

INTRODUCTION: VITAMIN D

Vitamin D is a lipid soluble hormone that acts on numerous tissues within the body. Technically speaking it is not a vitamin, but a prohormone; however, it is still commonly referred to as a vitamin. Vitamin D is generally absent from the food supply with little in both plants and animals (Deluca, 2004). Most of the vitamin D in the body comes from a photolytic process in the skin using cholesterol derivatives. Vitamin D is produced by both the epidermis and by absorption in the intestines from primarily fortified dairy products. There are two major forms of vitamin D. Vitamin D₂, also called ergocalciferol, is formed by UV irradiation of ergosterol in plants (Bikle, 2007). Vitamin D₃, or cholecalciferol, is more potent and produced by the UV irradiation of 7-dehydrocholesterol in the skin of animals (Bikle, 2007). The major difference between the two forms is the presence of a double bond between C22 and C23 and a methyl group on C24 for vitamin D₂ (Figure 1) (Bikle, 2007).

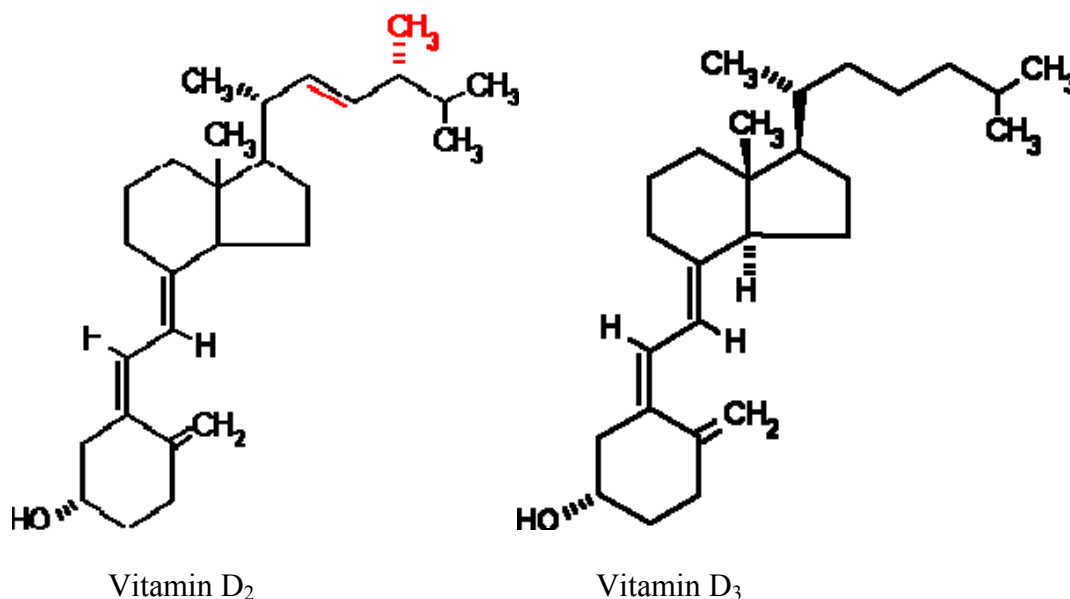


Figure 1: Structure of vitamin D₂ and vitamin D₃. (Stryer, 1995)

The metabolism of vitamin D₃ converts it into a biologically active hormone (Figure 2). Both liver and kidney metabolism is required for this conversion. First, 7-dehydrocholesterol is photolyzed by UV radiation to form pre-vitamin D₃. Pre-vitamin D₃ spontaneously isomerizes into vitamin D₃. Next, in the liver vitamin D₃ is hydroxylated to 25-hydroxyvitamin D₃ (25-(OH)D₃). 25-(OH)D₃ is the most prominent form of vitamin D₃ found in the blood (Bikle, 2007). In the kidney, a second hydroxylation forms 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) or 24,25-dihydroxyvitamin D₃ (24,25-(OH)₂D₃) in a strictly regulated reaction that depends on the conditions (Malluche *et al.*, 2007). 1,25-(OH)₂D₃ production is increased under low calcium, phosphate and the presence of parathyroid hormone (Barthel *et al.*, 2007). High calcium and phosphate increases production of the other metabolite 24,25-(OH)₂D₃. The enzyme responsible for the second hydroxylation has been found in other tissues besides the kidneys including the intestines, epidermis, macrophages, prostate, pancreas, breast, and the parathyroid gland (Reichrath *et al.*, 2007).

The biological effects of vitamin D₃ are generally produced by genomic mechanisms in which vitamin D₃ acts on a variety of tissues that contain the vitamin D receptor (VDR) (Reichrath *et al.*, 2007). However, only the active metabolite 1,25-(OH)₂D₃ is able to bind this nuclear receptor, which acts as a transcription factor (Barthel *et al.*, 2007). Almost all organs have been shown to contain VDRs that bind 1,25-(OH)₂D₃. This causes a conformational change that allows VDR to partner with a retinoic acid receptor (RXR). This complex then binds to vitamin D response elements (VDRE's) in the nuclear DNA, as well as coactivators that link VDRE's to transcription start sites (Bikle, 2007). Thus, vitamin D₃ acts genomically by changing gene transcription within cells. The VDR controls the transcription of at least 50 known genes responsible for multiple actions depending on the tissue, including cell cycle arrest, differentiation, apoptosis and anti-angiogenesis (Bikle, 2007).

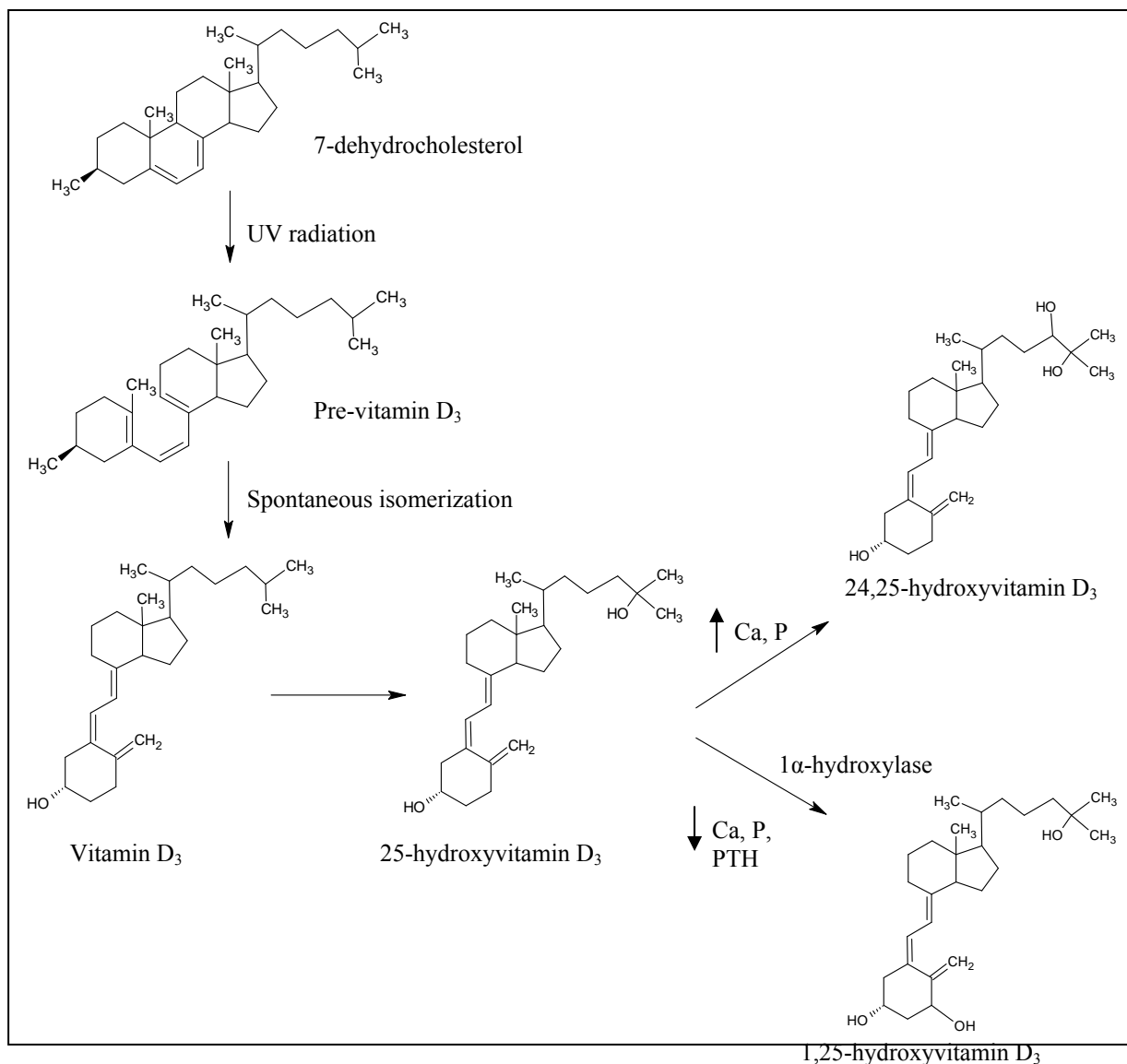


Figure 2: Major pathway for the metabolism of vitamin D₃ within the body (modified from Bouillon *et al.*, 1995).

The major role of vitamin D₃ is in calcium homeostasis and bone metabolism. In bone metabolism vitamin D₃ together with parathyroid hormone increases calcium re-absorption by activating the proteins involved in calcium absorption (Deluca *et al.*, 2001). Due to these activated proteins calcium and phosphorus concentrations increase in the plasma providing conditions necessary to support bone mineralization (Deluca *et al.*, 2001). During periods of little dietary calcium intake, vitamin D₃ aids in mobilizing calcium by interacting with osteoblasts

(DeLuca, 2004). These osteoblasts induce receptor activator nuclear factor- κ B ligand, which further activates osteoclastogenesis and causes bone resorption (DeLuca, 2004). The major disease caused by vitamin D deficiency is Rickets. Children with this disease have softened bones sometimes leading to deformation (DeLuca, 2004). In the early twentieth century researchers learned about the advantages of vitamin D₃ supplementation in patients with rickets, nearly eliminating the disease from non-developing countries.

Other physiological roles of active vitamin D₃ include growth and differentiation of cells in multiple tissues (Reichrath *et al.*, 2007). These include prostate, keratinocytes and breast cells. In keratinocytes, low concentrations of 1,25-(OH)₂D₃ have been shown to stimulate cell proliferation *in vitro*, while inhibition occurs after high concentration supplementation (Gniadecki, 1996). This is accomplished by altering the transcription of genes involved in proliferation. In prostate tissue it was found that the addition of 1,25-(OH)₂D₃ to prostate led to the inhibition of proliferation and invasiveness in both animal and cell-based studies of the disease (Bonjour *et al.*, 2007). This control of proliferation has led researchers to believe that vitamin D₃ may act as an anti-cancer agent.

The role of vitamin D₃ in immune modulation has become an emerging topic in recent years. This has been particularly as a result of finding that immune cells contain both the hydroxylase enzyme responsible for activating 25-(OH)D₃ to 1,25-(OH)₂D₃, as well as the VDR receptor (Reichrath *et al.*, 2007; Cantorna, 2006). Vitamin D₃ has been found to inhibit maturation of dendrites, suppress stimulation of major histocompatibility complex II (MHC-II) molecules (Reichrath *et al.*, 2007), and regulate the proliferation and differentiation of B cells (Chen *et al.*, 2007). In addition, vitamin D₃ has been found to affect immune function by increasing the production of regulatory-T cells and decreasing the activation of T_H1-cells allowing for the maintenance of a crucial T-cell balance (Cantorna, 2006). Over-activation of

T_H1 cells has been implicated in autoimmune disease, so consequently much research has gone into the effects of vitamin D₃ deficiency on autoimmune diseases (Cantorna, 2006). Multiple sclerosis is one such disease.

VITAMIN D₃ AND MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (Lassmann *et al.*, 2007). It is a complex neurodegenerative disease for which the cause and pathogenesis remain largely unknown. There are three types of MS: relapsing-remitting, primary progressive, and secondary progressive. Relapsing-remitting is the most common type of MS, and is often followed after several years by secondary progressive MS (Lassmann *et al.*, 2007). Symptoms of MS include spasticity, weakness, fatigue, cognitive dysfunction, depression, bladder dysfunction, bowel dysfunction, sexual dysfunction, and pain (Crayton and Rossman, 2006). Severity of symptoms varies widely; some patients show only mild symptoms over many years, while others require a wheelchair within the first year of diagnosis (Gregory *et al.*, 2007). The disease is most commonly diagnosed in young adulthood and is two to three times more prevalent in females than males (Gregory *et al.*, 2007).

The pathology of MS is currently described as an inflammatory process, which is associated with demyelination of white matter in the brain and spinal cord causing lesions. However, more recent evidence suggests that it is more complex than this, as progressive MS involves neurodegeneration in some lesions that is independent of inflammation (Lassmann *et al.*, 2007). Most active lesions are accompanied by a disturbance of the blood brain barrier, localized expression of proinflammatory cytokines, chemokines, and their receptors, and recruitment of T cells, activated macrophages, and microglia (Lassmann *et al.*, 2007). While it is not known for

certain, it is believed that the inflammation observed in MS is the consequence of an autoimmune process (Noseworthy *et al.*, 2000).

It is hypothesized that both genetics and environmental factors play a role in the risk for developing MS. Two loci have been linked to increased risk of developing MS, human leukocyte antigen (HLA) (Smestad *et al.*, 2007) and interleukin 7 receptor α chain (IL7R) (Gregory *et al.*, 2007). Twin studies have shown that there is a 25.3% chance of both monozygote twins developing MS, while in dizygote twins the concordance is only 2.9% (Willer *et al.*, 2003). This implicates genetics in playing a role in MS risk, but not as the sole contributor.

Infection with Epstein-Barr virus (EBV) has also been found to increase risk of developing multiple sclerosis. Ascherio and Munch (2000) showed that the odds ratio of an EBV seropositive patient developing MS compared to an EBV seronegative patient was 13.5 (95% CI = 6.3-31.4). Alotaibi *et al.* (2004) found a similar trend in pediatric MS patients, with 83% showing evidence of EBV compared to 42% in the control population.

Another environmental factor that MS strongly correlates with is latitude and the duration and intensity of sunlight. Average annual hours of sunshine and average December solar radiation are both strongly and inversely correlated with MS (Ascherio and Munger, 2007). In addition, monozygote twin studies have shown that increased sun exposure during childhood activities results in protection against MS within the twin pair (Islam *et al.*, 2007). Why this correlation with sunlight exists is uncertain; however, the most widely accepted hypothesis is that it is linked to the amount of vitamin D₃ circulating in the body (Ascherio and Munger, 2007).

There is a growing amount of evidence in the literature that vitamin D₃ can reduce both the risk and severity of MS. In a study performed in the United States, Munger *et al.* (2006) showed that increasing levels of 25-(OH)D₃ significantly reduced to the risk of MS. A similar study in Australia showed that while there was a high prevalence of vitamin D₃ deficiency in both

MS patients and controls, increased disability in MS patients was strongly correlated with reduced sun exposure and 25-(OH)D₃ in the blood (Van der Mei *et al.*, 2007). In a third study, Soilu-Hanninen *et al.* (2005) found that in relapsing-remitting MS patients 25-(OH)D₃ levels were lower during times of relapses than during periods of remission.

It has also been noted that there is a significant difference in the frequency of VDR polymorphisms in MS patients. Two VDR alleles (named Taq and Apa variants after the restriction enzymes used to identify them) were found to be in strong and significant linkage disequilibrium in MS patients (Tajouri *et al.*, 2005). The disequilibrium was most apparent in primary progressive MS patients. It was suggested by the authors that these polymorphisms could affect the affinity of vitamin D₃ for its receptor, thus altering its activity within the immune system.

Based on the hypothesis that MS is an autoimmune disease, the research supporting the role of vitamin D₃ in immune system modulation, and the evidence correlating vitamin D₃ deficiency with MS, it has been predicted that vitamin D₃ supplementation could be used as a treatment to decrease both the risk of developing MS and the severity of its symptoms. Current research is now focusing on the mechanisms by which vitamin D₃ reduces the inflammation associated with the development and increase severity of the disease. The following two studies by Pedersen *et al.* (2007) and Cohen-Lahav *et al.* (2007) investigated the pathways involved in the anti-inflammatory action of the activated vitamin D₃ metabolite 1,25-(OH)₂D₃ using an animal-model and cell-based study respectively.

HOW DOES VITAMIN D₃ REDUCE INFLAMMATION IN NEURODEGENERATIVE DISEASE?

Animal Study (Pederson et al, 2007)

Experimental Autoimmune Encephalomyelitis (EAE) Mouse Model of MS: Pederson et al. (2007) explored the mechanisms by which 1,25-(OH)₂D₃ reduced inflammation in the CNS using the EAE mouse model for MS. EAE is one of the most commonly used models of immune disease (Baxter, 2007). It is induced by injecting the mouse with brain-specific antigens mixed with dead bacteria, which results in the creation of antibodies that specifically target the CNS causing demyelination and inflammation (Baxter, 2007). CD4⁺ T_H1 myelin-specific T cells and macrophages are the most prominent immune cells found in the resulting lesions and are therefore implicated in initiating the disease (Friese et al., 2006). Several different models exist that differ based on the CNS antigen utilized. In MS models, the most commonly used antigens are myelin basic protein (MBP), proteolipoprotein (PLP), myelin oligodendrocyte glycoprotein (MOG), myelin associated glycoprotein (MAG), and S-100 protein (Sriram and Steiner, 2005). In this study EAE was induced with pertussis toxin mixed with MBP isolated from guinea pig spinal cord.

Experimental Design: It was hypothesized that 1,25(OH)₂D₃ reduced inflammation by inhibiting chemokine synthesis, nitric oxide (NO) synthesis, and inflammatory cell recruitment. To test this hypothesis, mice with induced EAE were injected with 200ng of 1,25-(OH)₂D₃, as well as fed a diet supplemented with 100ng/day of 1,25-(OH)₂D₃. The authors used PCR, RNase protection assays, and immunostaining to quantify the amount of chemokines, inducible nitric oxide synthase (iNOS), and arginase transcripts present at various time points from spinal cord samples. In addition, monocyte recruitment and amount of CD4⁺ T cell apoptosis was measured. All results were compared to those of a control group not given 1,25-(OH)₂D₃.

Results: Within 48 hours of injection with 1,25-(OH)₂D₃, a decline in clinical disease could be observed in the EAE/1,25-(OH)₂D₃ mice. This decline was found to be preceded by a reduction of chemokines in CNS lesions. Chemokines are molecules responsible for the recruitment of inflammatory cells. Six hours post-treatment EAE/placebo and EAE/1,25-(OH)₂D₃ mice had comparable levels of chemokines present in spinal cord RNA samples. At 18 hours post-treatment levels of CXCL10, CCL2, and CCL3 had begun to decrease in the EAE/1,25-(OH)₂D₃ mice, and by 24 hours post-treatment were 85% lower (P<0.02), 80% lower (P<0.02) and 88% lower (P<0.001) respectively compared to the EAE/placebo group. In addition the amount of CCL4 and CXCL2 was also reduced. CXCL10, CCL2, CCL3 are believed to play important roles in attracting T cells and macrophages to EAE and MS lesions, while CXCL2 attracts neutrophils. Similar results were not observed in cultured monocytes from EAE mice treated with 1,25-(OH)₂D₃. This indicated that 1,25-(OH)₂D₃ did not have a direct effect on chemokine synthesis and must therefore rely on other CNS cells for its anti-inflammatory action.

Another factor that plays a role in immune cell recruitment is the permeability of the blood brain barrier. In cases of MS and EAE, the blood brain barrier is believed to be more permeable to inflammatory cells than in normal individuals due to increased nitric oxide (NO) production. The enzyme responsible for NO production is iNOS. At 6 hours post-treatment with 1,25-(OH)₂D₃, the amount of iNOS was equivalent in EAE/placebo and EAE/1,25-(OH)₂D₃ mice. However, at 24 hours post-treatment iNOS concentrations had decreased by 87% (P<0.01) in EAE/1,25-(OH)₂D₃ mice, while no change was observed in the EAE/placebo mice.

Monocyte recruitment to CNS lesions was also found to decrease in EAE/1,25-(OH)₂D₃ mice. This was predicted to be a result of the reduced amount of chemokines and iNOS in the CNS. After 24 hours the EAE/1,25-(OH)₂D₃ mice had 92% (P<0.01) fewer CFSE⁺CD11b⁺

monocytes in the spinal cord and 30% fewer ($P < 0.05$) in the spleen compared to EAE/placebo mice. These reduced levels were comparable to those observed in healthy control mice.

$CD4^+$ T_H1 cells levels were also measured and found to decrease prior to chemokine and iNOS reduction. Therefore, it was hypothesized that $CD4^+$ T_H1 cells were destroyed by inducing apoptosis. At 7 hours post-treatment it was found that the number of apoptotic $CD4^+$ T_H1 cells was 2-fold higher in EAE/1,25-(OH) $_2$ D $_3$ mice compared to EAE/placebo mice and by 18 hours post-treatment the number of $CD4^+$ T_H1 cells present had reduced by 63% ($P < 0.05$). IFN- γ levels were also found to decrease by 52%, confirming the loss of IFN- γ -producing $CD4^+$ T_H1 cells.

One method to induce apoptosis is by the production of arginase. Arginase is an enzyme that depletes L-arginine in the cell. Arginine starvation results in the production of peroxynitrite by iNOS, which is toxic to the T cell signaling for apoptosis. At 6 hours post-treatment arginase transcripts had increased 2.5-fold in the EAE/1,25-(OH) $_2$ D $_3$ mice compared to the EAE/placebos. Therefore, it was predicted that this could be the mechanism by which $CD4^+$ T_H1 cell apoptosis was induced.

Critical Analysis of Results: The results of this paper suggest that the anti-inflammatory action of 1,25-(OH) $_2$ D $_3$ results from stimulating a two-part anti-inflammatory pathway (Figure 3). In an early response, T cells were sensitized to apoptosis signals, which were hypothesized to be driven by arginine starvation and peroxynitrite formation. These events were later followed by a decrease in chemokines and iNOS, which reduced monocyte recruitment to the CNS lesions. These results provide valuable insight into how vitamin D $_3$ reduces inflammation in the CNS. However, pieces of the puzzle are still missing. For example, it was shown that chemokine levels in cultured monocytes from EAE spinal cords did not decrease in the presence of 1,25-(OH) $_2$ D $_3$. Therefore, it is believed that other CNS-cells play an important role in 1,25-(OH) $_2$ D $_3$ -mediated reduction of inflammation; however, these cells and their roles have yet to be identified.

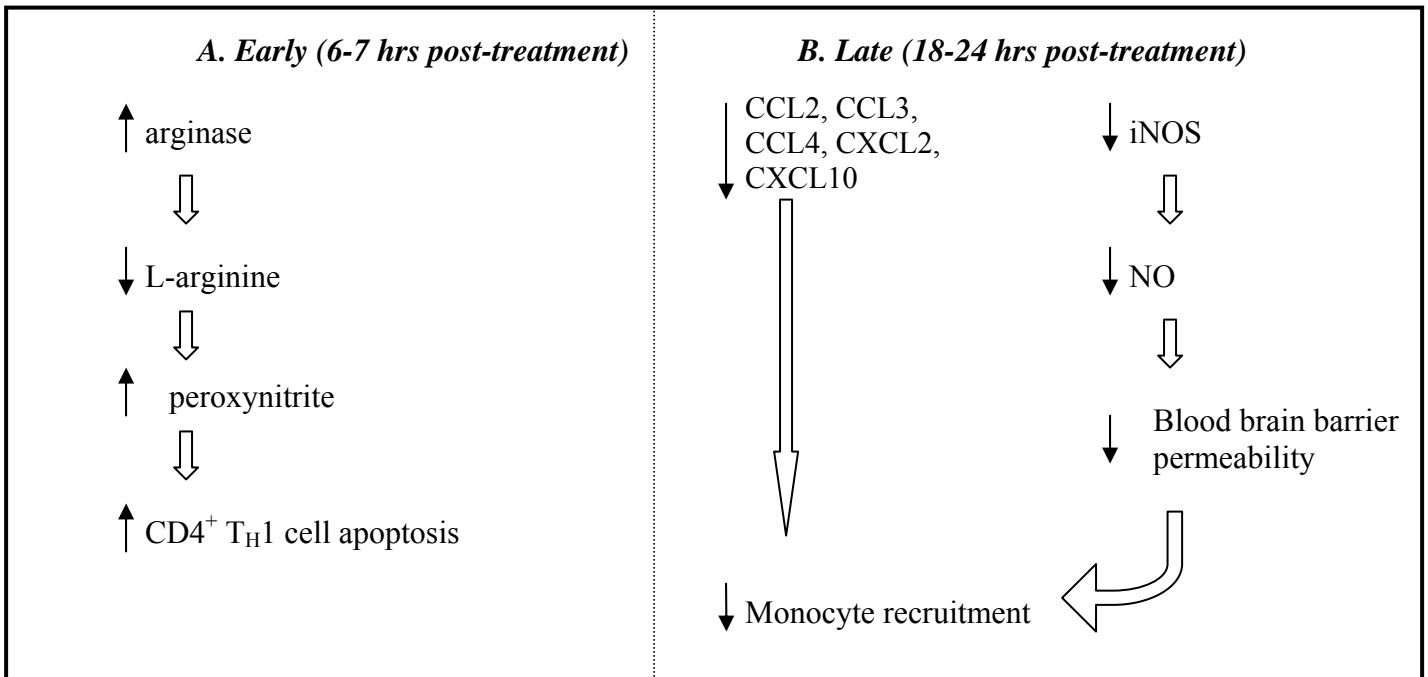


Figure 3: Hypothesized anti-inflammatory pathways observed in the CNS of EAE mice treated with 1,25-(OH)₂D₃.

This research could have implications in treating other autoimmune inflammatory diseases such as diabetes, arthritis, and inflammatory bowel syndrome. In fact, similar results were observed in the NOD mouse model for diabetes. Decallonne and Mathieu (2003) reported that treated of NOD mice with 1,25-(OH)₂D₃ increased apoptosis of T cells in the pancreas. Treatment with 1,25-(OH)₂D₃ was also shown to decrease CCL2, CCL5, and CXCL10 expression in pancreatic β-cells of NOD mice leading to reduced T cell recruitment (Giarratana *et al.*, 2004). The effect of this chemokine reduction on monocyte recruitment was not investigated. When treated with 1,25-(OH)₂D₃ within the first 14 weeks of life, diabetes could be prevented in the NOD mice (Giarratana *et al.*, 2004).

The results obtained using the EAE model strongly support the claim that vitamin D₃ supplementation can decrease the severity of multiple sclerosis by reducing CNS inflammation, and are supported by similar results obtained for 1,25(OH)₂D₃-mediated anti-inflammatory action

in the NOD model for diabetes. However, one must take a critical look at the experimental design and question whether similar results would be observed in humans. There is much controversy existing in the literature as to whether or not EAE is a valid model for MS, particularly when it comes to investigating therapeutic treatments.

There are several key differences between EAE and MS (Sriram and Steiner, 2005). Firstly, in EAE lesions are found predominately in the lumbar spinal cord, while in MS there are more lesions in the brain stem, optic nerves, periventricular area, cortical mantle and upper cervical cord. Secondly, in MS demyelination has been observed in the absence of inflammation, which has not been seen in EAE. Thirdly, CD4⁺ T cells and macrophages are the predominant immune cells in EAE cellular infiltrate, while in MS CD8⁺ T cells and macrophages predominate. Fourthly, EAE must be induced, while MS is a spontaneous disease. Finally, antibodies to myelin antigens are present in cerebral spinal fluid of mice with EAE, while in MS such antibodies are infrequent (Sriram and Steiner, 2005).

In addition, while over one hundred potential therapies for MS have been identified and proven effective in EAE models (Sriram and Steiner, 2005), only three of these therapies (glatiramer acetate, mixoxantrone and natalizumab) have been approved by the FDA as safe and effective for treating MS (Baxter, 2007). Natalizumab was later pulled off the market due to a rare but severe side effect called progressive multifocal leukoencephalopathy, an opportunistic infection of the CNS (Friese *et al.*, 2006). Therefore, it would appear that the pathology of EAE and MS are not as similar as once thought. While some researchers argue that EAE should not be used as an experimental model for MS (Sriram and Steiner, 2005), the majority of scientific community recognize it as a useful model for preliminary studies when appropriate critical analysis is utilized to identify the limitations (Baxter, 2007; Steinman and Zamvil, 2006; Friese *et al.*, 2006; Gold *et al.*, 2006). It is proposed that the model is most useful when used to further

investigate observations made in studies of humans with MS (Steinman and Zamvil, 2006). Such is the case in this paper, in which the EAE model was used to follow up on the observation that 1,25-(OH)₂D₃ reduced the severity of clinical symptoms in patients with MS.

Recent attempts to “humanize” the EAE model have resulted in improving it to create a more MS-like pathology. Transgenic mice have been created to include human MHC-II, as well as the T-cell receptor from a MS patient’s myelin-specific T_H1 clone (Gregersen *et al.*, 2004). These mice show more lesions in the brainstem compared to the traditional EAE model, as well as have a 4% incidence of spontaneous disease. In addition, other variations have been made to the EAE model to produce versions for modeling optic neuritis, relapsing-remitting MS, and progressive MS (Steinman and Zamvil, 2006). It has also been predicted that the use of human haematopoietic stem cells to reconstitute the immune system of mice may improve the model and its usefulness in identifying therapeutic treatments for MS (Friese *et al.*, 2006). The authors of this paper should consider using some of these newer models to confirm their results, as well as to determine whether 1,25-(OH)₂D₃ has the same effects in reducing inflammation in both relapsing-remitting and progressive types of models. There is also the need to create a model that better represents the high recruitment of CD8⁺ cells to MS lesions (Gold *et al.*, 2006), to support the proposed mechanism by which vitamin D₃ reduces inflammation in human patients.

Despite the drawbacks of using EAE as a model, this research does make a significant contribution to the literature in suggesting how 1,25-(OH)₂D₃ reduces inflammation. The fact that vitamin D deficiency has been linked to increased risk and severity of MS in patients supports that similar results may be observed in humans. Further investigation is now required to determine how chemokine and iNOS synthesis is reduced and to confirm the mechanism by which apoptosis is induced. In the next study presented, the authors determined that 1,25-(OH)₂D₃ initiated a pathway in macrophages that resulted in a decrease of NFκB translocation to

the nucleus (Cohen-Lahav *et al.*, 2007). NF κ B is a transcription factor that plays an important role in initiating the transcription of inflammatory genes including chemokines and iNOS, as well as inhibiting apoptosis (Escarcega *et al.*, 2007). Therefore a decrease in NF κ B could be used to explain the results observed in this study.

In Vitro Cell-Based Study (Cohen-Lahav et al., 2007)

TNF α produces a pro-inflammatory cascade when activated. Previously, Cohen-Lahav *et al.* (2001) found a down-regulation of TNF α mRNA and protein after the addition of 1,25-(OH) $_2$ D $_3$ or its analogue 1,24-dihydroxyvitamin D $_2$ (1,24-(OH) $_2$ D $_2$) to macrophages resulting from a decrease in NF κ B activity. The following paper investigated the processes leading to reduced NF κ B activity in the macrophage cell line P388D1.

Macrophages as a Model for the Anti-inflammatory Action of Vitamin D: Macrophages are important for the initiation and propagation of the immune response. They produce numerous activated molecules including tumour necrosis factor (TNF α), which act to modulate the immune system (Hakim and Bar-Shavit, 2003). In macrophages, TNF α is dependent upon the transcription factor NF κ B (Figure 4). For NF κ B to be activated I κ B must dissociate from it. After dissociation, I κ B proteins undergo a three step process of degradation where they are phosphorylated, ubiquitinated and finally degraded by a proteasome. This normally prevents its accumulation and inhibition. In order to activate TNF α chemically, lipopolysaccharide (LPS) can be added. LPS causes protein phosphorylation leading to increased TNF α production. Over-activated TNF α is found in a number of inflammatory diseases; therefore, a treatment to decrease TNF α could provide a strong anti-inflammatory therapeutic.

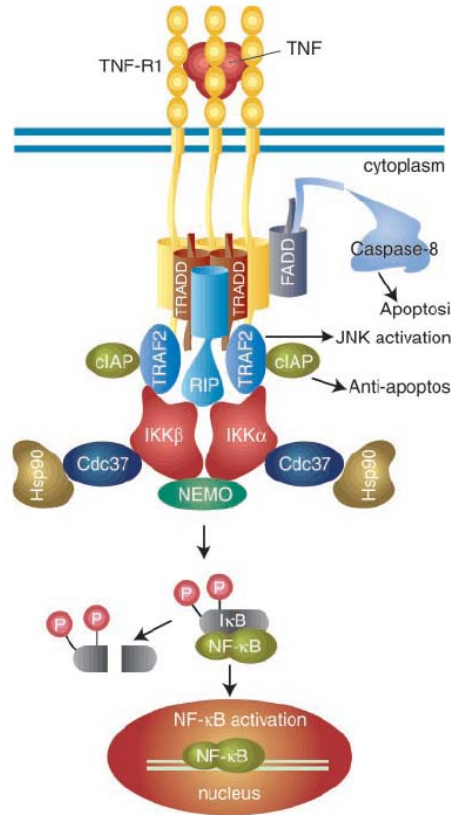


Figure 4: Tumour necrosis factor pathways in macrophages (Chen and Goeddel, 2002).

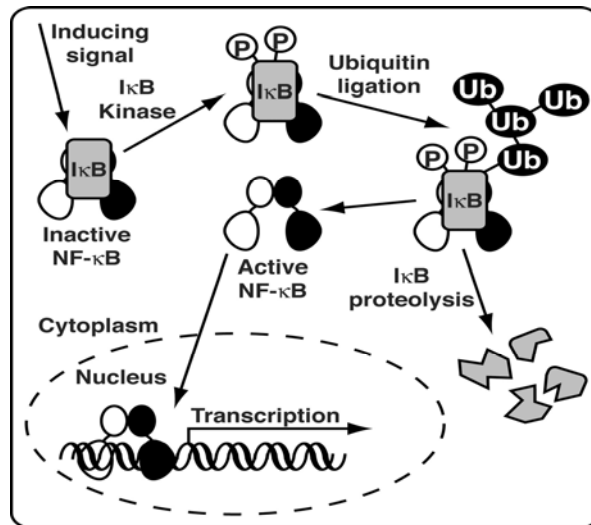


Figure 5: IκB degradation and activation of NFκB.(Ghosh et al., 1999)

Experimental Design: To test the mechanism of reduced NFκB activity that causes decreased TNFα, the levels of IκBα were analyzed. To determine the amount of IκBα after vitamin D₃ treatment, P388D1 cells were treated with either 1,25-(OH)₂D₃ or the analogue 1,24-(OH)₂D₂. Next, a known TNFα inducer LPS was added to the cells. The protein levels of IκBα were measured using Western Blot analysis. IκBα mRNA quantities were determined by RNA extraction using the RNeasy Mini Kit and RT-real time PCR amplifications with specific primers.

The translocation of NFκB in macrophages after 1,25-(OH)₂D₃/1,24-(OH)₂D₂ treatment was determined by collecting cytoplasmic, whole-cell, and nuclear extracts. Western blotting was used to analyze the protein levels in each extract. RT-PCR was used to determine the NFκB-p65 mRNA levels in support of the protein levels.

IκBα half-life after 1,25-(OH)₂D₃/1,24-(OH)₂D₂ treatment was analyzed by RT-PCR of P388D1 cells to which actinomycin D was added. Actinomycin D blocks RNA synthesis. Finally, the effect of treatment on IKK activity was examined by measuring the amount of phospho-IκBα produced using Western Blot analysis.

Results: After treatment with 1,25-(OH)₂D₃ or 1,24-(OH)₂D₂ IκBα protein levels increased approximately 4.5 times in comparison to LPS. Real time PCR showed an increase of 6.5 times for the IκBα mRNA levels. IκBα release and degradation is required for NFκB translocation and activation. Therefore, after supplement treatment the increase in IκBα was hypothesized to reduce the release and lower translocation of NFκB.

The upregulation of IκBα by vitamin D₃ and its analogue decreased the nuclear translocation of NFκB. In comparison to LPS, lower levels of NFκB were present in the nuclear extracts than cytoplasmic extracts as determined by Western Blot. Protein levels did not change in the total lysate. RT-PCR of NFκB-p65 mRNA confirmed protein levels did not change. This furthers the idea that the increase in IκBα protein and mRNA leads to more inhibition of NFκB

translocation and a decrease in TNF α levels. The authors found the decrease was not due to reduced NF κ B content because the supplements had no effect on the amount of total protein.

I κ B α -mRNA stability was increased by 1,25-(OH) $_2$ D $_3$ and its analogue as viewed by an increase in half-life from 110 to 190 minutes. Also, phospho-I κ B α was reduced after treatment. The upregulation of I κ B α was determined to be due to decreased phosphorylation of I κ B α protein because of lower IKK activity and greater stability of I κ B α mRNA. Therefore, I κ B α expression appears to be upregulated by at least two different methods.

Finally, the addition of 1,24-(OH) $_2$ D $_2$ as an analogue was tested and provided equivalent results to 1,25-(OH) $_2$ D $_3$. The analogue has increased benefits because it is not as hypercalcemic and therefore a better candidate to treat inflammatory diseases.

Critical Analysis of Results: The results of this paper provide evidence to support the claim that the active metabolite of vitamin D $_3$ and its analogue act in an anti-inflammatory fashion within macrophages. The metabolite acts by down-regulating NF κ B and decreasing TNF α . Cohen-Lahav *et al.* (2007) found that increased I κ B α -mRNA half-life and decreased phospho-I κ B α protein led to reduced NF κ B. However, the authors only identified two areas, increased mRNA stability and decreased phosphorylation of I κ B α , which led to TNF α inhibition. There may be other methods of modulation due to TNF α regulation via different pathways that should be analyzed. Largely, this research supports the claim that 1,25-(OH) $_2$ D $_3$ plays a role not only in bone homeostasis, but also macrophage regulation. In relation to the autoimmune disease MS, down-regulation of TNF α may lead to decreased inflammatory cascades and could potentially aid in the repression of MS symptoms or prevent the disease from developing.

The anti-inflammatory effects of 1,25-(OH) $_2$ D $_3$ on other cells, tissues and animals have been analyzed in the literature and there appears to be contradictory results. Hakim and Bar-Shavit (2003) found an increase in TNF α in bone marrow macrophages after supplementation

with 1,25-(OH)₂D₃. Their analysis suggested that TNF α was increased by a transcriptional mechanism, but provided no detailed hypotheses. Similar results were obtained by Hakim and Bar-Shavit (2003) using an *in vivo* mouse model. It appears that the cytokine modulation by 1,25(OH)₂D₃ differs even between similar cell types.

In a study by Guilietti *et al.* (2006) monocytes from diabetic patients were determined to have upregulated inflammatory cytokines including TNF α . Upon addition of 1,25-(OH)₂D₃ the relative amount of TNF α decreased drastically. One hypothesis for the differing responses to 1,25(OH)₂D₃ by various cells may be due to the maturation status of the cells (Hakim and Bar-Shavit, 2003). After treatment, immature cells including many bone marrow cells generally increase pro-inflammatory cytokine production. On the other hand, mature cells including peritoneal macrophages may experience a decrease in cytokine production. These contradictory results help to underline the importance of either producing a more specific analogue for therapeutic treatment and better understanding its action within multiple cells and tissues in the body.

Because the active vitamin D₃ metabolite has potential therapeutic benefits the authors also analyzed the action of its analogue 1,24-(OH)₂D₂. The use of an analogue for therapy is advantageous because 1,25-(OH)₂D₃ causes hypercalcemia at high concentrations (Peleg *et al.*, 2002). Hypercalcemia is caused when calcium intestinal absorption and mobilization is increased due to illness (or in this case a hormone) and can lead to a variety of problems within the body depending on severity. Calcium plays a major role in the functioning of the central nervous system. Symptoms of hypercalcemia include weakness, loss of muscle reflex, and decreased stamina (Edelson and Kleerekoper, 2006). Also, the heart does not function properly and increased calcium can lead to appetite loss, nausea, and vomiting along with other serious symptoms (Edelson and Kleerekoper, 2006). This has been one of the drawbacks of using

vitamin D₃ supplementation in MS patients since its use for anti-inflammatory purposes is likely to require unsafe, large doses. Therefore, finding less calcemic analogues is an attractive alternative to allow for safe use of this therapy.

In this study, one analogue was tested; however, there are other novel analogues that may provide similar or better results and should be considered. Daniel *et al.* (2005) tested the analogue 22-ene-25-oxa-vitamin D. They found it was also less calcemic and had similar effects as 1,25-(OH)₂D₃ when added to human peripheral blood mononuclear cells (PBMCs) (Daniel *et al.*, 2005). They tested regulation of TNF α , as well as interleukin-10 and IL-4 cytokines, and found a marked decrease in both TNF α and the interleukins after treatment (Daniel *et al.*, 2005). The vitamin D₃ analogues tested so far all show similar regulation of anti-inflammatory pathways; however, these constructs show no specificity to bodily tissues. Further investigation into the use of an analogue that is specific to a certain tissue within the body could be advantageous by having increased efficacy and fewer side effects.

Cohen-Lahav *et al.* (2007) utilized a cell-based study for their analyses. The use of cell-based studies is generally limited, but provides a good starting point for investigations. Cell-based studies take less time than whole animal or clinical trials and the supplement can be directly added to the cells. Also, direct analysis of the response is easily done because no other factors have changed. However, a potential problem is that cells isolated in tissue culture do not always behave the same as *in vivo*. More studies using *in vivo* models as demonstrated in the previous paper by Pederson *et al.* (2007) may provide more applicable results for use in therapy, particularly as the roles and effects on other cells and tissues are not ignored.

There were a few other problems specific to the methods of the paper. First the P388D1 cell line utilized is a macrophage-like cell line isolated from methylcholanthrene-induced lymphoid neoplasm of a DBA/2 mouse (Koren *et al.*, 1975). They have been experimentally

shown to possess most, if not all, the characteristics of macrophages (Koren *et al.*, 1975). The authors of this paper generalized macrophages as the equivalent to P388D1, which may not be the case. In a study by Overberg *et al.* (2000) both murine macrophages isolated directly from mice and the cell line P388D1 were investigated. They found similar data, but quantities were greatly reduced in the isolated macrophages indicating that the cell line may provide higher amounts of inflammatory products (Overberg *et al.*, 2000). These exaggerated results highlight the importance of *in vivo* testing. Macrophages in a human may behave differently and the affectivity of 1,25-(OH)₂D₃ or an analogue on these cells may be lessened.

Real-time PCR is used frequently to analyze the amount of mRNA produced. However, to be accountable this technique requires stringent optimization including normalization to a housekeeping gene (Guilietti *et al.*, 2001). It is often difficult to compare the samples accurately due to interassay variability, which becomes a major problem when carrying out multiple samples. While real-time PCR is often preferred because it is more sensitive than ELISA, if used improperly contradictory and incorrect results are easily obtained.

Despite the drawbacks in regards to methods and lack of variety, the authors were still able to find a strong positive correlation between NFκB inhibition and a decrease in TNFα in the cell line P388D1. This provides a basis for which future studies could expand on. Experiments revealed IκBα mRNA and protein levels were upregulated due to increased mRNA stability and reduced phosphorylation. This hindered NFκB translocation and protein distribution was shifted from the nuclear fraction to the cytoplasmic fraction. Also, the less calcemic 1,24-(OH)₂D₂ analogue was found to act similarly to 1,25-(OH)₂D₃ and could therefore be further investigated as a safer option than vitamin D₃ as treatment for MS. In the future different *in vitro* and *in vivo* analyses should be conducted to check the effects of 1,24-(OH)₂D₂ and 1,25-(OH)₂D₃ on

different tissues and cells within the body. Also, the testing of different analogues may provide more options towards therapeutic applications in the future.

SUMMARY OF IMPORTANT IMPLICATIONS OF STUDIES

The research shown in these two papers suggests a possible mechanism by which vitamin D₃ supplementation could lead to reduced inflammation. While the papers by Pedersen *et al.* (2007) and Cohen-Lahav *et al.* (2007) investigated different potential anti-inflammatory pathways, these two pathways complement each other and could be connected. Pedersen *et al.* (2007) determined that 1,25-(OH)₂D₃ increases apoptosis of T_H1 cells and suggested arginine starvation was responsible for the additional activation of apoptotic signals. They also found that chemokines and iNOS were down-regulated resulting in reduced monocyte recruitment. Cohen-Lahav *et al.* (2007) determined that 1,25-(OH)₂D₃ up-regulated IκBα, which prevented the translocation of NFκB from the cytoplasm to the nucleus. This resulted in decreased expression of TNFα. The possible connection between these two papers is that NFκB also plays an important role in initiating the transcription of inflammatory genes including chemokines and iNOS, as well as in inhibiting apoptosis. Therefore, the down-regulation of NFκB identified by Cohen-Lahav *et al.* (2007) could also be responsible for the observations made by Pedersen *et al.* (2007).

These two studies effectively show how two different methods (animal models and cell based studies) can be used to approach the same question. At the same time they demonstrate that no one method is optimal for displaying the entire picture. When different studies and experimental approaches are put together they can complement each other and overcome the disadvantages of using one method alone. The more research that is done in regards to the anti-

inflammatory actions of vitamin D₃, the better understanding investigators will gain on how this mechanism occurs.

The findings of these studies support the hypothesis that vitamin D₃ supplementation reduces the risk and severity of MS by showing that 1,25-(OH)₂D₃ decreases inflammation. While it is not known for certain whether the anti-inflammatory pathways observed in the EAE animal model or in tissue culture after treatment with 1,25-(OH)₂D₃ are the same responses that would be observed in human patients, the results do support a possible mechanism to explain the inverse correlation between vitamin D₃ and risk and severity of MS. Based on the widely accepted hypothesis that MS is an autoimmune disease caused by an over-active immune response, it is logical that compounds that down-regulate the immune system and inflammation could decrease risk of its development and severity of symptoms. It is concluded that the use of vitamin D₃ supplementation for treatment and prevention of MS holds great potential and that further research should be pursued in this area.

As it appears that vitamin D₃ acts on the autoimmune aspect of MS, this research could also have important applications in treating other autoimmune diseases. Vitamin D₃ supplementation has been correlated with a decreased risk of developing rheumatoid arthritis (Cutolo *et al.*, 2007), inflammatory bowel disease (Cantorna, 2006), and type-1 diabetes (Hyponen *et al.*, 2001). It has also been shown to reduce pro-inflammatory cytokines and increase anti-inflammatory cytokines in chronic inflammation found in cardiovascular disease (Schwalfenberg, 2007). *In vivo* mice models for diabetes and inflammatory bowel disease have correlated increased vitamin D₃ with reduced inflammation associated with these diseases and their symptoms (Gysemans *et al.*, 2005; Cantorna, 2006). In addition, studies have shown a correlation between polymorphisms in the enzyme that activates vitamin D₃ and susceptibility to type-1 diabetes (Baily *et al.*, 2007).

In conclusion, vitamin D₃ is believed to decrease the risk and severity of MS by decreasing or inhibiting the inflammation associated with the disease. It is proposed that vitamin D₃ supplementation could be used as prevention and treatment of MS; however, further research is still required to determine if the anti-inflammatory pathways activated in EAE mice and cultured macrophages are similar in humans and to find analogs that are safer to use in high doses.

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