Is there a window of opportunity for the therapeutic use of vitamin D in multiple sclerosis?

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Abstract
Multiple sclerosis is an autoimmune treatable but not curable disease. There are a multiplicity of medications for multiple sclerosis therapy, including a class entitled disease-modifying drugs that are mainly indicated to reduce the number and severity of disease relapses. Not all patients respond well to these therapies, and minor to severe adverse effects have been reported. Vitamin D, called sunshine vitamin, is being studied as a possible light at the end of the tunnel. In this review, we recapitulated the similar immunopathogenesis of multiple sclerosis and experimental autoimmune encephalomyelitis, the immunomodulatory and neuroprotective potential of vitamin D and the state-of-art concerning its supplementation to multiple sclerosis patients. Finally, based on our and other groups’ experimental findings, we analyzed the need to consider the relevance of the central nervous system and the different time-point administration aspects for a more rational indication of this vitamin to multiple sclerosis patients.

Key Words: central nervous system; cytokines; experimental autoimmune encephalomyelitis; immunomodulation; immunopathogenesis; inflammation; multiple sclerosis; neuroprotection; regulatory T cells; vitamin D

Introduction
The simplest and widely disseminated concept of multiple sclerosis (MS) is that it is a heterogeneous, autoimmune and neurodegenerative disease characterized by inflammation, demyelination and axonal damage (Lassmann et al., 2007). It is believed that myelin-specific T cells from distinct subsets orchestrate an autoimmune attack to the central nervous system (CNS). However, the complexity of this pathology seems to go beyond the autoimmune phenomenon and includes a pivotal contribution of genetic background and environmental circumstances. Human leukocyte antigen (HLA) class II and I genes, which encode molecules crucial for antigen presentation, activating T helper and T cytotoxic lymphocytes, respectively, are relevant modifiers of disease risk. The class II HLA variants have a striking association with an increased risk of MS (odds ratio (OR)=3), particularly HLA-DRB1*15:01 in Europeans, HLA-DRB1*15:03 in African Americans and HLA-DRB1*04:05 in the Japanese population, whereas the class I HLA-A*02 variant is associated with protection from the disease (OR~0.6) (Moutsianas et al., 2015; Chi et al., 2019). There are approximately 200 non-HLA single nucleotide polymorphisms conferring modest risk of MS, predominantly inflammation-related genes (Consoritum, 2017). These single nucleotide polymorphisms associated with MS are located close to these genes and regulate both adaptive and innate immune responses. These researchers found an enrichment of MS genes in microglia but not in astrocytes or neurons, suggesting that these brain-resident immune cells play a role in MS susceptibility. This contribution of genes located outside the major histocompatibility complex has been more recently sustained by the findings of an International Multiple Sclerosis Genetics Consortium, 2019 (Consoritum, 2019).

Concerning the relevance of the environment, special emphasis has been placed on smoking, obesity and infectious agents such as Chlamydia pneumoniae, human herpes virus 6, Epstein-Barr virus, influenza A, measles, parainfluenza 2, varicella zoster (Olsson et al., 2017; Saberi et al., 2018) and fungi and their toxins (Benito-León et al., 2010; Saroukolaei et al., 2016). As the disease is more common in women and there are clear gender differences in terms of progression and severity, it is believed that hormonal factors contribute to regulate the evolution of this pathology (Avila et al., 2018; Ysraelit and Correale, 2019). Another strong environmental risk factor for MS development and severity is vitamin D (VitD) levels. According to epidemiological evidence, lower serum VitD levels are associated with a higher risk of developing disease and with more severe clinical manifestations (Munger et al., 2006; Alharbi, 2015; Sintzel et al., 2018; Bäcker-Kodua et al., 2020). The name sunshine vitamin derives from the fact that most of this hormone is produced by the skin under the influence of sunlight. During exposure to ultraviolet light, 7-dehydrocholesterol is converted to pre-VitD3 by keratinocytes and dermal fibroblasts (Mostafa and Hegazy, 2015). Detailed information about its metabolism, interaction with the specific receptor (VitD receptor-VDR) and numerous biological functions are available in the literature (Bikle, 2014; Khammissa et al., 2018). This essential participation of sunlight in VitD production and its relevance in the context of MS is supported by strong and recent epidemiological evidence (Tremlett et al., 2018; Gallagher et al., 2019). Most of the protective effect of VitD in MS has been attributed to its immunomodulatory capacity and to direct neuroprotective effects (Wrozek et al., 2013; Wei and Christakos, 2015; Sintzel et al., 2018).

Evidence from prospective studies is consistent with the hypothesis that high circulating levels of VitD are associated with a lower risk of MS. A study by Munger et al. (2006), for example, studying MS cases identified among more than 7 million US military personnel between 1992 and 2004, evidenced a decreased risk of MS among white population, but not among black and Hispanic populations,
with high VitD serum levels. Another prospective study conducted in Sweden identified an association between high VitD levels during the years preceding disease onset and a decreased risk of MS (Salzer et al., 2012). Interestingly, sunlight exposure during childhood and adolescence also interferes with the risk of developing MS in adulthood. A multinational case-control study reported an association between infrequent summer outdoor activity and increased MS risk in Norway and Italy (Bjørnevik et al., 2014). Comparable findings were recognized in Tasmania (van der Mei et al., 2003), Sweden (Bäärnhielm et al., 2012) and the USA (Tremlett et al., 2018). Ethnicity also interferes with the beneficial effects of VitD or sun exposure on MS risk. In a more recent study conducted in California, higher lifetime ultraviolet radiation exposure and serum VitD levels were associated with a lower risk of MS in whites but not in Hispanics or blacks (Langer-Gould et al., 2018).

In this review, we will address the related immunopathogenesis underlying MS and EAE development and the relevance of VitD as an environmental immunomodulator, including its role in the CNS. Finally, we will cover experimental and clinical studies concerning its use as an adjunct therapy for MS control. Our experience with EAE added to that of other researchers suggests that the preclinical application of VitD in MS patients could be more effective in controlling the disease.

Search Strategy and Selection Criteria

A literature review was electronically performed using the PubMed database. The following search term combinations were used to select the articles: vitamin D and immunomodulation; multiple sclerosis and experimental autoimmune encephalomyelitis; immunopathogenesis; vitamin D and neuroprotection; vitamin D and multiple sclerosis clinical trials; vitamin D and experimental autoimmune encephalomyelitis. Most elected studies (approximately 80% of all references) were published from 2011 to 2021. The remaining articles were published before 2011 and were included due to their relevance in the area.

Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

Immunopathogenesis

Much of the scientific knowledge concerning the immunopathogenesis of MS derives from investigations performed with EAE. This disease is artificially induced in rodents, mainly mice and rats, by immunization with myelin-derived proteins and peptides in the presence of complete Freund’s adjuvant and pertussis toxin (Robinson et al., 2014; Bjelobaba et al., 2018). Despite clear differences between human and animal diseases (Lassmann and Bradl, 2017), this experimental model is considered suitable for studying MS immunopathogenesis. The main stages of this pathology are briefly outlined in Figure 1.

Periphery

1. Activation of myeloid reactive cells
2. Licensing in the lymph
3. Th17 expansion in the intestine

CNS

4. Inflammation and neurodegeneration
5. Activation and expansion of Th17 cells
6. Regulatory mechanisms
7. T cell barriers and migration

Figure 1 | Multiple sclerosis/experimental autoimmune encephalomyelitis immunopathogenesis.

The following sequence of events has been suggested for multiple sclerosis and experimental autoimmune encephalomyelitis immunopathogenesis: activation of self-reactive T cells in secondary lymphoid organs, probably in cervical lymph nodes (1), possible licensing in the lungs but perhaps in other places (2), Th17 differentiation in the intestine (3), breakdown of central nervous system (CNS) permeability barriers (4), local reactivation and expansion of T cells and release of proinflammatory mediators (5), demyelination and neurodegeneration triggered by the inflammatory reaction (6), and regulatory mechanisms usually activated but not strong enough to control the disease (7).

Peripheral activation of myelinspecific lymphocytes

Activation of myelinspecific T cells could occur in peripheral lymphoid organs by recognition of microbial epitopes that share homology with self-antigens (molecular mimicry) (Cusick et al., 2012), release of myelin from the CNS by a local infection (Stohlm an and Renders, 2003) or by bystander activation (Waldner et al., 2004). These possibilities are probably facilitated by the higher degree of T cell receptor degeneracy that was confirmed in myelin T cells by Hemmer et al. (1998). Apart from Th1/Th17 subsets that have been more classically associated with MS (Fletcher et al., 2010), Th6, CD8 T cells, B lymphocytes and macrophages/microglia have also been considered essential in the immunopathogenesis of MS and EAE (Yadav et al., 2015; Zarobkiewicz et al., 2019).

Presumptive T cell licensing for pathogenicity

In the last few years, a new concept has emerged from EAE studies concerning the “education” of T cells to become pathogenic and therefore to be able to reach and damage the CNS. This phenomenon has been called “licensing” or “licensing for pathogenicity” (Tan et al., 2017). Despite some controversies, the lungs and spleen seem to be the main licensing environments in the context of EAE/MS (Odoardi et al., 2012; Tan et al., 2017). This licensing process, which allows T cells to acquire the capability to cause damage when they reach the CNS, is basically a change in the pattern of gene expression characterized by downregulation of genes related to proliferation/activation and upregulation of migration-promoting genes (Ransohoff, 2012). Human Th17 lymphocytes stand out among encephalitogenic effector cells because they are initially involved in the opening of the blood-brain barrier (BBB) (Kebir et al., 2007) and later in neurodegeneration (Ransohoff, 2012). In humans, this subset is found in lesions, and transcriptomic research reveals that interleukin-17 is the highest-ranking gene expressed in active plaques (Lock et al., 2002).

Expansion of Th17 in the intestine

The immune system associated with the intestinal mucosa has recently been recognized as a potential source of MS and EAE development. The intestinal environment plays a crucial role in the pathogenesis of these two diseases mostly by promoting activation and acquisition of the Th17 phenotype (Ivanov et al., 2009; Cosorich et al., 2017). Th17 cells present a considerable degree of plasticity involving not only the Th17 phenotype but also other functions. It is well documented that these cells act in the defense against a variety of pathogens and in a myriad of inflammatory pathologies (Stockinger and Omenetti, 2017). It is assumed that activation of effector Th17 cells occurs mostly in the murine small intestine, independent of their ensuing function (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Interestingly, the development of steady-state or pathogenic Th17 cells is critically determined by microbiota composition. Segmented filamentous bacteria, for example, induce brain autoimmunity in mice by selectively promoting Th17 differentiation (Lee et al., 2011). More recently, Cosorich et al. (2017) confirmed the contribution of the intestinal milieu in promoting Th17 expansion in MS patients. They described that a higher Th17 frequency correlated with more accentuated disease severity and dysbiosis characterized by an elevated Firmicutes/Bacteroidetes ratio, relative abundance of Streptococcus, and a decreased abundance of Prevotella strains. These findings emphasize the relevance of investigating targeted gut therapies such as dietary intervention, helminth therapy, and, more recently, fecal microbiota transplantation (Budry et al., 2017; Brown et al., 2021). It is well established that certain dietary modifications, such as reduced intake of fats and salt and probiotic supplementation, can ameliorate EAE/MS symptoms (Valburg et al., 2021). Conversely, a high-fat/high-salt diet led to increased disability in the EAE model with augmented immune cell infiltration and oxidative stress in the CNS (Valburg et al., 2021). A probiotic mixture (VSL#3) including several strains of Lactobacillus, Bifidobacterium, and Streptococcus was also tested in patients with relapsing remitting MS. This procedure reversed MS-like symptoms but not disease severity.

Breakdown of the blood-brain barrier and cell migration to the CNS

In the past few years, it became evident that the crosstalk between the gut microbiota and brain has a crucial impact on neurodegenerative processes (Ma et al., 2019). CNS integrity and functionality are preserved by the BBB and the blood-cerebrospinal fluid barrier. These two barriers are located in...
distinct CNS compartments, and damage and/or activation of their various components will allow leukocyte infiltration and ensuing neurodegeneration in MS and EAE (Alvarez et al., 2011). The breakdown mechanisms of such barriers in these pathologies are incompletely understood but appear to involve many molecular and cellular factors. Inflammation during MS/EAE experimental models (interferon (IFN)-γ, TNF-α and interleukin (IL)-1β) disorganizes T cells in the CNS (Koutrolos et al., 2014). Tregs are also involved in the remission phase of an acute monophasic disease in Lewis rats (Almolda et al., 2011). These animals totally recovered from leg paralysis through elimination of inflammatory cells in the CNS by apoptosis mediated by NK cells, macrophage polarization toward M2 and Treg activation (Shin et al., 2012). Similarly, in a chronic mouse model of EAE, Tregs mediated the recovery phase of the disease by controlling cytokine production, proliferation and motility of effector T cells in the CNS (Koutrolos et al., 2014). Tregs are also involved in the differential susceptibility of male and female SJL/J mice to develop relapsing remitting EAE. Male SJL mice are more resistant to autoimmune diseases by favoring the development of Th2 immune responses through augmented expression of the inhibitor markers CTLA-4 and CD62L by IL-10-secreting Tregs (Hussain et al., 2011).

Immunomodulatory Skills of Vitamin D

Vitamin D is a powerful hormone derived from cholesterol that is primarily produced by the skin under the catalytic effect of ultraviolet B rays (Plum and DeLuca, 2010). Its classic role is to allow calcium and phosphate absorption to maintain adequate serum levels and to promote bone growth (Dereje et al., 2017). However, the widespread expression of VDR (Kongsbak et al., 2013) and of the enzyme 1α-hydroxylase (CYP27B1) led to the discovery that it is also fundamental in many other physiological circuits, including immunomodulation and neuroprotection.

Vitamin D from the diet or synthesized by the skin is biologically inactive. To perform immunomodulatory or other functions, it needs to be hydroxylated twice, one in the liver and another in the kidneys (Hewison et al., 2000). At the 25(OH)D level, vitamin D has also been demonstrated in normal human tissues, including the gastrointestinal tract, skin, vasculature, placenta, brain and lymph nodes, through the expression of CYP27B1 (Hewison et al., 2007). Classical VDR is a member of the nuclear receptor family, and is found in the proximity of regulatory elements that control the expression of cell types such as macrophages, DCs, activated T cells, neurons and glial cells (Smolders and Damaoisaux, 2011), and can regulate the expression of more than 1000 genes. After interaction with 1,25(OH)2D3, VDR heterodimerizes with the retinoid-X receptor. Activated VDR binds to vitamin D-responsive elements, which are located close to gene promoters, allowing VDR complexes to regulate the expression of approximately 3% of the human genome (Clark and Mach, 2016). Vitamin D can also act through the putative membrane receptor membrane-associated, rapid response steroid-binding to induce nongenomic actions (Landel et al., 2018).

Enhancement of phagocytic and antimicrobial ability

Broadly speaking, Vitamin D has distinct effects on innate and specific immunity. Most of the antimicrobial monocyte/macrophage activities are upregulated by Vitamin D. Under the effect of this vitamin, macrophages increase their phagocytic ability, produce more defensin β2 and cathelicidin antimicrobial peptide (Youssef et al., 2011) and synthesize more reactive oxygen intermediates (Ghosh et al., 2016). Vitamin D also induces autophagy, which is a potent innate antibacterial defense mechanism. Interestingly, autophagy is upregulated via cathelicidin, which activates the transcription of autophagy-related genes such as Beclin-1 and Atg5 (Fabri and Modlin, 2009). The VitD/VDR heterodimer (Gao et al., 2008) also maintains optimal expression of defensin and tight junction genes, therefore supporting intestinal integrity and eubiosis (Su et al., 2016).

Tolerance of dendritic cells

A great deal of research has been dedicated to the effect of Vitamin D on antigen presenting cells, especially DC differentiation. This initial stage of specific immunity is critical because it will shape much of the T cell polarization into distinct subsets. It is well established that Vitamin D has long been proposed to be able to imprint DCs with a tolerogenic profile. DCs differentiated in the presence of calcitriol produced lower amounts of IL-12 and TNF-α and higher levels of IL-10 (Griffin et al., 2001). These cells also expressed reduced amounts of class II MHC and costimulatory molecules (Széles et al., 2009). Under the influence of VitD, DCs display higher expression of inhibitory molecules, including immunoglobulin-like transcript 3 and PD-L1 (Chambers and Hawrylowicz, 2011). These tolerogenic DCs are poor inducers of T cell proliferation and activation, and they also trigger autoreactive T cell apoptosis (Colotta et al., 2017). On the other hand, Vitamin D-modified DCs are very efficient in inducing Tregs, as will be further described.

Promotion of T lymphocyte polarization

Vitamin D can also directly modulate T cells. These effects are complex, diverse among T cell subtypes and require TCR stimulation; that is, naïve T cells do not respond to interaction with VitD (Colotta et al., 2017). Calcitriols inhibits Th1 and Th17 differentiation and the...
production of their related cytokines (Chang et al., 2010; Zhang et al., 2018). Despite some controversial findings, it has been shown that VitD promotes Th2 polarization (Sloka et al., 2011).

The ability of VitD to induce the differentiation of Tregs is being viewed and explored as a highly promising strategy to control autoimmune diseases (Kurniawan et al., 2020). The most well-characterized Treg lineage is CD4\(^+\)CD25(high)Foxp3\(^+\) T cells (Vignali et al., 2008). This vitamin can promote Treg differentiation by acting directly on CD4\(^+\) T cells, downmodulating the production of proinflammatory cytokines such as IFN-γ, IL-17 and IL-21. Interestingly and very promising from a therapeutic point of view, these cells acquire phenotypical and physiological characteristics of Treg cells. They express high levels of Foxp3 and CTLA-4 and are also able to suppress the proliferation of resting cells (Jeffery et al., 2009). As stated above, Tregs can also be indirectly induced by VitD with the assistance of DCs. DCs differentiated from monocytes in the presence of VitD displayed a semimature phenotype identified by low expression of costimulatory molecules together with increased indoleamine 2,3 dioxygenase, IL-10, TRAIL and PDL-1 expression levels (Raker et al., 2015). These are very encouraging findings because these molecules are intrinsically related to the immunoregulatory ability of Treg cells. Indoleamine 2,3 dioxygenase, for example, triggers tryptophan starvation to inhibit T cell responses through the expansion of Tregs (Yan et al., 2010). Additionally, TRAIL and PDL-1 are critically involved in the induction of these classical Treg markers. CD4\(^+\)CD25(high)Foxp3\(^+\) Treg cells. VitD is also able to induce Foxp3-Tr1 cells; preferential induction of Treg Foxp3\(^+\) or Tr1 cells is attributed to interaction with distinct DC subsets (van der Aar et al., 2011).

**Role of Vitamin D in Neurogenesis and Neuroprotection**

A growing number of studies performed in vitro, ex vivo, and employing animal models provide evidence that VitD is crucial for the healthy function of neuronal pathways. Fundamental findings in this area indicated that VitD signaling affected both the developing and adult brain (Eyles et al., 2013; Groves et al., 2014). Studies have also indicated that high levels of VitD stimulates the synthesis of several neuroprotective mediators, including nerve growth factors and neurotrophins, which are essential for neurite outgrowth, neuronal growth and survival, neurotransmission, and synaptic plasticity. By using a rat model, Pardridge et al. (1985) demonstrated that VitD enters the CNS through the blood-brain barrier. The local presence of enzymes involved in VDR metabolism and VDRs sustains local bioactivation, catabolism and biological activity (Almokhtar et al., 2016; Landel et al., 2018). This pivotal role of VitD in brain development triggered attention to diseases that were linked to VitD deficiency in early life as autistic spectrum and schizophrenia (Eyles et al., 2013) and later to pathologies more frequently linked to adult life as MS or aging as a process, Alzheimer’s disease (AD), Parkinson’s disease and amyotrophic lateral sclerosis. Epidemiological data have supported this possibility. There is, for example, a striking association between low VitD levels and dementia and AD (Chai et al., 2019). By using a mouse model of AD, (Morello et al., 2018) thoroughly investigated the role of VitD and found that its supplementation was able to improve neurogenesis and cognition. The evidence of a relationship between VitD levels and Parkinson’s disease is less strong and waits for additional investigations (Rimmer et al., 2016). Numerous studies support the notion that VitD insufficiency contributes to higher-risk MS (Pierrot-Deseilligny and Souberbielle, 2017). As MS involves a complex interplay of genetic factors and environmental factors, VitD efficiently controls EAE development, but its usage in MS patients is still a matter of contention.

According to Berridge’s hypothesis, Vit D deficiency accelerates aging and age-related diseases (Berridge, 2017). This author proposes that this hormone controls aging by acting in a series of metabolic pathways, such as autophagy, mitochondrial dysfunction, oxidative stress, inflammation, calcium signaling, and telomere shortening. In fact, many of these processes are disturbed in these pathologies, and VitD has been able to restore their physiological status. VitD is able, for example, to reestablish cellular calcium homeostasis through the synthesis of protein binding calcium ions (Pendo and DeGiorgio, 2012) and by regulation of L-type voltage-sensitive Ca\(^{2+}\) channel expression that together will avoid the excessive accumulation of Ca\(^{2+}\) in nerve cells. The increase in cytoplasmic and mitochondrial membrane damage through increased release of neurotransmitters, activation of lipases and activation of nitric oxide synthase and formation of ROS (Zündorf and Reiser, 2011). Low levels of VitD have also been associated with lower antioxidative potential, as recently demonstrated by (Fan et al., 2020). According to these authors, the exacerbation observed in experimental Alzheimer-like pathologies was due to enhanced oxidative stress via the downregulation of superoxide dismutase 1, glutathione peroxidase 4 and cysteine/glutamate exchanger.

Several neurodegenerative disorders have been more recently associated with mitochondrial dysfunction (Wako et al., 2020). Interestingly, silencing VDR in different cell types initially triggered impairment of mitochondrial integrity and then cell death (Ricca et al., 2018). In addition to these more straightforward effects, VitD controls CNS homeostasis through its previously mentioned immunomodulatory ability (Fernandes de Abreu et al., 2009). An encouraging and enlightening review concluded that the use of VitD to control the brain decay that accompanies aging and neurodegenerative conditions was recently published (Farghali et al., 2020).

**Vitamin D Efficacy in Multiple Sclerosis: A Controversial Issue**

Available data suggest that VitD levels play a role in both the risk of developing MS and the degree of disease disability. Concerning the risk, several studies have indicated the existence of a latitudinal gradient in MS prevalence. A lower risk of developing this disease was found in geographical areas with higher exposure to sunlight (Pierrot-Deseilligny and Souberbielle, 2017). This finding is easily explained by the need for ultraviolet light skin exposure for VitD synthesis (Jeon and Shin, 2018). Many studies have revealed VitD deficiency in these patients (Suresh Kumar et al., 2013; Martellini et al., 2014). It is important to highlight, however, that not all MS patients presented VitD deficiency. In contrast, negative associations with VitD deficiency have been described before IFN-β treatment, as observed in RRMS patients from the original OFAMS study. In these patients, the serum levels of VitD were inversely associated with radiologic disease activity (Løken-Amsrud et al., 2012) and were negatively correlated with magnetic resonance imaging (MRI) activity (Røsjø et al., 2015) only before IFN-β treatment. During IFN-β therapy, similar alterations were observed in these patients, suggesting that VitD status has no influence on IFN-β treatment effects.

Concerning the degree of disease activity, many studies also reinforced the direct relationship between lower VitD levels and more severe disease or more severe comorbidity manifestations (Thouvenot et al., 2015; Brola et al., 2016; Wawrzyniak et al., 2017). Interestingly, the possible contribution of VitD to lower MS disability rates seems to be affected by the underlying disease modifying therapy treatment; this was clear in patients receiving IFN-β treatment. However, until now, there has also been no consensus regarding the effect of VitD on disease severity (Nikanfar et al., 2015; Muris et al., 2016). The scientific community is still asking if this deficiency is contributing to trigger MS or if the deficiency is being caused by the disease itself. In this scenario, it has been debated whether VitD supplementation is therapeutically recommended for MS patients. A recent study based on the clinical trial called BENEFIT (Betaferon/Betaseron in Newly Emerging Multiple Sclerosis for Initial Treatment) that completed the 11-year assessment (BENEFIT-11) indicated that higher VitD serum levels predicted better cognitive performance in MS patients. Higher 25(OH)D (50 nM) levels in the first 2 years were related to 65% lower odds of poorer Paced Auditory Serial Addition Test-3 performance at year 11 (Cortese et al., 2020). Analogously, and based on the phase 3, double-blind, placebo-controlled study, called FREEDOMS (FTY720 Research Evaluating Effects of Daily Oral therapy in Multiple Sclerosis), it was verified improved MRI outcomes on percent brain volume change, proportion of patients free of new/enlarging T2 lesions, and a trend of less depression in the ‘daily users’ of VitD versus ‘non-users’ (Hongell et al., 2018).

Most of the recent systematic reviews and meta-analyses indicate that VitD supplementation had no therapeutic effects on MS patients (Jagannath et al., 2010; Mostafa and Hegazy, 2015; Zheng et al., 2018). This was partially reinforced by the study of (Kampman et al., 2012), in which weekly supplementation with 20,000 IU vitamin D3 did not result in beneficial effects on the measured MS-related annualized disability progression and expanded disability status scale (EDSS). However, some papers have shown that VitD is safe and is at least able to promote some
degree of immunomodulation. With respect to safety, a single center open-labeled randomized, controlled clinical Phase II/I pilot study conducted with 15 pregnant women with confirmed MS and low serum 25(OH)D levels reported no adverse effects after VitD supplementation (Etemadifar and Janghorbani, 2015). These pregnant women received 50,000 IU/week of VitD from 12 to 16 weeks of gestation until delivery and had fewer relapse events during pregnancy and within 6 months after delivery. In addition, the mean EDSS score did not change 6 months after delivery, whereas in the nonsupplemented group, the mean EDSS score increased from 1.3 to 1.7 (Etemadifar and Janghorbani, 2015). Concerning immunomodulation, VitD administration was able to increase TGF-β serum levels (Mahon et al., 2003) and to reduce IL-17 production by T lymphocytes, the proportion of effector memory T cells (Sotirchos et al., 2016) and TNF-α concentration in culture supernatants of anti-CD3 stimulated PBMC cultures from VitD treated RRMS patients but not in the placebo group (Rolf et al., 2019). In addition, 48 weeks of VitD supplementation preserved CD25 expression in Tregs and circulating soluble CD25 levels, contrasting with a significant reduction in the placebo group (Rolf et al., 2018).

From 2018 to the current year, at least three trials were published and showed positive results regarding VitD supplementation in MS patients. In a double-blind, placebo-controlled parallel-group study named CHOLINE (Cholecalciferol in Relapsing-Remitting MS: A Randomized Clinical Trial), conducted for 2 years, enrolled RRMS patients whose inclusion criteria were low serum 25(OH)D concentration (< 75 nM) and treatment with IFN-β-1a. All efficacy parameters were better for cholecalciferol. In this study, patients who received oral 100,000 IU of cholecalciferol every other week for 96 weeks presented an ARR reduction, fewer new hypointense T1-weighted lesions, a lower volume of hypointense T1-weighted lesions, and a lower progression of EDSS (Camu et al., 2019). Based on the aforementioned findings, these authors suggested a potential therapeutic effect of cholecalciferol in RRMS patients who were being treated with IFN-β-1a and presented an original low serum level of 25(OH)D.

The SOLAR study was performed by following a group of patients similar to the CHOLINE trial, that is, RRMS subjects with low serum 25(OH)D levels (< 150 nM in this case) who were also being treated with IFN-β-1a. This phase II, randomized, double-blind, placebo-controlled, supplemented with Vigantol Oil, showed no significant difference based on evidence of disease activity (NEDA-3) status (Hupperts et al., 2019). These authors also reported that oral treatment with 14,007 IU/d cholecalciferol for 48 weeks protected the patients against the development of new MRI lesions (Hupperts et al., 2019). However, high-dose vitamin D3 as an add-on to IFN-β-1a showed no established benefit based on the primary outcome of NEDA-3, and these patients showed no difference in circulating levels of neurofilament light chain, which is a biomarker of activity in RRMS (Smolders et al., 2020). A similar absence of effect over the levels of neurofilament light chain had already been published by (Holmøy et al., 2019), conducted with 71 RRMS patients weekly supplemented with 20,000 IU of VitD3.

In contrast, a recent multicenter randomized stratified actively controlled explorative phase 2 pilot trial with a double-blind intervention period of 18 months, called EVIDIMS (Efficacy of Vitamin D Supplementation in Multiple Sclerosis), enrolled RRMS patients with IFN-β-1b treatment and compared the effects of every other day high- (20,400 IU) versus low-dose (400 IU) cholecalciferol supplementation. No differences were observed in these two treatments concerning clinical (relapse rates, disability progression) and radiographical (T2-weighted lesion development, contrast-enhancing lesion development, brain atrophy) parameters. The authors concluded that the results neither support nor disprove a therapeutic benefit of high-dose VitD supplementation and suggested that a larger sample size was necessary to prove the hypothesis (Dörri et al., 2020).

To explore the association between dietary intake and physical capacity and fatigue in MS, a randomized controlled trial assessed the dietary records of patients for 4 days and correlated them with the 6-minute walk test, a VO₂ max test, and self-reported questionnaires assessing fatigue severity (Fatigue Severity Scale) and impact (Modified Fatigue Impact Scale) (Albrechtsen et al., 2020). In the multiple regression analyses, the absolute intake of VitD (average of 41.6 µg/d) by RRMS patients showed trends towards a positive association with VO₂ max but not with other parameters. The main immunological or clinical outcomes observed in patients supplemented with VitD are summarized in Table 1.

VitD supplementation in MS patients has been revealed to be an amazing complex subject to be addressed and elucidated. Some of the constraints that prevent this clarification are the wide and intricate activity of VitD in the body, the expected variability in each patient’s response to this hormone, the obstacles to establishing homogenous and safe clinical trials, the variation in doses, how long a patient should be treated, and what disease modifying therapy or other drugs are being used by the patient. In our opinion, the stage of the disease, as will be approached next in this review, is one of the parameters that deserves major attention.

It is important to keep in mind that mild and severe side effects have been verified in both experimental animal studies and clinical trials. The most well-described manifestations include neuropsychiatric, gastrointestinal, cardiovascular, and renal symptoms. This subject has been addressed in detail by experts in the field (Jones, 2008; Galior et al., 2018; Taylor and Davies, 2018; Feige et al., 2019). Despite these side effects and many disappointing outcomes during clinical trials, the scientific community still believes that the usage of VitD is worthwhile to be further evaluated.

Table 1  Clinical trials with vitamin D supplementation

| Type of study | Patients | Dose | Period | Effect |
|--------------|---------|------|--------|--------|
| Double-blind design study | 17 patients | 1000 IU/d | 6 mon | Increased serum TGF-B1 levels |
| Phase II/I randomized trial (Completed) | 15 pregnant patients | 50000 IU/wk | 12 to 16 wk | Fewer relapse events and unchanged EDSS |
| Clinical trial (Completed) | 40 RRMS patients | 10400 IU or 800 IU/d | 6 mon | Reduction in the proportion of IL-17+CD4+ cells, CD161+CD4+ cells, and effector memory CD4+ cells in high-dose group |
| Phase 3 FREEDOMS and FREEDOMS II trials (Completed) | 1953 patients | Not registered | 674.5±181.61 d | Improved MRI on brain volume and patients free of T2 lesions |
| Clinical trial (Terminated) | RRMS female patients | 4000 IU/d | 16 wk | Decreased TNF-α concentration in culture supernatants of CD3+CD4+ cells |
| Clinical trial (Completed) | 53 RRMS patients | 14000 IU/d | 48 wk | Decreased Treg CD25-expression and circulating soluble-CD25 levels period |
| Clinical trial CHOLINE (Completed) | RRMS patients with low serum VitD and therapy with IFN-β-1a | 100000 IU every other week | 96 wk | ARR reduction, less new T1-weighted lesions, lower volume of T1-weighted lesions, and a lower EDSS period |
| Clinical trial SOLAR (Completed) | RRMS patients with low serum VitD and therapy with IFN-β-1a | 14007 IU/d | 48 wk | Protective effects on development of new MRI lesions |
| Clinical trial BENEFIT (Completed) | 278 patients with clinically isolated syndrome | 50 nM | 2 yr | 65% lower odds of poorer PASAT-3 |

ARR: Annualized relapse rate; CD: cluster of differentiation; d: daily; EDSS: expanded disability status scale; IFN: interferon; IL-17: interleukin-17; IU: international unit; MRI: magnetic resonance imaging; PASAT-3: paced auditory serial addition test-3; RRMS: Relapsing Remitting Multiple Sclerosis; TGF: transforming growth factor; TNF: tumor necrosis factor; Treg: regulatory T cells; VitD: vitamin D; mths: months; wk: week; yr: year.
Vitamin D: Lessons from Experimental Encephalomyelitis

Despite the differences between EAE and MS, a great deal of information obtained from this model helps to understand MS immunopathogenesis and to adopt new therapeutic mechanisms. EAE has also been widely employed to uncover the potential mechanisms by which VitD could control EAE development and, eventually, be translated to MS. Table 2 summarizes the main findings showing that the in vivo administration of this hormone protected mice against EAE development.

One of the promising research lines in this scenario is the tolerogenic DC/Treg axis. Most of the investigations regarding the effect of VitD on DCS indicate that its in vitro addition promotes a tolerogenic profile. The adoptive transfer of these cells to EAE rats and mice was able to induce regulatory Foxp3 T cells and to reduce disease severity (Farias et al., 2013; Mansilla et al., 2015; Xie et al., 2017). Cryopreservation of DCS generated in the presence of VitD conserved their tolerogenic properties, which are relevant for human application (Mansilla et al., 2015). This strategy is very elegant and appealing and generates robust knowledge in adoptive cell transfer technology. However, it is very expensive and laborious and would be interesting to consider other alternatives based on this tolerogenic phenomenon. Leichner et al. (2017) demonstrated that topical, but not systemic, application of a VitD analog increased the proportion and absolute number of Tregs and attenuated EAE clinical scores. The literature also points to other pathways and components that can boost this VitD & Foxp3 Treg intersection. Estrogen, for example, synergizes with VitD, promoting higher CD4+Helios+Foxp3+ Treg differentiation (Spanier et al., 2015). Interestingly, it was recently described that higher VitD levels were protective in females but not in C57BL/6 male mice. In contrast to this enhancing effect on Treg cell differentiation, VitD downregulates multiple signaling and metabolic pathways critical for T lymphocyte activation and further polarization towards Th1 and Th17 subsets (Nanduri et al., 2015; Zeitelhofer et al., 2017). In addition, downmodulation of proinflammatory cytokines and key chemokines and their specific receptors has been observed.

Table 2 | Preclinical studies with vitamin D supplementation

| Reference | Experimental model | Mechanism of VitD action |
|-----------|-------------------|--------------------------|
| Lemire and Archer, 1991; Cantorna et al., 1996 | SIL/J and B10.PL mice | VitD prevented the induction and blocked the progression of relapsing EAE. |
| Cantorna et al., 1998 | B10.PL mice | Increased the levels of IL-4 and TGF-β1 transcripts in LN and CNS. |
| Mattner et al., 2000 | Biozi AB/H mice | Reduced T cell proliferation and lower IFN-γ levels in popliteal LN. |
| Nashold et al., 2000 | B10.PL mice | Reduced the inflammatory infiltrates, demyelinated areas and axonal loss in brains and spinal cords. |
| Meehan and DeLuca, 2002 | VDR null and B10.PL mice | Decreased macrophage accumulation in the CNS. |
| Muthian et al., 2006 | C57BL/6 and SIL/J mice | VDR was necessary for preventing the onset of EAE development. |
| Spach et al., 2006 | C57BL/6 and SIL/J mice with a disrupted IL-10 or IL-10R gene | Inhibited JAK-STAT pathway in IL-12/IFN-γ axis leading to differentiation of neural antigen-specific T cells. |
| Chang et al., 2010 | BALB/c, C57BL/6 mice, Rag2−/−, DO11.10, MOG-TCR (2D2), Il-10−/− and Stat1−/− transgenic mice | Down-regulated CCR6 expression, inhibited the differentiation and migration of Th17 cells to the CNS. |
| Sokola et al., 2011 | C57BL/6 and STAT6−/− mice | Reduced the total infiltration of CD4+ T cells in the CNS. |
| Grishkan et al., 2013 | C57BL/6, CD45.1, B6(Cg)-Tyrc-2J/J and 2D2 mice | Increased the levels of GATA-3 and STAT6. |
| Li et al., 2013 | C57BL/6 mice | Reduced the oxidative stress and expression of NLRP3, caspase-1, IL-1β, CX3CR1, CCL17, CCL22 and CCR4. |
| Nashold et al., 2013 | B10.PL mice | Reduced the expression of inflammatory parameters, and demyelination at the CNS. |
| Mohammadi-Kordkhayli et al., 2015 | C57BL/6 mice | Down-modulated the expression of IL-27 and IL-33 in the CNS. |
| Sokola et al., 2015 | C57BL/6 mice | Reduced the axonal and neuronal loss. |
| Waddell et al., 2015 | C57BL/6 mice | Protection from EAE mediated by VitD was partially regulated by NKT cells. |
| Zhen et al., 2015 | C57BL/6 mice | Increased the proportion and absolute number of Tregs and attenuated EAE clinical scores. |
| Shirai et al., 2017 | C57BL/6 mice | Down-regulated the expression of some Th17 cell-related cytokines, key inflammatory chemokines, and chemokine receptors in the CNS (IL-17, IL-23 P19, IL-23 P40, CCL20, CCL22 and CCR4). |
| Jafarzadeh et al., 2019 | C57BL/6 mice | Down-regulated the expression of some Th17 cell-related cytokines, key inflammatory chemokines, and chemokine receptors in the CNS (IL-17, IL-23 P19, IL-23 P40, CCL20, CCL22 and CCR4). |
| de Oliveira et al., 2020 | C57BL/6 mice | Down-modulated IL-12 and TGF-β expression in the CNS and serum. |
| Gomez-Pinedo et al., 2020 | C57BL/6 and B6. Ly2-Cre+Cypr27b1f/f mice | Increased glucocorticoid efficacy via inhibition of mTORC1. |
| Spanier et al., 2020 | C57BL/6 mice | Reduced the oxidative stress and expression of NLRP3, caspase-1, IL-1β, CX3CR1, CCL17, RORc and Tbx21 in the CNS. |
| Mimura et al., 2021 | C57BL/6 mice | Reduced the expression of some Th17 cell-related cytokines, key inflammatory chemokines, and chemokine receptors in the CNS (IL-17, IL-23 P19, IL-23 P40, CCL20, CCL22 and CCR4). |

CCL: Chemokine ligand; CCR: chemokine receptor; CD: cluster of differentiation; CNS: central nervous system; CTLA: cytotoxic T-lymphocyte associated protein; EAE: experimental autoimmune encephalomyelitis; FoxP3: forkhead box P3; GR: glucocorticoid receptor; IFN: interferon; IL: interleukin; IAK: Janus kinase; LC3: microtubule-associated protein light chain 3; LN: lymph node; MOG: myelin oligodendrocyte glycoprotein; mTOR: mammalian target of rapamycin; NKT: natural killer T cell; NLRP: nucleotide-biding oligomerization domain, leucine rich repeat and pyrin domain containing; STAT: signal transducer and activator of transcription; TGF: transforming growth factor; Th: T helper cell; TLR: toll-like receptor; TNF: tumor necrosis factor; Treg: regulatory T cell; VDR: vitamin D receptor; VitD: vitamin D; WT: wild-type.
in VitD-treated EAE mice (Ahangar-Parvin et al., 2018; Jafarzadeh et al., 2019). Other EAE protective mechanisms, such as enhanced DC lysis by NK cells (Al-Jaderi and Maghazachi, 2015) and release of IL-4 by NKT cells (Waddell et al., 2015), have been attributed to VitD.

One of the key questions being addressed by our research team refers to the potential of VitD to work as a tolerogenic adjuvant. Initially, we demonstrated that VitD associated with MOG was able to clinically control EAE development in both prophylactic and therapeutic scenarios. Lower production of encephalitogenic cytokines and inhibition of DC maturation in peripheral lymphoid organs and less inflammation at the CNS were concomitant with this tolerogenic effect (Chiuso-Minicucci et al., 2015; Mimura et al., 2016). Interestingly, this tolerogenic vaccination with MOG plus VitD was able to control paralysis even in mice whose EAE was aggravated by previous infection with Candida albicans (Fraga-Silva et al., 2016).

More recently, we reported that the association of MOG with a VitD analog (paricalcitol), applied by an epicutaneous route, was also effective in controlling EAE evolution. An enhanced proportion of Treg cells and a direct downmodulatory effect on microglial cell activation were associated with this protection (Zorzella-Pezavento et al., 2017). Among the putative benefits of VitD in the control of EAE is its ability to act directly on neural cells. In vitro and in vivo assays indicated that VitD was able to induce the proliferation of neural stem cells, enhance their differentiation into neurons and oligodendrocytes and protect neurons from killing by T cells (Gu et al., 2015; Slioka et al., 2015; Shirazi et al., 2017).

When we tested the effectiveness of VitD administered at different stages of the disease to control paralysis, a clear picture emerged, indicating that the sooner the disease was treated, better was the result. Significant control of disease disability was observed when VitD was provided 1 or 7 days after disease induction, being the earlier even more efficient (de Oliveira et al., 2020; Mimura et al., 2021).

However, when therapy was postponed to 10 days postinfection, VitD lost its effectiveness (not shown). The protective outcome coincided with a stabilizing effect on BBB permeability, which is an interesting finding because a turning point in MS development is exactly BBB disruption and the subsequent transendothelial migration of activated leukocytes to the CNS. We verified that VitD stabilizing ability over the BBB is also crucially dependent upon the stage of the disease; only very precocious intervention with VitD can preserve its normal level of permeability (de Oliveira et al., 2020; Mimura et al., 2021). This competence of VitD to stabilize the BBB was already demonstrated in other scenarios, such as in vivo in a hypoxia/reoxygenation model (Won et al., 2015) and in vitro with human endothelial cells (Takahashi et al., 2017). Some of the immunomodulatory effects of VitD in experimental models of MS are depicted in Figure 2.

As the results concerning VitD were observed by its intraperitoneal administration, we recently tested its per-oral efficacy. We presumed that this approach could better mimic the physiological immunoregulatory role of this hormone and considered that this is a more friendly procedure that facilitates patient adherence to therapy. As illustrated in Figure 3, per-oral delivery of VitD was also able to control paralysis development when administered 1 or 7 days after disease induction. Interestingly, unlike previous findings that indicated high calcium levels in the blood after delivery of VitD by the intraperitoneal route (de Oliveira et al., 2020), the oral route determined only a nonsignificant increase in serum calcium levels. However, efficacy was again more pronounced when therapy was delivered earlier. Taken together, our results employing the experimental model indicated that VitD administration in the early phase is clearly more effective in reducing disease severity. The translation of these and of other authors’ findings to MS patients would require that their future disease development could be already predicted during the prodromal phase. Even though this still seems a challenging task, increasing evidence suggests that MS patients present various clinical manifestations, such as anxiety, depression, migraine, and lower cognitive performance, several years before diagnosis (Giovannoni, 2017; Disanto et al., 2018). According to those authors, the identification of a prodromal syndrome in MS can positively impact patient management.

Conclusion

Considering the aspects reviewed above, we consider that the potential use of VitD as an adjunct therapy for MS deserves to be investigated. Based on our experience with the EAE model and the plethora of data from other authors in both the experimental model and the clinical trials, we believe that better results could be obtained by a more precocious administration of this hormone. We certainly agree with other authors that emphasize the relevance of nontoxic doses, duration of the therapy and the maintenance of classical treatments during VitD supplementation.

Acknowledgments: The authors thank the Brazilian site for fCiências - Ciência e Tecnologia for providing a free image bank that was used in the assembly of Figures 1 and 2.

Author contributions: All authors contributed to the content of the review and approved the final manuscript.
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