in the short run, supplementation of vitamin D₃ does not evoke a decompensation of calcium metabolism, and is safe in a selected patient population.

Figure 2 The mean amount of vitamin D effective ultraviolet radiation (J/M^2) adjusted for clouds and ozone in Sittard between January 1st 1979 and 31st 1990, stratified for months. Between October and March, vitamin D effective UV radiation is almost absent (Courtesy of Ola Engelsen, Norwegian Institute for Air Research, The Polar Environmental Centre, Norway).

The second goal of the intervention study, was to assess whether we could measure a difference in peripheral CD4^+ T cell characteristics before and after supplementation. As discussed into detail in **chapter 6**, we did not find any effect on Treg numbers in the circulation, no significant improvement of Treg function in the circulation, but a shift of CD4^+ T cell cytokine profile after stimulation of PBMC directly ex-vivo. A limitation of our dataset is that we did not assess the suppressive effect of Treg on cytokine production in the proliferation suppression assays. Besides the true absence of an effect in this pilot, Treg suppressive capacity may be a too complex and difficult outcome measure to monitor an immune modulating effect of vitamin D supplementation in upcoming trials. The discrepancy between **chapter 4** and **chapter 6** is difficult to explain. A provoking hypothesis is that the $\text{CD25}^+\text{CD127}^-$ or $\text{CD25}^+\text{FoxP3}^+$ Treg may not be the most important cell in the immune modulation by vitamin D_3 . The increased proportion of circulating $\text{IL-10}^+ \text{CD4}^+$ T cells could represent an expansion of the Tr1 compartment. An increased proportion of these cells in the periphery could also drive the increased Treg suppressive capacity and decreased Th1/Th2 ratio in patients with an adequate vitamin D status as described in **chapter 4 and 5**. DC are potent targets of $1,25(\text{OH})_2\text{D}$ *in vitro*, and have been argued to be of vital interest for the actions of vitamin D_3 on the adaptive immune response. Tolerogenic $1,25(\text{OH})_2\text{D}$ -treated DC induce a skewing from Th1 to Th2 cells, and have been shown to promote $\text{IL-10}^+ \text{CD4}^+$ T cell development. In **chapter 4**, however, we assessed the proportion of circulating $\text{IL-10}^+ \text{CD4}^+$ T cells, and found no correlation with vitamin D status. However, this may be attributable to a technically inferior analysis due to the unpaired design and the older generation flowcytometer. It will be a challenge for future studies to capture the interaction between vitamin D and Tr1 (function) in patients with MS. Alternatively, in the cohort described in **chapter 4**, 6/29 patients displayed an especially poor suppression. In the participants of the pilot study, a similar pronounced poor suppression was not observed.

Therefore, the discrepancy may be a sampling effect. Additionally, in several patients control studies, a substantial proportion of MS patients shows a Treg suppressive capacity comparable to healthy controls (Viglietta *et al.*, 2004; Venken *et al.*, 2006). It is uncertain whether vitamin D supplementation can be expected to improve Treg suppressive capacity when it is already within the physiological range at baseline. A trial design which could take this limitation into account, is selection of patients with a poor Treg function at baseline, and allocation towards either vitamin D₃ or placebo supplementation afterwards. Alternatively, since we found more frequently a poor Treg suppressive capacity in patients with a poor vitamin D status (**chapter 4**), inclusion of patients with a poor vitamin D status (< 50 nmol/L) would also result in a study cohort which is enriched in patients with a poor Treg function. In **chapter 4**, it also appears that a certain proportion of patients displays a normal Treg function, despite a poor vitamin D status. This observation suggests that certain factors may make an individual more susceptible to immunological complications of a poor vitamin D status. This might be genetic background. Both immune- and vitamin D-related genes have been argued to affect the interaction between vitamin D and the immune response in MS (Colin *et al.*, 2000; Ramagopalan *et al.*, 2009; van Etten *et al.*, 2007), and the response of an individual on vitamin D supplementation (Orton *et al.*, 2008). When developing definitive studies on the effects of vitamin D supplementation on the peripheral immune system, these variables should be taken into account. Additionally, the choice of immunological assays to assess the effects of vitamin D should also be carefully made. Based on our results, a focus on T cell cytokine-profile directly ex-vivo could be a logical choice. Others have chosen T cell proliferation on specific stimulation by CNS proteins (Burton *et al.*, 2010). The most important limitation of this system is that only a subset of patients responds to these stimuli (Banwell *et al.*, 2008). Although the stimulation observed is specific, the effect of the intervention is not necessarily so. An increase in regulatory/ anti-inflammatory cells in the culture system may also affect the response. Studies like the one presented in **chapter 6** provide more data for a rational choice in this perspective.

Although the numbers are small and the study is unblinded and uncontrolled, the results show at least that the peripheral immune modulating potential of vitamin D₃ is a plausible underlying mechanism of the associations between vitamin D status and MS disease activity in the investigated population of patients. Furthermore, it provided directions to design an immunological substudy to the large randomized placebo controlled trial (**chapter 8**).

5. Genes and vitamin D

In **chapter 7**, we discussed the effect of a genetic polymorphism of the vitamin D receptor (VDR) on serum 25(OH)D levels in patients with MS. Apparently, the polymorphism Fok-I (RS10735810) interfered with the regulation of vitamin D metabolism. However, the risk for developing MS was unaffected.

When seasonal variation of serum 25(OH)D was eliminated by measuring all subjects in the same month, 77% of the variation of serum 25(OH)D has been estimated to be attributable to a variation in genetic factors in a population of MS patients and healthy controls (Orton *et al.*, 2008). As candidate genes for these genetic factors, genes involved in vitamin D metabolism have been frequently investigated with various results. The most well established predictors of vitamin D status, are two genetic polymorphisms within the vitamin D binding protein gene, i.e. RS4588 and RS7041 (Engelman *et al.*, 2008; Sinotte *et al.*, 2009). Additionally, polymorphisms of the CYP27B1 gene have been associated with serum 25(OH)D levels, albeit with variable results (Orton *et al.*, 2008; Engelman *et al.*, 2008). Concerning the effects of single nucleotide polymorphisms (SNP's) within the VDR gene, the results are even less clear (**Table 1**). When analyzing exclusively genetic contributions to vitamin D status, all other contributors to vitamin D status should most preferably be eliminated. The level of exposure to environmental sources of vitamin D is probably the important one. The seasonal fluctuation of serum 25(OH)D-levels can be dealt with by limitation of the timeframe of inclusion, or by introducing month of serum sampling in regression models. However, even when seasonal fluctuation is eliminated, 23% of the variation in serum 25(OH)D levels has been estimated to be caused by other factors than genes (Orton *et al.*, 2008). Variation in diet and sun-behavior is not taken into account. The most appropriate way to address the issue of exposure is to sample serum in a short time-frame and additionally to quantify vitamin D exposure with questionnaires regarding diet and sun-behavior (Van der Mei *et al.*, 2007), or with a sun-exposure meter (Simpson *et al.*, 2010). Even when the extent of sun-exposure is standardized, the efficiency of sunlight to induce synthesis vitamin D differs between geographic locations (**Figure 3**), making populations in different geographical areas hard to compare (Wang *et al.*, 2010). Additionally, the distribution of vitamin D related SNP's is likely to be affected in different populations by natural selection (Jablonski and Chaplin, 2010), since an allele associated with increased 25(OH)D levels may be more essential for survival to someone living near the polar circle, than for someone living near the equator. Lastly, clear differences can be observed in the vitamin D metabolism of Caucasians when compared to African Americans (Harris 2006). Beside these factors, there are also other contributors to vitamin D status, including body mass index (BMI), skin melanin density, age, and drug use. All these factors introduce noise in the analysis of a relationship between vitamin D status and genetic polymorphisms, and the different

ways by which studies cope with these confounders, may also contribute critically to their different results (Table 1).

Figure 3 The mean amount of vitamin D effective ultraviolet radiation (J/M^2) adjusted for clouds and ozone in several countries between January 1st 1979 and 31st 1990, stratified for months. (Courtesy of Ola Engelsen, Norwegian Institute for Air Research, The Polar Environmental Centre, Norway)

Table 1 Vitamin D receptor gene polymorphisms and serum 25(OH)D levels

Study	# Subjects	# SNPs	Associated with 25(OH)D	Adjustments in model	Inclusion timeframe
d' Alesio <i>et al.</i> , 2005	185	3	Hap. RS4516035 & RS7139166	Age, month, BMI, PTH, calcium intake.	None
Wjst <i>et al.</i> , 2006	872	12	None	Age, month	None
Engelman <i>et al.</i> , 2008	229	14	RS10783219	Age, Sex	None
Orton <i>et al.</i> , 2008	198	30	RS10735810	None	Mar-Apr
Smolders <i>et al.</i> , 2009a	212/289	1	RS10735810	Age, Sex, Disability	May-Oct/ Nov-Apr
Smolders <i>et al.</i> , 2009b	212/ 289	2	None	Age, Sex, Disability	May-Oct/ Nov-Apr

In our dataset, we reduced the effect of season of serum sampling by including individuals during either summer or winter period. Additionally, we took sun exposure into account by introducing EDSS-score in the analysis. Van der Mei *et al.*, showed that EDSS-score correlates negatively with the level of sun-exposure (Van der Mei *et al.*, 2007). In our population, vitamin D status correlated negatively with EDSS-score (Smolders *et al.*, 2008b, **chapter 3**). The reproducibility of the predictive effect of the polymorphism on serum 25(OH)D levels in MS patients in summer and winter, and in healthy controls, supports its relevance, as well as the reproduction in a Canadian cohort of MS patients and healthy controls (Orton *et al.*, 2008). Therefore, we can conclude that the Fok-I VDR polymorphism affects at least serum 25(OH)D levels, and therefore the interaction as is indicated by arrow 1 in **Figure 1**.

Subsequently, an effect of genetic polymorphisms on the circulating 25(OH)D levels would also be expected to affect the risk on developing MS. Poor serum 25(OH)D levels have been associated with an increased risk for developing MS

(Munger *et al.*, 2006). However, vitamin D related genes seldom surface in genome wide screens (International MS Genetics Consortium, 2007), and only one large study convincingly showed an association of CYP27B1 SNP's with the risk on developing MS in a Swedish population (Sundqvist *et al.*, 2010). A conceivable explanation is, that genetic background is only one of many factors which affect serum 25(OH)D levels. An effect of a polymorphism on vitamin D status (*vide supra*) should be very strong to overrule all these other factors. Additionally, other genetic and environmental risk factors might also interfere. The genotype of HLA-DR alleles has also been reported to interact with vitamin D status (Ramagopalan *et al.*, 2009). Therefore, it is not too surprising that we do not find an association of the Fok-I VDR gene polymorphism with MS in our population. The uncorrected difference in mean serum 25(OH)D level was only 10 – 16 nmol/L between homozygous carriers of the major and minor allele, and 13 – 17 nmol/L when corrected for age, sex, and disability. Considering the wide range of serum 25(OH)D levels found in our studies, this contribution is quite modest. Another mechanism could be, that a polymorphism affects the functionality of the VDR (arrow 2 in **Figure 1**). A genetic defect within the CYP27B1 gene, which causes vitamin D dependent rickets type 1, has been found in several relatives with familial MS (Torkildsen *et al.*, 2008; Ramagopalan *et al.*, 2010). In addition, common genetic variants of the CYP27B1 gene have also been associated with the odds on MS (Sundqvist *et al.*, 2010). In vitamin D dependent rickets type 2 (VDDR-2), the VDR pathway in the cell is disturbed. Interestingly, 1,25(OH)₂D reduces IFN- γ production in PBMC from healthy donors, but not in PBMC from VDDR-2 patients (Reichel *et al.*, 1987). Additionally, treatment with 1,25(OH)₂D induces no up-regulation of CYP24A1 in fibroblasts of VDDR-2 patients (Gamblin *et al.*, 1985). An association of VDDR-2 with MS has not been described, but an association of genetic variants of the VDR with MS is conceivable. The Fok-I polymorphism leads to a longer isoform of the VDR, which has been argued to be less transcriptionally active (reviewed in Smolders *et al.*, 2009c). However, consistent effects of the Fok-I VDR polymorphism on immune response to 1,25(OH)₂D have not been described (Colin *et al.*, 2000; van Etten *et al.*, 2007).

6. The clinical trial in preparation

Some researchers and clinicians have been skeptical about the need for designing clinical trials on vitamin D₃ supplementation in MS, since the therapeutic efficacy might not match the efficacy of newly appearing MS drugs. To determine the theoretical efficacy of vitamin D supplementation, association studies can be used. In the assumption that trials will supplement moderate- to high-doses of vitamin D₃, it is reasonable to assume that an elevation of serum 25(OH)D levels of at least 50 nmol/L will be achieved in each participant supplemented with vitamin D₃ (Burton

et al., 2010, Kimball *et al.*, 2007, **chapter 6**). An estimation of effect-size based on our own retrospective data is extremely inaccurate. These data would suggest a massive effect, with an 51% increase in the odds on remaining relapse-free for every 10 nmol/L increase of vitamin D (Smolders *et al.*, 2008b, **chapter 3**). The prospective Tasmanian study calculated a 9–12% increase in the proportion of relapse-free patients for each 10 nmol/L increase (Simpson *et al.*, 2010). Interestingly, a prospective trial on vitamin D status and relapse-risk in children with MS also calculated a 13% increase in relapse-free proportion of patients for each 10 nmol/L increase of vitamin D status (Mowry *et al.*, 2010). When assuming a linear relationship, this would mean at least a 45–60% increase in the relapse-free proportion of patients between both treatment-arms in the trial. However, it remains to be seen whether treatment effects will really be this large. Observational studies acquired their estimations of effect-size mostly by extensive modeling and making several assumptions. It must be realized that any estimation of effect size is surrounded by large confidence intervals. Vitamin D status itself is dependent on sun-exposure and outdoor physical activity. There is some evidence available that both these factors contribute individually to disease activity of MS (Becklund *et al.*, 2010). Additionally, the inverse linearity of the relationship between vitamin D status and the hazard on relapses in the supra-physiological range of serum 25(OH)D levels is uncertain. For the design of the trial, we performed a power-analysis from a worse-case scenario. The RNF study showed a relapse-free proportion of 52.8% after 2-year treatment with Rebif® (Giovannoni *et al.*, 2009). Interestingly, data from our own patient population displayed approximately the same proportion of 53.3%. Therefore, we expect in the placebo arm, at the end of the study, 53% of all participants to remain relapse-free. The minimal increase that we want to be able to measure is to 69% relapse-free, which means a percentual increase of 30%. It should be noted that this increase is only half of the increase predicted by association studies assuming an elevation of serum 25(OH)D of 50 nmol/L.

What are the most obvious challenges faced when designing a trial on vitamin D₃ supplementation in MS? The first challenge is, that treatment and placebo arms do not compare either the presence or absence of the intervention (i.e. vitamin D), but rather exposure to vitamin D in the physiological vs. the super-physiological range. This problem can be approached in two ways: either only patients with a poor vitamin D status should be included, or patients should be supplemented with sufficiently high doses of vitamin D₃ to induce a larger difference between the two arms. Both approaches have their downsides. Including selectively patients with low serum 25(OH)D levels, leaves patients in the placebo group exposed to a poor vitamin D status and a sub-optimal calcium homeostasis for a long period of time (Dawson-Hughes *et al.*, 2005). Alternatively, the safety of long-term high-dose vitamin D₃ supplementation is not yet fully consolidated (Hathcock *et al.*, 2007; Kimball *et al.*, 2007), and epidemiological evidence on the relationship between these supra-

physiological 25(OH)D levels and disease activity of MS is at present lacking. A potential U-shaped relationship cannot be excluded, yet our own data and the data of Burton *et al.* do not point in that direction (Burton *et al.*, 2010; **chapter 6**). In the upcoming SOLAR study, we choose the latter approach, since a beneficial effect of high-doses vitamin D₃ on MS activity has been suggested (Burton *et al.*, 2010). Safety aspects will be carefully monitored, and an independent data safety monitoring board has been installed. The second challenge is the incorporation of genetic background in a trial. As described in **chapter 7**, several vitamin D related genes are likely to interact with the effects of vitamin D supplementation (Orton *et al.*, 2007). Additionally, interaction of vitamin D status with the genotype of the MHC class II receptor has been proposed (Ramagopalan *et al.*, 2009). Studies should be designed in such a way, that influences of these genes on treatment outcomes can be analyzed. In the SOLAR study, DNA will be sampled for this purpose. The last challenge is the careful choice of outcome measures. Primarily, the SOLAR study will focus on classical MS outcome measures, as relapse activity, EDSS progression, and MRI activity. Other symptoms frequently found in MS patients are cognitive deterioration, fatigue, and depression (Bol *et al.*, 2010). For instance, the presence of depressive symptoms in MS patients has also been associated with a poor vitamin D status (Knippenberg *et al.*, 2010). Including also these parameters could provide a complete overview of the impact of vitamin D supplementation of the disease process of MS.

With our research as presented in **chapters 4–6**, we made the interaction between vitamin D status and peripheral T cell homeostasis a likely driver of a therapeutic effect of vitamin D. The SOLAR study provides the opportunity to test this hypothesis in a blinded, placebo-controlled design. The patients which will be included, match the patients which we included in the studies described in **chapters 4–6**. However, it should be kept in mind that an immune modulating effect of vitamin D in the periphery might not be the only mechanism of action of vitamin D. Vitamin D is present in the circulation, but also in CNS of MS patients (Holmøy *et al.*, 2009). The role that vitamin D plays in this inflammatory environment, is at present still unexplored. An up-regulation of the VDR and CYP27B1 in rodents with EAE when compared to control animals has been reported (Spach *et al.*, 2005). Whether vitamin D also plays a role in local immune homeostasis in the CNS of MS patients is uncertain. If so, experimental evidence would provide more reason to also assess the effects of vitamin D status on disability progression in patients with multiple sclerosis.

7. Conclusions

This thesis is not the end of a story, but reports rather first investigations of a new research line. We started with a review of the existing literature on vitamin D and MS, and are now performing a large international multi-center randomized placebo-controlled clinical trial on vitamin D₃ supplementation in MS. At present, the evidence from our work is not conclusive, but provided rather tools for further research.

From the research presented in this thesis, we can draw the following conclusions: Vitamin D is *in vitro* an important regulatory molecule within the adaptive immune response, but whether vitamin D is an immune and disease modulator *in vivo* in MS is uncertain (**chapter 2**).

In the MS population in South Limburg (The Netherlands), a poor vitamin D status is associated with a high level of disability, and with an increased relapse activity (**chapter 3**).

It is plausible that vitamin D act as a peripheral immune modulator in MS patients, and that low 25(OH)D levels result in a loss of regulation of peripheral CD4⁺ T cell homeostasis (**chapters 4–6**).

Patients with MS should be supplemented with vitamin D in trials, and not with 1,25(OH)₂D (**chapters 3 and 5**).

Supplementation of high doses vitamin D₃ without a dose-escalation scheme for 12 weeks does not induce a decompensated calcium metabolism (**chapter 6**).

Genetic polymorphisms of vitamin D related genes contribute to 25(OH)D concentrations in the circulation of patients with MS and of healthy subjects (**chapter 7**).

There is a large clinical trial underway, which addresses several key questions in the field of vitamin D and MS research (**chapter 8**).

8. Future directions and concluding remarks

In the end, the research presented in this thesis generated more questions than answers. This provides many entries for further studies.

T cells are the most important effector cells in MS (Bar-Or 2009). The correlation between vitamin D status and peripheral T cell homeostasis is not necessarily a direct effect of 25(OH)D on the T cell (Correale et al. 2009), but might be the result of an immunological chain of events. Many candidate targets can be proposed. Firstly, monocytes and dendritic cells are potent targets of 1,25(OH)₂D *in vitro*, and 1,25(OH)₂D-treated dendritic cells skew the T cell compartment to a less pro-inflammatory state (**chapter 2**). Additionally, experimental research suggested that for a therapeutic effect of 1,25(OH)₂D in EAE, a RAG-dependent (Nashold *et al.*, 2001), IL-10 producing cell (Spach *et al.*, 2006) is mandatory. Although we focused

on T cells, the regulatory B cell is also a theoretical option (Heine *et al.*, 2008). Interestingly, more studies are currently focusing on regulatory B cells in MS. Further characterization of the interaction between vitamin D and the adaptive immune response *in vivo* could lead to a more specifically directed therapeutic intervention in MS.

The interaction between vitamin D and the adaptive immune response is likely not specific. Since we included no control groups in **chapters 4–6** of this thesis, we cannot validate this claim with our own data. However, an increased incidence of other autoimmune diseases has also been associated with correlates of a poor vitamin D exposure, including diabetes type 1 (Hypponen *et al.*, 2001), rheumatoid arthritis (Cutolo *et al.*, 2006), systemic lupus erythematoses (Müller *et al.*, 1995), and vasculitis (Gatenby *et al.*, 2009). Since a disturbed T cell homeostasis has also been argued to play a role in these diseases, observational and intervention studies on vitamin D (supplementation) and disease activity are warranted. Another therapeutic strategy with vitamin D has been proposed in diabetes mellitus type 1. Vaccination of epitope-loaded DC after tolerogenisation with 1,25(OH)₂D has been proposed as a treatment option in transplant and autoimmune disease patients (Unger *et al.*, 2009). This approach differs in essence from supplementation of vitamin D, since it aims to force the adaptive immune response in a tolerant state, rather than optimizing a biological homeostatic mechanism. In MS, the vaccination strategy may prove difficult, since the exact epitope against which the autoimmune response is directed, is not known.

The relationship between vitamin D status and several disease activity measures of MS has been assessed. The association between relapse activity of MS and vitamin D status is the most well consolidated (Mowry *et al.*, 2010; Simpson *et al.*, 2010; Smolders *et al.*, 2008b, **chapter 3**). An association between EDSS-score and vitamin D status is known for the longest time (Smolders *et al.*, 2008b, **chapter 3**; van der Mei *et al.*, 2007), yet prospective studies on disability progression in MS are lacking. Additionally, we described a correlation between depressive symptoms of MS and vitamin D status (Knippenberg *et al.*, 2010), which also requires consolidation. In a prospective follow-up of the (extended) cohort as described in **chapter 3**, several of these issues will be addressed. With clinical trials ahead, it is important to consolidate outcome measures of interest.

Vitamin D research is important. Supplementation can be safe, without side-effects, and cheap. Although the efficacy in MS patients is difficult to estimate, a small additive effect to current or future disease modulating drugs in MS is worthwhile. Especially, since drug treatment costs for MS patients would be elevated by a negligible fraction, and the safety profile appears at present favorable. Not only a role in MS, but also in many other (autoimmune) disease has been proposed for vitamin D. In these days of rising healthcare costs, a potential benefit of a cheap

intervention which could be applied on a public scale in the prevention and treatment of invalidating diseases is extremely important to investigate.

To conclude, the most relevant question for MS patients is at present whether they should be supplemented with vitamin D₃. Although we cannot relate our data to healthy control data, we showed in **chapters 3–6** that about 50% of MS patients displays a vitamin D status <50 nmol/L. There is a considerable amount of agreement among experts that these levels are too poor to maintain a healthy calcium metabolism (Dawson-Hughes *et al.*, 2005). Therefore, patients should be encouraged to supplement themselves with moderate amounts of vitamin D₃. Although cross-sectional studies show promising results, it should be made clear to patients that a therapeutic effect of vitamin D on the disease course of MS is at present uncertain. Therefore, supplementation of (high) doses of vitamin D₃ for the sake of modulating the disease course of MS, is at present premature. How does this idea relate to government policy? In 2008, the national Dutch Health Counsel advised the minister of public health on supplementation of vitamin D₃ to the Dutch public (Weggemans *et al.* 2009). In their much criticized report (Muskiet *et al.*, 2010), advises for supplementation were mostly based on large national cohort studies. Supplementation of 20 µg/d was advised to institutionalized elderly with or without osteoporosis and to subjects older than 50 (females) or 70 (males) which have a dark skin-type or are not frequently exposed to sunlight, and to veiled females older than 50 years. The studies on which these advises are partly based (the Longitudinal Aging Study Amsterdam and the Hoorn study) show that 47–51% of ambulatory elderly has a vitamin D status <50 nmol/L. From the perspective of the Dutch Health Council committee, patients with MS (regardless age) who are not much exposed to sunlight, should also be recommended supplementation of 20 µg/d vitamin D₃. This may also account for other chronic diseased cohorts. Hopefully, in the next report of the Dutch Health Council regarding vitamin D, this significant and vulnerable part of Dutch society will not be forgotten.

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Summary

11

Multiple sclerosis (MS) is a severe inflammatory disease of the central nervous system. In patients with MS, the immune system attacks and damages the surrounding isolation sheet of neurons, the myelin. This results in a dysfunction of these neurons, and subsequently the patient will experience loss of body function. The reason for this attack of the immune system is not clear.

In the inflammatory reaction of MS, $CD4^+$ T cells play an important role. These cells are central players in the adaptive immune response. When specialized antigen presenting cells encounter a pathogen (for instance a bacteria), they present this to $CD4^+$ T cells. If a $CD4^+$ T cell recognizes this pathogen, it reacts by starting to proliferate and to produce inflammatory mediators (cytokines). These cytokines mediate clearance of the pathogen by other cells. It is important that T cells only start an inflammatory response upon presentation of an intruder from outside the body, and not upon presentation of self-antigens. To ensure this, T cells are carefully selected for being not auto-reactive during their development, and, additionally, specialized regulatory T cells (Treg) control the remaining peripheral autoimmune T cell response when needed. Current opinion is that, in patients with MS, these regulatory mechanisms fail, and that T cells start an inflammatory response against self-antigens (autoimmune disease). The auto-antigen which causes this response, is at present unknown. In patients with MS, a relatively disturbed T cell compartment is frequently found, with less functional Treg and $CD4^+$ T cells which produce more pro-inflammatory cytokines.

Vitamin D is acquired both from dietary intake and from exposure of the skin to sunlight. It is predominantly known as an important molecule in calcium homeostasis. Several studies show an association between a limited exposure to vitamin D, and an increased level of disability of MS. This could indicate that vitamin D acts as an inhibitor on the disease process and disease activity of MS. With the vitamin D project of which this thesis is the start, we want to investigate i) if disease activity of MS can be inhibited by supplementation of vitamin D, and ii) which biological mechanisms mediate this effect.

In **chapter 2**, we describe a literature study. We review the evidence for a disease modulating effect of vitamin D in MS, and the role of vitamin D in the immune response. Experimental studies in animals and plastic dishes (*'in vitro'*) show that vitamin D plays an essential role in the adaptive immune response as a strong promoter of T cell homeostasis. T cell proliferation and pro-inflammatory cytokine production are inhibited, and the production of anti-inflammatory cytokines and the development of Treg are promoted. In animal models of MS, vitamin D prevents and inhibits symptoms of disease. Interestingly, vitamin D status, or correlates of vitamin D status, correlate negatively with the risk on developing MS. An increased disease activity of MS has also been associated with an impaired vitamin D status. However, besides a few safety studies, large intervention-studies with vitamin D are

lacking. We conclude that vitamin D is an immune modulator, but it is uncertain whether it can also be considered as an immune and disease modulator in MS.

In **chapter 3**, we investigated in a cross-sectional study among 267 patients from our MS population an association between vitamin D status and disease activity of MS. We showed that poor vitamin D statuses are especially found in patients with a high level of disability and in older patients. Additionally, we showed that RRMS patients with a short disease duration (<5 years) who remained relapse-free prior to serum sampling displayed a better vitamin D status when compared to patients with relapses prior to serum sampling. The latter observation suggests that a good vitamin D status might lower the hazard for relapses in this cohort.

Because a disturbed peripheral CD4⁺ T cell homeostasis is especially found in RRMS patients with a short disease duration, and vitamin D is an important promoter of T cell homeostasis *in vitro*, we assessed in this cohort cross-sectionally if a correlation between vitamin D status and both function and composition of the peripheral CD4⁺ T cell compartment was present (described in **chapter 4**). In 29 RRMS patients, not the number of Treg in the circulation, but rather the function of Treg was negatively correlated with vitamin D status. This means that Treg were less capable of controlling T cell proliferation in patients with a poor vitamin D status. CD4⁺ T cells in the circulation produced relatively less pro- and more anti-inflammatory cytokines in patients with a good vitamin D status. We concluded that vitamin D appears to promote the regulation of T cell homeostasis *in vivo* in MS.

To investigate whether the role of vitamin D in calcium homeostasis was of primary importance, we investigated in **chapter 5** in the same 29 patients whether T cell regulation was also correlated with the effects of vitamin D on calcium homeostasis. There was no correlation of either Treg suppressive function or CD4⁺ T cell cytokine balance with serum levels of 1,25-dihydroxyvitamin D, total calcium, and parathyroid hormone (PTH) levels. This observation suggests that the interaction between vitamin D status and T cells *in vivo* is not dependent on calcium metabolism.

The associations between vitamin D status and CD4⁺ T cell function suggest that supplementation of vitamin D might be used to skew the T cell compartment to a more regulatory state in patients with MS. In preparation of a large intervention study, we performed a pilot study to assess both the safety of vitamin D supplementation and the effects of vitamin D supplementation on the T cell compartment (described in **chapter 6**). We observed in 15 RRMS patients that supplementation of 500 µg/day (20.000 IU/day) vitamin D₃ for 12 weeks did not affect the proportion of Treg in the circulation. The suppressive capacity of Treg was not enhanced significantly, and only a marginal trend towards improvement could be observed. However, after vitamin D supplementation, relatively more CD4⁺ T cells produced anti-inflammatory cytokines (IL-4, IL-10), and relatively less cells produced pro-inflammatory cytokines (IFN-γ). This study suggests that the cytokine profile of pe-

peripheral CD4⁺ T cells may form an interesting marker to monitor the immune modulating effects of vitamin D in randomized placebo-controlled clinical trials. Additionally, we conclude that supplementation of high doses of vitamin D₃ was without side-effects in our cohort. Participants reported no side-effects, and the calcium levels in the circulation were unaffected.

In **chapter 7**, we assessed whether a functional genetic polymorphism of the vitamin D receptor (VDR) gene was associated either with MS, or with vitamin D status in patients with MS and healthy subjects. A genetic polymorphism is a common variation in the genome. In the case of this specific polymorphism, it leads to a longer or shorter VDR protein. We found no difference in the distribution of the polymorphism between 289 healthy subjects and 212 patients with MS. However, both in summer and in winter, lower vitamin D statuses were observed in carriers of the allele encoding the shorter VDR protein. The association was found both in patients and in healthy controls. We concluded that vitamin D related genetic variation may be an important confounder in intervention studies on vitamin D in MS, and should therefore be taken into account.

All the knowledge collected in the previously described chapters was combined in the development of an international trial on vitamin D supplementation in MS, of which the protocol is described in **chapter 8**. In a multi-center trial in several European countries, 348 RRMS patients with a disease duration <5 years will be included. In a double-blind, randomized, placebo-controlled design, we will assess whether supplementation of either vitamin D₃ or placebo aside regular MS therapy (Beta Interferon) has an additive inhibiting effect on disease activity of MS. In several sub-studies, the effects of this intervention on the peripheral immune system will be assessed. Additionally, potential genetic confounders of treatment effects will be taken into account.

In this thesis, we did not answer the question whether vitamin D reduces disease activity of MS. However, this thesis did contribute significantly to the development of a trial which might provide answers to this question. Additionally, a mechanism was conceptualized by which vitamin D may influence the pathophysiology of MS. We conclude (**chapter 9**) that a poor vitamin D status is associated with a high level of both relapse rate and disability in our MS population. Furthermore, vitamin D status correlates with the composition and function of the peripheral CD4⁺ T cell compartment in patients with MS. In a small pilot study, patients displayed after supplementation a less pro-inflammatory cytokine profile, which at least supports the idea that vitamin D supplementation may restore the imbalance in the peripheral CD4⁺ T cell compartment as is frequently found in MS. In a large clinical trial, we will investigate whether vitamin D can be used as a disease and immune modulator in MS.

Dutch summary

Nederlandse samenvatting

12

Multipele sclerose (MS) is een ernstige ontstekingsziekte van het centrale zenuwstelsel. In MS patiënten valt het immuunsysteem de isolatielaag rond zenuwen, het myeline, aan en zorgt ervoor dat deze isolatielaag beschadigd wordt. Hierdoor kunnen deze zenuwen niet meer goed functioneren en ervaart de patiënt uitval van lichaamsfuncties. De reden waarom het afweersysteem in de aanval gaat is niet duidelijk.

In de ontstekingsreactie bij MS spelen $CD4^+$ T cellen een belangrijke rol. Deze cellen zijn een essentieel onderdeel van de verworven immuun respons. Als een antigeen presenterende cel een pathogeen (bijvoorbeeld een bacterie) oppikt, laat hij deze aan de $CD4^+$ T cel zien. Als de $CD4^+$ T cel dit pathogeen herkent, reageert deze hierop door te gaan delen en door ontstekingsstoffen (cytokines) te maken. Dit zorgt ervoor dat het pathogeen door andere cellen opgeruimd wordt. Het is belangrijk dat een T cel normaal gesproken alleen bij indringers van buiten voor ontsteking zorgt, en niet bij het zien van lichaamseigen eiwitten. Hiervoor worden T cellen in hun ontwikkeling zorgvuldig geselecteerd, en zijn er speciale regulerende T cellen (Treg) in het lichaam, die de T cel respons in bedwang houden wanneer nodig. Bij MS patiënten denkt men dat dit proces mis gaat, en dat het lichaam een afweerreactie tegen een lichaamseigen stof veroorzaakt (auto-immuunziekte). Welke stof dit precies is, is onbekend. Wel wordt bij MS patiënten een instabiel T cel compartiment gevonden, met minder goed werkende Treg en $CD4^+$ T cellen die relatief gezien meer ontstekingsstoffen maken.

Vitamine D komt uit het voedsel en het zonlicht, en kennen we vooral als een belangrijke speler in de calcium huishouding. Verschillende studies suggereren een verband tussen een beperkte blootstelling aan vitamine D, en een toegenomen mate van beperking door MS. Dit zou kunnen betekenen dat vitamine D wellicht een remmende werking op de ziekteactiviteit van MS zou kunnen hebben. Met het vitamine D project waar dit proefschrift de start van is, willen we onderzoeken i) of we met het toedienen van vitamine D de ziekteactiviteit van MS kunnen remmen, en ii) welke biologische mechanismen hieraan ten grondslag liggen.

In **hoofdstuk 2** beschrijven we een literatuurstudie. Hierin hebben we onderzocht hoe stevig de aanwijzingen voor een ziektemodulerend effect van vitamine D op MS zijn, en welke rol vitamine D in het afweersysteem heeft. Experimentele studie in proefdieren en plastic schaaltes ('*in vitro*') laten zien dat vitamine D een essentiële rol in de verworven immuun respons speelt, en een sterke promotor van T cel homeostase is. T cel proliferatie en productie van ontstekingsbevorderende cytokines worden geremd, de productie van ontstekingsremmende cytokines en de ontwikkeling van Treg worden gestimuleerd. In proefdiermodellen van MS voorkomt en remt vitamine D ook de ziekteverschijnselen. Interessant genoeg correleert vitamine D status, of afgeleiden hiervan, negatief met het risico op het ontwikkelen van MS. Ook een grote ziekteactiviteit van MS is geassocieerd met een beperkte vitamine D status. Echter, naast veiligheidsstudies zijn geen grote interventiestudies

met vitamine D verricht. We concluderen dat vitamine D een immuun modulator is, maar of vitamine D ook een immuun en ziekte modulator in MS is, is op dit moment onzeker.

In **hoofdstuk 3** hebben we in een cross-sectionele studie onder 267 patiënten uit onze MS populatie onderzocht of er een verband tussen ziekteactiviteit van MS en vitamine D status is. Lage vitamine D statussen werden gevonden in oudere patiënten en in patiënten met een grote mate van beperking. Hiernaast hadden RRMS patiënten met een korte ziekteduur (<5 jaar) die relapsvrij waren voor bloedafname, een betere vitamine D status dan patiënten die relapsen hadden. Deze laatste observatie is suggestief voor het idee dat een goede vitamine D status het risico op relapsen van MS zou kunnen verlagen.

Omdat in het cohort RRMS patiënten met een korte ziekteduur vooral een verstoring van perifere CD4⁺ T cel homeostase wordt gevonden, en vitamine D *in vitro* een belangrijke promotor van T cel homeostase is, onderzochten we in dit cohort cross-sectioneel of er een correlatie tussen vitamine D status en functie en samenstelling van het perifere CD4⁺ T cel compartiment was (beschreven in **hoofdstuk 4**). In 29 RRMS patiënten hing het aantal Treg in de circulatie niet samen met vitamine D, maar de Treg leken wel minder goed te werken in patiënten met een beperkte vitamine D status. CD4⁺ T cellen in de circulatie maakten bij patiënten met een hoge vitamine D status relatief minder ontstekingsbevorderende en meer ontstekingsremmende cytokines. Vitamine D lijkt dus *in vivo* in MS patiënten dus de regulatie van T cel homeostase te bevorderen.

Om te onderzoeken of de rol van vitamine D in de calcium huishouding voor deze interactie primair belangrijk is, bekeken we in **hoofdstuk 5** in dezelfde 29 patiënten of T cel regulatie ook samenhangt met afgeleiden van de effecten van vitamine D op de calcium huishouding. Zowel serum waarden van 1,25-dihydroxy vitamine D, totaal calcium, en parathyroïd hormoon (PTH) correleerden niet met Treg functie en CD4⁺ T cel cytokine balans. Deze observatie suggereert dat de interactie tussen vitamine D en T cellen ook *in vivo* onafhankelijk van het calcium metabolisme is.

De associaties tussen vitamine D status en CD4⁺ T cel functie suggereren dat met vitamine D suppletie het T cel compartiment gestuurd zou kunnen worden. Als voorbereiding op een grote studie verrichtten we een pilotstudy om naar zowel de veiligheid van vitamine D, als naar de effecten van vitamine D op de T cellen te kijken (beschreven in **hoofdstuk 6**). We vonden in 15 RRMS patiënten dat suppletie van 500µg/dag vitamine D voor 12 weken geen effect had op het aantal Treg in de circulatie. Hiernaast werd ook de Treg functie niet significant beïnvloed, en leek deze hooguit marginaal gestimuleerd te worden. Wel vonden we na vitamine D suppletie relatief gezien meer CD4⁺ T cellen die ontstekingsremmende cytokines maakten (IL-4, IL-10), en minder die ontstekingsbevorderende cytokines maakten (IFN-γ). Deze studie suggereert dat perifere CD4⁺ T cellen een interessante marker vormen om de immuun modulerende gevolgen van vitamine D suppletie in grote

gerandomiseerde en placebo gecontroleerde studies te onderzoeken. Verder concluderen we dat suppletie van hoge dosis vitamine D zonder bijwerkingen was. Deelnemers rapporteerden geen bijwerkingen, en de calcium waarden in het bloed bleven onveranderd.

In **hoofdstuk 7** onderzochten we de associatie van een functioneel vitamine D receptor (VDR) gen polymorfisme met MS, én met vitamine D status in MS patiënten en gezonde proefpersonen. Een genetisch polymorfisme is een veel voorkomende variatie in het erfelijk materiaal, dat in het geval van dit polymorfisme tot een langer of korter VDR eiwit leidt. Er was geen verschil in het voorkomen van het polymorfisme tussen 289 gezonde proefpersonen en 212 patiënten met MS. Wel werden zowel in de zomer als in de winter lagere vitamine D statussen gemeten in patiënten met het allel dat met een korter VDR eiwit geassocieerd is. In de gezonde proefpersonen vonden we dit verband ook. We concluderen dat vitamine D gerelateerde genetische variatie mogelijk een belangrijke confounder is in interventiestudies met vitamine D en daarom in deze studies meegenomen moet worden.

Alle opgedane kennis uit de verschillende hoofdstukken hebben we gecombineerd in de ontwikkeling van het protocol voor een grote internationale studie naar vitamine D suppletie in MS dat beschreven staat in **hoofdstuk 8**. In een Multicenter studie in verschillende Europese landen wordt in 348 patiënten met RRMS en een korte ziekteduur onderzocht of suppletie van vitamine D of placebo naast reguliere MS therapie (Interferon Beta) een toegevoegd remmend effect op de ziekteactiviteit van MS heeft. Hiernaast zal in verschillende substudies het effect op het perifere immuunsysteem onderzocht worden. Verder worden in de trial ook genetische variabelen meegenomen.

Met dit proefschrift hebben we niet de vraag beantwoord of vitamine D de ziekteactiviteit van MS kan remmen. Wel heeft dit proefschrift bijgedragen aan de ontwikkeling van een klinische studie die dit wel kan doen. Daarnaast heeft het een mechanisme uitgekristalliseerd waardoor vitamine D de pathofysiologie van MS kan beïnvloeden. We concluderen (**hoofdstuk 9**) dat een slechte vitamine D status samenhangt met een grote relaps activiteit en een grote mate van beperking in onze patiëntenpopulatie. Verder hangen de samenstelling en functie van het perifere CD4⁺ T cel compartiment samen met vitamine D status in de circulatie van MS patiënten. In een kleine openlabel interventiestudie hadden patiënten na vitamine D suppletie een minder ontstekingsbevorderend CD4⁺ T cel cytokine profiel, wat op z'n minst de suggestie wekt dat vitamine D suppletie de imbalance in het perifere CD4⁺ T cel compartiment bij patiënten met MS kan herstellen. In een grote interventiestudie gaan we onderzoeken of vitamine D als ziekte en immuun modulator in MS ingezet kan worden.

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Ook buiten de onderzoekssetting hebben velen bijgedragen aan dit onderzoek. In het bijzonder te noemen mijn collega hoornisten van de Koninklijke Harmonie van Heer en het Universiteitsorkest Maastricht: Frank, Jaap, Olav, Stijn, Willemien, Carmen en Paul. Mijn irritatiedrempel is laag, maar mijn waardering voor jullie des te hoger! Frank (en Linda en Rolo), Jaap (en Elena), ik waardeer onze vriendschap zeer en ben blij dat dit ook op pubmed vastgelegd is. Dank ook aan alle andere musici van beide orkesten, in het bijzonder de 'studenten' van de Koninklijke Harmonie van Heer: de weekendjes-weg waren een feest!

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Als laatste Lies. ¡Hola! Dit stukje is af, nu kunnen we verder gaan.

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Curriculum Vitae

Joost Smolders werd op 28 maart 1983 geboren in Tilburg. Hij behaalde zijn VWO diploma in 2001 aan het Koning Willem II college te Tilburg. Hierna studeerde hij geneeskunde aan de Universiteit Maastricht, waar hij in 2007 cum laude afstudeerde. Tijdens zijn laatste studiejaar startte hij bij professor Hupperts (Maastricht Universitair Medisch Centrum (MUMC), Maastricht) met het onderzoek naar vitamine D in MS patiënten. Inmiddels is dit project uitgegroeid tot een voorname onderzoekslijn van het Academisch MS Centrum Limburg (Orbis MC, Sittard en MUMC) en de onderafdeling Immunologie van de afdeling Interne Geneeskunde (MUMC). In dit proefschrift is het onderzoek uiteengezet dat hij verrichtte bij de *School for Mental Health and Neuroscience* (MUMC) onder supervisie van professor Hupperts en professor Cohen Tervaert. Joost is mede-initiator en *steering committee* lid van de SOLAR trial, een internationale klinische studie naar vitamine D suppletie in MS. Als *coordinating investigator* werkt hij aan een immunologische substudie van SOLAR, de SOLAR(IUM). Momenteel onderzoekt hij de activiteit van vitamine D in het centrale zenuwstelsel van MS patiënten bij de Neuroimmunologie onderzoeksgroep van dr. Huitinga (Nederlands Instituut voor Neurowetenschappen, Amsterdam). Joost woont samen met Marlies Kaal in Utrecht.

Joost Smolders was born on March 28th, 1983 in Tilburg (The Netherlands). He graduated from grammar school at Koning Willem II College (Tilburg) in 2001. Subsequently, he studied medicine at Maastricht University (Maastricht, The Netherlands), where he graduated with distinction in 2007. During the last year of his medical training, he started under supervision of prof. dr. Hupperts (Maastricht University Medical Center (MUMC), Maastricht, The Netherlands) his research on vitamin D in MS. This vitamin D project has become an important research-line of the Academic MS Center Limburg (Orbis Medical Center, Sittard, The Netherlands; MUMC) and the division Immunology of the department of Internal Medicine (MUMC). This thesis comprises the research he performed at the School for Mental Health and Neuroscience (MUMC) under supervision of professor Hupperts and professor Cohen Tervaert. Joost is co-initiator and steering committee member of the SOLAR study, a clinical study on vitamin D supplementation in MS. As coordinating investigator, he contributes to an immunological sub-study of the SOLAR trial, the SOLAR(IUM). At present, he works on the activity of vitamin D in the central nervous system of MS patients at the Neuroimmunology research group of dr. Huitinga (Netherlands Institute for Neuroscience, Amsterdam, The Netherlands). Joost lives together with Marlies Kaal in Utrecht (The Netherlands).