VITAMIN D IN CHRONIC INFLAMMATORY AND AUTOIMMUNE DISEASES AND VITAMIN D SUPPLEMENTATION: RECONCILING CONTRADICTORY LITERATURE

Sergio Ballesteros, B.Eng. (October the 3rd, 2015); serbaso@alumni.uv.es
VP of the NPO MERCURIADOS (national Spanish association of patients injured by dental amalgam mercury and by mercury from other sources);
Owner/administrator of the ME/CFS-research forum: www.sfc-em-investigacion.com

DISCLAIMER: *I have no official credentials (i.e. university degrees in science) that would confer on me the recognized scientific expertise to elaborate on and interpret scientific articles. It follows that any conclusions I might have reached do not have validity unless confirmed by someone with appropriate scientific expertise. Lastly, as English is not my first language and the current text has not been reviewed by a qualified translator, I take no responsibility for any misunderstandings or misinterpretations that might arise from my grammatical errors. This text must therefore be viewed as a layman's interpretation of the sources that have been noted.*

ABSTRACT

Low levels of the pre-hormone 25-hydroxyvitamin D (VD25) are normally shown in many chronic inflammatory diseases, and many studies show symptomatic improvements, lower rates of autoimmune diseases and fewer inflammatory markers, from taking vitamin D supplements. This has led many physicians and governments to argue that low levels of VD25 lie at the core of the pathogeneses of many conditions, and therefore vitamin supplementation is broadly recommended. However, some physicians and researchers defend the so called "alternate hypothesis", which describes the low levels of vitamin D as a consequence of the chronic inflammation, instead of the cause. If the proponents of the alternate theory were right, vitamin D supplementation could be really dangerous; therefore, it is of imperative importance to review the available evidence and draw a solid conclusion on the matter.

One of the most important functions of vitamin D is exerted on the immune system. In this regard, when certain Toll-Like-Receptors (TLR) are activated, mainly on monocytes and macrophages, the circulating VD25 is converted into the active form 1,25-dihydroxycholecalciferol (VD1,25) by the cells, which in turn induces the expression of antimicrobial peptides (AMPs) by binding to the nuclear vitamin D receptor (VDR). These peptides (mainly cathelicidins and beta defensins) constitute a major component of the innate immune system, showing activity against bacteria, fungi and viruses.

While the most accepted effect of vitamin D on the immune system is to enhance the innate immune response and to inhibit the acquired immunity, *in vitro* essays as well as interventional studies show contradictory results. In this vein, VD1,25 has demonstrated to block the conversion of Th1- and Th17-cytokines while promoting the Th2-immune response and the formation of Treg-cells. VD1,25 has also shown to inhibit the maturation and proliferation of dendritic cells (DC), to halter the antigen-presentation capacity of DC and other antigen-presenting-cells, and to promote the anti-inflammatory and tolerogenic phenotypes of macrophages and DC. Furthermore, it has shown to induce hyporesponsivity in T cells and in peripheral blood mononuclear cells (PBMC), and to inhibit antibody secretion and autoantibody production by B-lymphocytes. Moreover, and in respect to the innate immunity, it has been reported that VD1,25 inhibits TLR2, TL4 and TLR9 as well as NK-cells.

There is a significant paradox, in that vitamin D is necessary for an efficient innate response against numerous intracellular pathogens, but at the same time, it has shown to impair both the innate and the Th1-mediated immune responses. In this vein, some of these discrepancies shown in the literature could be explained by the different doses of VD1,25 or VD25 used in the experiments. Thus, the studies using supraphysiological doses are those which show the most immunosuppressant effects of VD1,25, while

some studies using physiological doses report, for example, that VD1,25 does not impede the Th1-mediated response or that the TLR and NK-cells are not inhibited by VD1,25 when lower doses are used. In any way, it seems clear after reviewing some of the literature, that the high VD1,25 serum levels found in patients with many chronic inflammatory and autoimmune conditions, can actually suppress significantly the immune system. In this regard, the anti-inflammatory properties and potency of VD1,25 (in considered physiological doses) and dexamethasone have been shown to be quite similar. Likewise, the inhibitory effect of VD1,25 on DC was shown to be very similar to the effect of glucocorticoids. In anyway, the effects of vitamin D supplementation observed in interventional studies are very useful in elucidating the effects of vitamin D on the immune system. In this regard, vitamin D supplementation in patients with multiple sclerosis, confirmed the promotion of tolerogenic DC, which in turn induced regulatory T cells and produced a shift toward T-helper-type 2 response. In this vein, PBMC responsiveness to disease-associated antigens was significantly reduced, while the signs and symptoms of the disease improved. Likewise, supplementation of vitamin D in patients with cystic fibrosis showed similar results.

Finally, at the end of this article, the "alternate theory" is described in detailed. This model proposes that, in chronic inflammatory and autoimmune diseases, intracellular microbes invade nucleated cells, inhibiting the VDR. This leads to high levels of VD1,25, low levels of VD25 and low-grade chronic inflammation and autoimmune processes produced by cross-reactivity, what would explain the symptoms of these diseases. When these conditions take place, AMPs cannot be properly expressed, what renders the immune system unable to eradicate the perpetuated infections. Thus, in this proposed-disease-model, extra vitamin D supplementation would be really harmful, as it can displace the VD1,25 from the VDR, blocking this receptor even more. In addition, vitamin D supplementation can further hamper AMPs production, what inhibits the innate response towards the intracellular pathogens even further.

If the alternate theory is accurate, the vitamin D supplementation in chronic inflammatory and autoimmune diseases can be detrimental and dangerous, as it would allow the intracellular pathogens to spread, and the chronic situation would become even worse. Paradoxically, this can lead to transient symptomatic relief, in a way similar to that of many anti-inflammatory drugs.

Is the alternate theory supported by evidence? After a thorough review, we could conclude that it is. In this respect, low levels of VD25 are clearly associated with a wide variety of conditions; similarly, in many of these diseases the VD1,25 have been found to be elevated. In a similar vein, various pathogens have shown to down-regulate the VDR (including mycobacterium tuberculosis, mycobacterium leprae, aspergillus fumigatus, Epstein–Barr virus, HIV and borrelia burgdorferi). Finally, low levels of AMPs have been reported in some autoimmune conditions.

In summary, after an exhaustive review of the literature, we can conclude that low levels of VD25 are possibly due to the effects of chronic inflammation, and not the other way around. In this respect, and given the fact that the so called "alternate theory" seems to be well supported by the literature, it seems advisable to be very careful when it comes to vitamin D supplementation, especially in those with chronic inflammatory diseases.

1. VITAMIN D AND CHRONIC INFLAMMATORY AND AUTOIMMUNE DISEASES. INTRODUCTION:

It seems clear that low levels of the pre-hormone 25-hydroxyvitamin D (VD25) are normally shown in many chronic inflammatory diseases,[1] including multiple sclerosis,[2] systemic lupus erythematosus,[3] rheumatoid arthritis (RA), scleroderma or systemic sclerosis (SSc), type 1 diabetes mellitus,[4] psoriasis, or Inflammatory Bowel Disease (IBD).[5] In addition, many studies show symptomatic improvements, lower rates of autoimmune diseases and fewer inflammatory markers, by taking vitamin D. These two facts have brought concern about vitamin D deficiency.[6] However, the question of whether vitamin D deficiency plays a vital role in the pathogenic processes of these conditions or whether this alteration is just a downstream consequence of the disease, needs to be properly addressed. In addition, vitamin D supplementation is recommended and advised by many physicians and governments, while some

researchers and other public health organizations do warn against it. Throughout the present text, these questions will try to be answered.

2. VITAMIN D AND THE IMMUNE SYSTEM. BASIC PHYSIOLOGY:

One of the most important functions of the 1,25-dihydroxycholecalciferol (VD1,25)—the hormonally active metabolite of vitamin D—is exerted on the immune system. Thus, activation of Toll-Like-Receptors (TLR) on monocytes and macrophages, induces the expression of the enzyme CYP27B1, which locally converts circulating VD25 into the active form VD1,25. This hormone, in turn, regulates the immune system by activating the VDR (vitamin D receptor), found in most cells of the body, particularly in antigen-presenting cells (APCs), such as monocytes, macrophages and dendritic cells (DC). The binding of VD1,25 to the VDR, promotes the expression of antimicrobial peptides (AMPs) such as cathelicidin and beta defensins, which belong to the large group of cationic peptides; they constitute a major component of the innate immune system, showing activity against bacteria, fungi and viruses, and modulating innate and adaptive responses. They also display a potent capacity for altering inflammatory cytokine responses and apoptotic pathways in healthy and infected cells.[6, 7, 8, 9, 10]

3. VITAMIN D EFFECTS ON THE IMMUNE SYSTEM:

The general recognized effect of VD1,25 is to **enhance the innate immune system** by promoting monocyte phagocytosis and autophagy, and to **inhibit the adaptative response** by decreasing MHC and co-stimulatory molecules expression, leading to a reduced ability to activate T cells. In this vein, vitamin D has shown to **block the formation of Th1 cytokines**, specially IFNγ, and to **promote a Th2-mediated-immune response** by haltering the synthesis of IFNγ and promoting IL-4 production. There is some controversy on this point, as other observations have shown inhibition of both Th1 and Th2 cell cytokines production, including inhibition of IL-4.[56]

As pointed by Rode et al.[11] in a recent study, these results are paradoxical: on the one hand, VD1,25 promotes cathelicidin expression, which plays an important role in the killing of intracellular pathogens, such as M. tuberculosis (TB), by macrophages. On the other hand, IFNγ (produced by Th1-lympocytes and critical for innate and adaptive immunity) is also necessary to stimulate macrophages to kill TB and other intracellular microbes. Also, IFNγ increases the expression of cathelicidin in human monocytes and macrophages, and vitamin D has shown to be necessary for this process. Therefore, vitamin D should be beneficial for TB prevention and treatment. However, some studies have reported that vitamin D inhibits the production of IFNγ in T cells, creating a contradiction, as pointed out by Rode et al: "This creates a significant **paradox in which vitamin D is required for efficient innate immune responses against M. tuberculosis but at the same time impairs Th1-mediated immune responses against M. tuberculosis** [emphasis added]". In order to solve this puzzle, the authors explain that most **previous studies have used non-physiological, high concentrations (1–100 nM) of VD1,25** (being the physiological concentrations established by the authors of 60-110 pM). They also indicate that the effects of physiological concentrations of vitamin D on T cells under Th1-inducing conditions have not been studied yet. In this respect, Rode et al. found that VD25 does not inhibit differentiation of naïve CD4+T cells into Th1 cells, and that, even though VD25 decreases IFNγ production by T-cells, when the conditions of Th1-induction are set, i.e., when IL-12 and anti-IL-4 are added, the production of IFNγ by T cells is partially restored. They also show that, even though TB does inhibit cathelicidin expression in DC, as a survival mechanism, the addition of vitamin D counteracts this by upregulating cathelicidin expression. From these results, it seems clear that **the effects of vitamin D observed on the immune system can yield sharply contradictory results according to whether physiological versus pharmacological doses of the hormone have been used.**

With the above conclusion in mind, we can continue with the review of the effects of vitamin D on the immune system. In this regard, VD1,25 has also shown to **down-regulates the Th17-mediated responses**,

probably by inhibiting the production of IL-6 and IL-23. On the other hand, VD1,25 downregulates the expression of many proinflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α, in many cell types.[16]

The effect of **vitamin D on different immune cells** is large as well. In accordance with its capacity to inhibit the T cell proliferation and effectiveness (something highly dependent on the dose used, as reviewed above), VD1,25 has demonstrated to **induce Foxp3+ Treg-cells** differentiation. Furthermore, VD1,25 **promotes the M2-phenotype of macrophages** over the "inflammatory" or M1-phenotype.[5,6,12] As for the Dendritic Cells (DC), the effect that vitamin D exerts on them is also quite extensive . As demonstrated by Piemonti et al.[13] VD1,25 interferes with differentiation of monocytes into DC in culture. The **DC formed under the influence of this hormone show enhanced antigen (Ag) uptake capacity and inhibited immunostimulatory abilities**. Therefore, DC's **capability to present Ags to T cells seems to be impaired**, showing, on the other hand, an augmented endocytic activity. In contrast to the inhibition of the antigen-presenting function exerted by VD1,25 on APCs, the chemotactic and phagocytic capacity of monocytes and macrophages necessary for the **anti-tumour and anti-microbial activity of these cells, is enhanced by exposure to VD1,25**.[41] For this study, the authors used 10 nM of VD1,25, what they explain is the **highest concentration considered physiological**. This dose is ten times higher than the maximum physiological dose established for VD1,25 by Rode et al. (110 pM or 0.11 nM). In this respect, Hewison[14] indicates that VD1,25 is effective at the physiological concentrations of 10^{-10} to 10^{-8}mol/l (0,1 to 10nM), which correspond to the accepted affinity values for its receptor. This contradiction calls again for caution when interpreting the effects of vitamin D on the immune system.

Piemonti et al. results further show that DC treated with VD1,25, when exposed to LPS, induce a state of non-alloantigen-restricted **hyporesponsivity in T cells**, meaning that T cells were forced into the anergic state, by DC obtained from either the same donor or from an unrelated one. This experiment also showed **reduced IL-12** production, a cytokine involved in the differentiation of naive T cells into Th1 cells,[15] after exposure to CD40L, LPS, or TNF-α. Piemonti et al. conclude: "**The inhibitory effect of 1α,25-(OH)2D3 on DC maturation and differentiation is very similar to that of IL-10, an anti-inflammatory cytokine, and to that of glucocorticoids** [emphasis added] (…) because DC have the unique property to activate naive T cells and are required for the induction of a primary response, the suppression of DC function may very efficiently control the specific immune response". (Piemonti et al, 2000). Additionally, and following the same pattern of immunosuppression, VD1,25 has been reported to significantly **reduce abnormal PBMC responsiveness to multiple sclerosis-associated antigens,** (with serum doses reached by daily vitamin D supplementation),[16] and to downregulate T cell-driven IgG production.[17] In a similar vein, vitamin D has been reported to **inhibit antibody secretion and autoantibody production by B-lymphocytes**.[56]

Interestingly, VD1,25 also **stimulates *in vitro* production of the suppressive cytokine prostaglandin E2 by monocytes**.[41] This is of relevance, as prostaglandins are important mediators of inflammation, for example, in Inflammatory Bowel Disease.[18]

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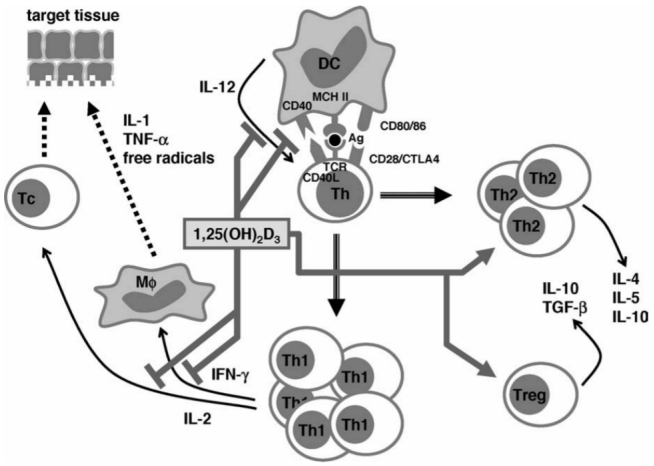

3.1 VITAMIN D IMPROVES MULTIPLE SCLEROSIS SIGNS AND SYMPTOMS:

While many studies have shown significant improvements from taking vitamin D, in both symptoms and inflammatory markers in patients with a variety of chronic conditions, it is clarifying to examine the particular effect on one of these condition, such as **multiple sclerosis** (MS), for which numerous small studies have found how vitamin D intake exerts a profound impact on patients, as described by Kimball et al:[16] (1) tolerogenic dendritic cells promoted by VD1,25 can **induce regulatory T cells and produce a shift toward T-helper-type 2**

(Th2) predominance in proinflammatory tissue lesions, (2) in patients with MS, **serum VD25** concentrations **are lower during relapse in comparison with remission, and correlate inversely with disease severity**, (3) uncontrolled clinical trials of vitamin D supplementation show **lower rates of exacerbation** and **reduced disease activity on magnetic resonance imaging**, (4) elevated steady-state VD25 levels after oral administration of cholecalciferol, had **desirable clinical effects**, (5) vitamin D supplementation has shown to **reduce circulating TGF-β1**. (Kimball et al, 2011).

In the same Kimball et al. study from 2011, the authors found that the rise of mean serum VD25 concentrations in the treated group, from 78 nmol/liter at baseline to 179 nmol/liter, significantly **reduced abnormal PBMC responsiveness to disease-associated antigens** (neuron, milk, and one islet antigen), after a year of cholecalciferol supplementation (4,000–40,000 IU/d plus calcium, followed by intake of 10,000 IU/d). The authors concluded: "cholecalciferol treatment resulted in stable increases in serum 25(OH)D levels that were associated with selective, significant attenuation of MS-associated T cell pools thought to drive progression of proinflammatory lesions in the CNS" (Kimball et al, 2011).

3.2. HIGH VITAMIN D LEVELS ASSOCIATED WITH EBV REACTIVATION AND CANCER:

Further evidence on immunosuppressive effects of vitamin D was demonstrated by Agborsangaya et al. in 2011.[19] The authors age-matched 111 women with pregnancy-associated breast cancer (PABC), to controls who had have the same number of pregnancies, and measured levels of VD25 and markers of EBV reactivation. Although they found no association between pre-existing EBV infection and risk of PABC development, they did find that markers of **EBV reactivation were associated with increased risk of breast cancer development** (during or soon after pregnancy), **in those women with sufficient levels of VD25 (≥75 nmol/l)**. It is worth noting that **EBV reactivation occurred almost exclusively in individuals with the considered sufficient levels of vitamin D**. (Agborsangaya et al, 2011). In accordance, this team had previously demonstrated that **higher levels of VD25 were associated with a 2 to 4-fold increased risk of PABC**, in a follow-up-longitudinal study.[20] However, the authors note that, even though their results agree with some studies finding association between high VD25 levels and increased risk of prostate cancer, these results contradict some experimental evidence. In fact, a recent meta-analysis concluded that high VD25 intake was inversely correlated with lung cancer risk, while, on the other hand, high vitamin D intake was shown to provide limited protection against lung cancer carcinogenesis.[21]

Digging deeper into the vitamin D and cancer relationship, numerous biologic functions have been reported for vitamin D, including control of proliferation, inflammation, autoimmunity, DNA damage/repair and tumorigenesis. Therefore, **vitamin D immune regulation is thought to be crucial for inhibiting cancer progression**.[22]

Perhaps, a general **"U-shaped theory"** extrapolated from the results published by Tuohimaa et al.[23] in 2003, **could reconcile the above contradictory findings**. In fact, focusing on prostate cancer, the authors are able to lay out a viable explanation for their results (i.e., both low and high VD25 serum levels are associated with higher prostate cancer risk) and the following apparently contradictory evidence: (1) prostate cancer has been linked to low vitamin D, (2) vitamin D metabolites have shown to be protective against cancer occurrence *in vitro*, (3) normal and malignant prostate cells contain VDR, which mediates the antiproliferative action of VD1,25, (4) VD1,25 also causes apoptosis, induces differentiation, inhibits telomerase expression and tumor cell invasiveness, and suppresses tumor-induced angiogenesis, (5) some

epidemiologic studies have shown that high serum vitamin D may protect against prostate cancer, (6) in prostate and breast cancer cell cultures, the effect of VD1,25 is concentration-dependent so that low concentrations are mitogenic whereas high ones are antiproliferative. (Tuohimaa et al, 2003).

Tuohimaa et al. longitudinal study used data from 200,000 men from Finland, Norway and Sweden, from which 622 patients with prostate cancer and 1,451 matched-controls were finally selected. The lag time between sampling and diagnosis ranged from ≤9 to >14 years. The authors found that **both low (<19 nmol/l) and high (>80nmol/l) VD25 serum levels were associated with higher prostate cancer risk**, while the average levels of VD25 (40–60 nmol/l) comprised the lowest risk of developing prostate cancer. In order to explain these surprising results, the authors highlight the fact that serum VD25 reflects its availability in the body, and that the dominating hypothesis has been that low levels of vitamin D increases the risk of prostate cancer. However, they explain that the association of low vitamin D status with the higher cancer incidence should be taken with caution, because one alternative explanation could be that **an existing cancer yet to be detected could actually lower vitamin D levels**. In this regard the authors emphasize the importance of the lag time from blood vitamin D measurements and cancer diagnoses, especially when considering the relationship between low levels of vitamin D and higher incidence of cancer, given that only with long lag times used, they were able to significantly link these two variables. On the other hand, in order to explain the relationship that they found between high levels of VD25 and higher probabilities of developing prostate cancer, Tuohimaa et al. point out that **high vitamin D concentrations may lead to** increased 24-hydroxylation, enzyme which would in turn **reduce the available local levels of the active VD1,25, what would finally lead to weak antiproliferative capacity** (Tuohimaa et al, 2003).

3.3. COMPARISON BETWEEN VD1,25 AND DEXAMETHASONE:

Unger et al. trial from 2009,[24] compared the anti-inflammatory properties of VD1,25 and those of the corticosteroid dexamethasone. They found that when monocytes are treated with either VD1,25 (at concentrations of 10^{-8}, considered physiological by some authors[14]) or dexamethasone, the DCs formed correlate with stable and semi-mature phenotypes with intermediate levels of co-stimulatory and MHC class II molecules, what supports the previous findings of VD1,25 **impairing the Ag-presentation-capacity of DCs**. Furthermore, the authors found that both **VD1,25 and dexamethasone possess the capacity to convert CD4+T cells into IL-10-secreting Tr1-like Treg-cells**, an indication of a potent suppressive effect of VD1,25 on T-cells proliferation; however, only Treg-cells induced by VD1,25-treated-DC exhibited antigen specificity. Additionally, the DC treated with both VD1,25 and dexamethasone, secreted less IL-12p70, but the amount of IL-10 was unchanged, what **demonstrates the Th1-inhibition properties of both substances** compared in this study.

From Unger et al. results, it is possible to observe that the **potency of VD1,25 and that of dexamethasone in suppressing T cell proliferation is pretty similar** (75% and 60% of suppression respectively). Delving deeper into this comparison, the authors note that both VD1,25 and dexamethasone show anti-inflammatory properties achieved also by means of gene expression. Finally, the authors mimicked *in vivo* DC activation by activated T cells using CD40 triggering, and found similar results. In conclusion, it seems that **the anti-inflammatory properties and potency of VD1,25 (in considered physiological doses) and dexamethasone are quite similar**. In agreement with this, **VD1,25 is thought to be the most potent steroid hormone in the human body**.[6] These assertions are in line with the conclusion quoted above from Piemonti and collegues' study, stating that **the inhibitory effect of VD1,25 on DC is very similar to the effect of glucocorticoids**.[13] Therefore, it is not surprising that Vitamin D is considered a secosteroid with a close resemblance in structure to immunosuppressive steroids.[25]

3.4 VITAMIN D INHIBITS TOLL-LIKE RECEPTORS:

Toll-likereceptors (TLR) are innate immune pattern recognition receptors that enable vertebrates to deal quickly and efficiently with invading foreign microorganisms such as bacteria, virus or fungi.[26] These

sensors play a critical role in the early innate immune responses to invading pathogens.[27] Activation of TLR results in initiation of innate and adaptive immune responses;[5] therefore TLR expression and function may ultimately predict the outcome of innate immunity response.[26] Although TLR play a vital role in the initiation of protective responses, the massive release of pro-inflammatory substances triggered by these receptors may produce harm in the host, both in the form of acute damage (such as in sepsis) and of chronic conditions such as autoimmune diseases.[26] In this vein, Hausmann and colleagues[28] found that **TLR2 and TLR4 levels in intestinal macrophages were up-regulated in patients with Crohn's disease, ulcerative colitis and diverticulitis, compared with non-inflamed mucosa of healthy controls**, highlighting this way the role of TLR in the inflammatory process. Accordingly, the stimulation of B cells via the TLR pathway, leads to an increase in antibody production and induces other events typical of autoimmunity processes, such as cytokine production and an enhancement of B cells as APCs. Finally, signaling via TLR7 and TLR9 seems to be predominantly involved in breaking tolerance in autoimmune diseases.[29]

Kambis et al.[26] showed that **VD1,25 at considered physiological doses of 1 nM, down-regulates TLR2 and TLR4** expression on human monocytes in a time- and concentration-dependent manner; therefore, according to the authors, the impaired inflammatory response to bacterial conserved pathogen-associated molecular patterns (PAMP) in monocytes treated with VD1,25 is, at least in part, due to TLR downregulation. The results obtained prompted the authors to suggest that **TLR down-regulation require functional VDR activity**. Moreover, they proposed that "the mechanism underlying the protective role of 1,25(OH)2D3 in Th1-mediated autoimmune disease involves down-regulation of antigen-presenting molecules, costimulatory CD40 and, most importantly, downregulation of TLR on APC" (Kambis et al, 2006).

It is important to note that the inhibition of TLR2 and TLR4 by VD1,25, was observed at doses of 1 nM, which, as explained above, are considered physiological by some authors, while it would be ten times higher than the maximum physiological levels established by others. In this regard, Radović et al.[30] pointed out that the inhibition of these TLR by VD1,25 was shown to be highest after 72h, and that it had been proposed that **this could constitute a negative feedback mechanism that prevents excessive TLR activation and inflammation in the late infection phase**.

Dickie et al[5] confirmed Kambis and colleagues' results and found, in addition, that the intracellular **TLR9 was also downregulated by VD1,25** at supraphysiological concentrations (100nM), and demonstrated that this might exert a clinical effect on certain autoimmune diseases in which IL-6 have been shown to be elevated (such as SLE), given that TLR9-induced IL-6 secretion was reduced by the TLR9 inhibition of VD1,25. Furthermore, the authors explain that the TLR9-inhibition by VD1,25 may further be beneficial for some autoimmune diseases such as SLE, because TLR9 is implicated in the autoimmune process of some of these conditions. (Dickie et al, 2010).

In a similar vein, there is evidence that **activation of TLR4 may play a critical role in** many diseases related to translocation of the gram-negative enterobacteria. In these conditions, the commensal bacteria would translocate from the gut milieu and activate in the blood stream the TLR4 complex, leading to the activation of intracellular signaling pathways, such as NF-κB, which induces in turn the production of ROS/RNS and pro-inflammatory cytokines. This process is thought to occur in clinical **depression, chronic fatigue syndrome (CFS), IBD, rheumatoid arthritis, cardiovascular disorders, psoriasis, HIV infection, Parkinson's and Alzheimer's disease, multiple sclerosis, and chronic alcoholism**. Other conditions in which activation of the TLR4 complex may underpin their pathophysiology include, **asthma, diabetes, obesity, metabolic syndrome, autoimmune disorders, neuroinflammatory disorders, schizophrenia, bipolar disorder, autism and toluene inhalation**.[31]

In summary, over-expression/activation of certain TLR are involved in the pathogenesis of numerous autoimmune diseases and other chronic inflammatory conditions. VD1,25 has shown to inhibit TLR2, TLR4 (possibly at physiological doses) and TLR9 (at supraphysiological doses). The inhibition of these receptors by VD1,25 seems to constitute one of the main pathways by which this hormone could inhibit the innate branch of the immune system, probably leading to a transient symptomatic relief. This could

certainly be achieved by pharmacological doses of VD1,25, but it is possible that even physiological doses could also exert the same effect.

3.5. VITAMIN D ON ACUTE AND SEMI-ACUTE CONDITIONS:

So far, we have described the effects of vitamin D on some immune cells from healthy donors, as well as from patients with chronic conditions. Would the effect of this hormone be somewhat different in preparing a healthy person for an acute infection? The results obtained by Kim et al[32] *in vitro* essay seem to answer this question affirmatively, as they found that **the presence of sufficient levels of vitamin D prior to infection with Mycobacterium leprae, contributes to effectively reduce the viability of the pathogen in macrophages (MΦ)** by inducing a vitamin D-dependent antimicrobial phenotype, in contrast to the phagocytic macrophages. As the authors explain, "the vitamin D-dependent antimicrobial pathway involves the induction of IL-15 and IL-32, the conversion of 25D3 to bioactive 1,25-dihydroxyvitamin D (1,25D3) and subsequent activation of the vitamin D receptor (VDR) to induce the expression of the antimicrobial peptides including cathelicidin, autophagy and phagolysosomal fusion. **This antimicrobial pathway is not induced in MΦ if the levels of 25D are not sufficient.**" (Kim et al, 2018). As the authors state, there have been **opposite findings by other groups, in which VD1,25 has shown to decrease phagocytic function, the release of pro-inflammatory cytokines, the antigen presentation capacity and the expression of DC-specific surface markers**. In these experiments, as the authors note, the concentration used of VD1,25 was supraphysiologic, while the concentration used of VD25 was within the physiologic range. One of these studies cited by Kim et al. has been reviewed above (Piemonti et al); they used 10 nM of VD1,25 and the authors state that this is the highest concentration considered physiological. Again, these contradictions call for caution when analyzing the effects of vitamin D on the immune system.

Accordingly, not always vitamin D seems to promote the pro-inflammatory macrophage phenotype. High VD1,25 levels impede fracture repair under sufficient vitamin physiologic conditions, because it suppresses the osteogenic functions of M1 macrophages (an inflammatory phenotype), as shown by Wasnik et al.[33] However, the authors explain that some studies show totally contradictory results, and therefore emphasize the importance of local tissue microenvironment for tissue repair, noting that "**1,25(OH)2D treatment is therapeutically beneficial for some inflammatory conditions but detrimental for others**" (Wasnik, 2018).

The above reviewed findings, necessarily bring up the following question: is Vitamin D helpful for chronic systemic inflammatory conditions?

3.6 IS VITAMIN D HELPFUL FOR CHRONIC SYSTEMIC INFLAMMATORY CONDITIONS?

Is Vitamin D helpful for chronic systemic inflammatory conditions? Do its anti-inflammatory effects cure or just palliate the symptoms at the expense of worsening the underlying pathophysiology? As reviewed above, it seems that **VD1,25 acts in a similar way to corticosteroids, which do not cure any chronic diseases, but only alleviates the symptoms**. In this regard, Chatenoud[34] says: "the prolonged use of immunosuppressants is linked with significant toxicity (i.e. over-immunosuppression leading to opportunistic infections and tumors). These long-term risks might become unacceptable in autoimmune patients because they affect both life expectancy and the quality of life".

Delving into this crucial question, in a double-blinded, placebo-controlled, randomized clinical trial, a single bolus of 250.000 IU of vitamin D3 was given for pulmonary exacerbation to adults with cystic fibrosis (CF). This strategy resulted in reduction of IL-6 and Tumor Necrosis Factor (TNF). However, there were no significant changes in IL-1β, IL-8, IL-10, IL-18BP and NGAL (neutrophil gelatinase-associated lipocalin). As the authors expected, the unique-high-dose of vitamin D improved clinical outcomes, but obviously this approach was not meant as a cure.[35] However, this was just an acute intervention and therefore an

imperative question remains: **does vitamin D regime stand a chance as a possible curative agent in chronic inflammatory conditions when given for a long time?**

The above analyzed open-label, 12-month, randomized controlled trial carried out by Kimball et al,[16] indicates that "Vitamin D supplementation may be beneficial alone or as an add-on to therapy in patients with MS"; in this vein they explain that, what they achieved after a year of vitamin D supplementation, is a significant attenuation of the T-lymphocytes associated with the CNS lesions. Finally, they describe how other studies had found improvement of MS clinical outcomes. (Kimball, 2011)

In the same vein, in a study carried out by Pincikova et al in 2017,[36] patients suffering from cystic fibrosis were given daily high doses of vitamin D, for 3 months, with the goal of achieving levels > 100nmol/l. In general, after 3 and or 5 months (3 months taking the hormone and 2 months of follow-up), **as the levels of VD25 raised, the levels of plasma LPS, haptoglobin, total immunoglobulins, and the frequency of CD8+ T cells co-expressing CD38 and HLA-DR, decreased**. Some of the immunological markers were reduced only during the period of vitamin D intake, while others remained changed for the 2 months follow-up. In short, in accordance with the previous analyzed trials, **daily levels of vitamin D decreased inflammatory humoral markers and also diminished to some degree the inflammatory and overactive acquired response in patients with cystic fibrosis**. Finally, the negative correlation between blood VD25 levels and LPS, were speculated by the authors to be a direct consequence of decreasing LPS translocation from the lungs, given the fact that active vitamin D has shown to restore tight junction proteins disruption in the airway epithelial barrier (Pincikova, 2017).

In summary, it seems clear that no curative effect is observed nor expected from vitamin D supplementation, but only relief of symptoms by means of downregulating to certain degree the pathological systemic inflammation thought to drive progression and cause symptoms of most, if not all, autoimmune and chronic inflammatory diseases.

3.7. VITAMIN D AND THE INNATE IMMUNE SYSTEM:

It seems clear after the revision made throughout the above text, that **VD1,25 downregulates the acquired immune response; however, does it actually enhance the innate immunity like it is generally thought?** As detailed above, VD1.25 activates the VDR, promoting this way the expression of antimicrobial peptides, which comprise a major component of the innate immunity. This is a well-known physiological process that takes place under physiological circumstances. However, does the same process apply when it comes to exogenous vitamin D supplementation for chronic inflammatory conditions?

As reviewed above, **VD1,25** (at doses considered physiological by some authors, but not by others)**, inhibits some of the main TLR, which constitute a major component of the first part of the innate response**. On the other hand, Natural killer cells (NK) are a type of cytotoxic lymphocytes, also critical to the innate immune system. The role NK cells play is analogous to that of cytotoxic T cells in the vertebrate adaptive immune response.[37]

How are the NK-cells affected by vitamin D? The answer to this question can be found in the study performed by Ota et al in 2015.[12] The authors carried out a comprehensive review of how VD1,25 affects NK, and performed an exhaustive and thorough analysis on **how vitamin D supplementation affects the NK status** of women with recurrent pregnancy losses (RPL). The authors summarize their findings by highlighting: "In this study, we have demonstrated that **1,25(OH)2D3 regulates NK-cell functions via various mechanisms. It suppresses NK-cell cytotoxicity** [emphasis added] regardless of presence or absence of cytokine secretion, upregulates killer inhibitory receptor expression while **downregulates killer activating receptors** [emphasis added] and increases the depolarization of perforin granules in conjugated NK cells. Furthermore, 1,25(OH)2D3 also modulates the innate immune system by reducing LPS-induced proinflammatory cytokine production and this action is mediated by blocking TLR4 pathways, which sequentially decreases TLR4 induced phosphorylation of NF-κB. Therefore, 1,25(OH)2D3 directly effects NK-cell function, and vitamin D deficiency may be associated with dysregulated NK cells, which is

present in women with RPL" (Ota et al, 2015). The authors also note "In this study, the **modulation of NK-cell phenotype and function by 1,25(OH)2D3 was specific for women with RPL** [emphasis added]. Lack of 1,25(OH)2D3 effect on NK cells from normal controls may be attributable to inactivated NK-cell status of normal controls, suggested by low level of CD107a+ NK cells and NK-cell cytotoxicity, and possible low level of VDR expression". Finally, it is important to remark that the authors state that the LPS-induced TNF-α and IFN-γ production suppression by VD1.25, is thought to be regulated via VDR.

A previous study carried out by Leung,[38] found, however, that both **vitamin D3 and VD1,25 inhibited NK-cells cytolytic activity in cultured of cells obtained from healthy volunteers**. Therefore, does VD1,25 inhibit cells of healthy individuals, as shown by this study, or it doesn't, as demonstrated by Ota et al? Paying attention at the concentration used by the two studies, Ota et used concentrations of 10 and 100 nM (equivalent to 4,16 and 41,6 ng/ml, respectively, knowing that the molecular weight of the VD1,25 is of 416.646 g/mol [39]) finding that 10 nM (equivalent to 10^{-8}M) were sufficient to inhibit NK cells form women with RPL; but this concentration didn't affect the NK-cells from healthy controls. On the other hand, Leung used concentration of 5 μg/ml (5000 ng/ml); which is around 1000 times more concentration of VD1,25 than that used by Ota et al. In this respect, Leung explains that NK cells activated by IL-2 were not sensitive to lower doses of vitamin D3. Actually, considering that normal serum levels of VD1,25 are 20-60 pg/ml (0.02-0.06 ng/ml), we might wonder whether the results of these two studies could actually apply to physiological doses of VD1,25. In this respect, Leung says that "**it is possible that under certain physiological and pathological conditions the local concentrations may approach the pharmacological doses** [emphasis added]". This makes sense, knowing that local autocrine/intracrine metabolism of vitamin D is predominant in extrarenal tissues[40]. In this regard, van Ettel et al,[41] explicate that "the *in vitro* observed immunomodulatory effects of 1,25(OH)2D3 only occur at concentrations of 10^{-10} M and higher". Therefore, given the fact that a concentration of 10^{-10} M equals 41,67 pg/ml and considering that serum levels of VD1,25 are 20-60 pg/ml, as described above, **we can infer that high VD1,25 serum levels found in patients with many chronic inflammatory and autoimmune conditions can actually be immune-suppressive**. Moreover, and in line with Leung assertions, van Ettel et al. point out that concentrations of 10^{-10} M (equivalent to 41,67 pg/ml) can probably be reached locally, by macrophages and other cells. In summary, **we should, at least consider the possibility that *in vitro* immune-suppressive observed effects of VD1,25 could apply to *in vivo* physiology and pathophysiology.**

Going back to Ota and colleagues' results, it is tempting to draw two conclusions:

1. On the one hand, given the fact that women with RPL have increased NK-cell levels and cytotoxicities, upregulated NK activity, downregulated killer inhibitory receptors and upregulated killer activating receptors on NK cells, and given that they also show vitamin D deficiency, the authors note how these findings raise a question about an immune modulatory role of vitamin D on NK-cell immunity. (Ota et al, 2015). So, **perhaps vitamin D levels correlates negatively with NKs function and activity**.

2. The second idea worth exploring arises from the fact that Ota et al study's results, show that VD1,25 doesn't have any effect on NKs from normal controls; the authors speculate that this could be attributable to inactivated NK-cell status of normal controls, and possible low level of VDR expression (Ota et al, 2015). Obviously, **vitamin D does display a pleitropic effect on the immune system** depending maybe on whether the person is healthy or sick, and therefore **we possibly should assume that the effects of vitamin D supplementation might be different for healthy population than to sick individuals.**

In summary, it seems that, even though vitamin D is thought to present immune-stimulant effects on the innate immune system under physiological or "ideal" circumstances, when vitamin D is cultured with certain immune cells, contradictory results are observed. Thus, doses considered by some authors to be physiological have demonstrated to inhibit NK-cells (at 10nM) and TLR2 and TLR4 (at 1nM). So, in view of this, it seems necessary to at least be aware that vitamin D supplementation might inhibit both the innate and the acquired immune responses at very different levels, being its immunosuppressive properties similar to those of exogenous corticosteroids. These effects might be somewhat different for sick people than to healthy population. The improvements achieved in several chronic inflammatory conditions after vitamin D supplementation, seem to constitute just a temporary effect due to the

strong immune-suppressive effects of this hormone, and in no way are they the result of a final resolution of the underlying pathophysiology of the trialed diseases.

4. MODELS TO EXPLAIN LOW LEVELS OF VD25: THE ALTERNATE THEORY FOR AUTOIMMUNE DISEASES:

As reviewed above, low serum levels of VD25 are normally found in many chronic conditions and autoimmune diseases, while high levels of serum VD1,25 has be found to be a hallmark of these inflammatory chronic conditions. In this regard, Blaney et al.[47] challenge the general assumption that low levels of VD25 are indicative of autoimmune conditions, and finds VD1,25 to be a very reliable marker of autoimmunity. This, as Mangin et al.[6] reports, has brought concern on the potential role of low levels of vitamin D as a possible contributor or direct cause of such inflammatory ailments. Furthermore, a large segment of the general population has shown vitamin D deficiency, what has opened a worldwide debate on whether vitamin D supplementation should be advised, either for chronically sick people, or for both, the sick and the healthy population. With this background, it seems appropriate to wonder whether low levels of VD25 should constitute a matter of concern, and weather low levels of VD25 are the cause or the consequence of chronic inflammation (Mangin et al, 2014).

4.1. SHOULD LOW LEVELS OF VD25 BE A MATTER OF CONCERN?:

As Mangin et al. explain in their study from 2014, **low VD25 levels are found in both, healthy individuals and people with chronic inflammatory and autoimmune conditions**. Two opposed explanations can be argued in this respect: (1) healthy people with low VD25 levels will eventually fall sick, or (2) in the absence of disease, low levels of VD25 should be considered normal (Mangin et al, 2014).

Mangin and collegues go on, arguing that **rickets**, often showed as a proof of vitamin D supplementation necessity, **has demonstrated to be actually due to insufficient calcium intake**, and consequently is cured with calcium supplementation. In the same way, **osteoporosis** is frequently cited as an example of the need of vitamin D supplementation. In this regard, Mangin et al. also dismantle this statement, explaining that **high levels of VD1,25 reduces the VDR competence in the intestinal mucosa, lowering this way calcium and phosphorus absorption, actually leading to osteoporosis by high levels of VD1,25**. Accordingly, there is ample evidence that **elevated VD1,25 leads to bone loss**. In fact, levels of VD1,25 ≥42 pg/ml stimulate bone osteoclasts, what leads to osteoporosis, **dental fractures and calcium deposition** into the soft tissues (Mangin et al, 2014).

A **meta-analysis performed in 2014** by Bolland et al,[42] further strengthen the position of low VD25 not being a real health-problem. The study analyzed and merged the data of a total of 98 trials and 378.839 patients, trying to elucidate **whether or not vitamin D supplementation with or without calcium, was beneficial for myocardial infarction or ischaemic heart disease, stroke or cerebrovascular disease, cancer, total fracture, hip fracture, and mortality**. The authors concluded that **the effect estimate for vitamin D supplementation lay within the futility boundary**, indicating that vitamin D supplementation does not alter the relative risk of any of these conditions by 15% or more. (Bolland et al. 2014). It is important to note that most trials were done in populations with VD25 levels lower than 50 nmol/L and achieved VD25 levels of 50 nmol/L or greater.

Finally, and continuing with this idea, Mangin et al. also quote the 2010 IOM report on the subject, which states: "**Outcomes related to autoimmune disorders, cancer, cardiovascular disease and hypertension, diabetes and metabolic syndrome, falls and physical performance, immune functioning, infections, neuropsychological functioning, and preeclampsia could not be linked reliably with calcium or vitamin D intake** [emphasis added] and were often conflicting."

In summary, even though this subject certainly needs a deeper review, it does seem reasonable to at least doubt the general believe that what is considered by certain standards to be low VD25 levels, do necessarily indicate a health problem, and that vitamin D supplementation is warranted for the general population and for people suffering from chronic inflammatory or autoimmune conditions.

4.2. ARE LOW VD25 LEVELS THE CAUSE OF, OR THE CONSEQUENCE OF CHRONIC INFLAMMATION? THE ALTERNATE HYPOTHESIS.

As Mangin et al explicates, Vitamin D advocates state that low VD25 causes chronic diseases; however, a proper explanatory pathogenesis has not been constructed. (Mangin et al, 2014). Several researchers defend an alternate theory. This hypothesis posits that **low vitamin D is the consequence of a chronic inflammatory process caused by persistent infection**.[43] The details of this theory would be as follows:

Intracellular bacteria (cell wall deficient bacteria) invade nucleated cells and, in order to survive, they wreck the normal vitamin D endocrine function. **Excess of VD1,25 is produced trying to activate the VDR in order to synthesize antimicrobial peptides (AMPs) to fight the infections**. This results in a rapid metabolism of VD25, leading to low **VD25 serum levels**.[6] When the immune system fails to eradicate these persistent intracellular pathogens, an **ineffective immunological response is perpetuated, causing low-grade inflammation** and **phagocyte-inflicted tissue damage**, which plays an important role in many chronic diseases. Thus, these persistent microbes release cytokines which signals T and B cells, provoking the production of numerous **auto-antibodies** directed to the intracellular pathogens, which **cross-react attacking human proteins**, what causes symptoms of chronic inflammatory diseases.[6] Furthermore, these persistent infections are able to modulate cytokine production, and in monocytes and macrophages **cytokine activation markedly inhibits 1,25D/VDR gene transcription**.[44] For example, **IL-4 release can inhibit the VDR expression of cathelicidin** in monocytes. This may have clinical implications, for instance, given the association between IL-4 and development of tuberculosis.[45]

The **inflammation caused by these intracellular microbes up-regulates the enzyme CYP27B1**, which converts the VD25 into VD1,25, **making VD1,25 to rise at the expense of lowering the levels of the VD25.** [1] In addition, some of these microbes **down-regulate the VDR in order to hinder the innate responses** (for example, mycobacterium tuberculosis, mycobacterium leprae, aspergillus fumigatus, Epstein–Barr virus or HIV, have shown to downregulate the VDR by different means). [6]. The VDR can, therefore, be downregulated by different mechanisms. For example, the bacteria **Borrelia burgdorferi** (which causes Lyme disease) was shown, using whole genome microarrays, to **down-regulate VDR gene expression by 50 times**, when live spirochetes were cultivated with PBMCs, and by 8.3 times, when Lysate of the bacteria was added to the culture instead. [46] Another possible mechanism by which bacteria downregulates the VDR, consists of **releasing certain ligands that compromise the activity of this receptor**. In this regard, *in silico* simulation showed that the sulfonolipid capnin, created by the biofilm bacterial species of the genera Cytophaga, Capnocytophaga, Sporocytophaga and Flexibacter, could bind the VDR and thereby reduce its activity. Therefore, **microbe-induced immunosuppression can occur as a result of VDR suppression**.[44] The **repressed VDR cannot express the enzyme CYP24A1**, which breaks down the VD1,25 into its inactive metabolites, **allowing this way VD1,25 levels to rise even further** without a feedback system to keep them in check.[47,48] Supporting the idea that down-regulated VDR leads eventually to high levels of VD1,25, Yoshizawa et al.[49] demonstrated that **generated VDR-deficient mice, showed a 10-times fold increase serum levels of VD1,25.**

High VD1,25 values are therefore the result of (1) chronic inflammation and, (2) VDR deactivation. Elevated VD1,25 further reduces VDR competence, suppresses macrophage activity, and inhibits the nuclear factor kappa-B pathway, **inhibiting this way the immune system** function, what results in **low grade chronic inflammation.** Moreover, **as the VD1,25 raises, it binds to the Pregnane X-receptor (PXR)** and down-regulates the amount of vitamin D3 converted into VD25, **reducing even further the concentration of VD25.** Additionally, **VD1,25 inhibits hepatic synthesis of its precursor VD25**. This is shown in a study performed by Bell et al,[50] in which supplemental VD25 significantly increased serum VD25 levels, but it fails to do so when given concomitantly with VD1,25. Consequently, **low VD25 levels may lead to chronic inflammation as this is in turn the result of high VD1,25**. This assertion is supported by Waldron et al,[51] who demonstrated that VD25 decreased after acute inflammation occurring after elective orthopaedic surgery.

The VDR deactivation, in turn, **prevents 1,25 from inducing the expression of AMPs**, such as cathelicidin and beta-defensin, what further inhibits the innate response.[6,44] Besides deactivation of the VDR, the persistent **microbes also give off proteases that degrade AMPs**.[6]

Interestingly, **as the VD1,25 rises, it doesn't seem to bind and activate the VDR**. This conclusion can be drawn from the Blaney et al. study from 2009.[47] In this study, **most patients with numerous chronic diseases showed serum levels of VD1,25 ranging from 110 pmol/L to 350 pmol/L**, while any patient had below-normal levels of VD1,25 (<40 pmol/L). However, **there were no apparent clinical manifestations of hypercalcemia**, suggesting that VD1,25 is not activating the VDR nor driving this way the expression of genes associated with calcium absorption. This could occur because the VDR may be already occupied by bacterial ligands; this concept is supported from a trial in which levels of VD1,25 in patients with autoimmune diseases, tended to drop down to normal values by taking a VDR agonist.[52]

As stated by Chen et al,[53] **the accepted innate mechanism by which VD1,25 activates the VDR to form AMPs in macropahes, doesn't explain the anti-inflammatory action of vitamin D**, in which VD1,25 down-regulates pro-inflammatory cytokines in macrophages and other cells. The authors concluded that "the **innate immune response is dysregulated and over-sustained in macrophages when the VDR signaling is inactivated** [emphasis added]" (Chen et al, 2013), after finding that more cytokines were produced in VDR-knocked-out mice's macrophages in presence of LPS.

Based on the known structural similarity between VD1,25, VD25, and the inactive metabolites 24,25-dihydroxycholecalciferol (VD24,25) and 25,26-dihydroxycholecalciferol (VD25,26), it becomes clear that **VD25; VD24,25; VD25,26 and even cholecalciferol** (the most supplemented form of vitamin D) **will displace the activating metabolite VD1,25 from the VDR, thus inactivating the innate immunity even more**. According to this, supplementation of vitamin D entails extra issues. Thus, **supplemental vitamin D** (normally cholecalciferol) intake increases both VD25 and VD1,25, which, at high levels, can **displace the endogenous ligands from** other nuclear receptors, including **thyroid-α1 (T3Ra), adrenal (AR), and glucocorticoid receptors (GR). This further disrupts the endocrine and the immune system** and also leads to more immune-suppression by reducing the ability of these receptors to induce production of antimicrobial peptides.[25] Thus, microbes which manage to block VDR transcription or high doses of vitamin D metabolites, will inhibit the expression of the 16 AMPs families expressed by that receptor. Moreover, the **vitamin D metabolites, including the resulting high VD1,25,** will competitively displace cortisol (and other glucocorticoids) and T3 from their respective nuclear receptors, what **will alter the expression of at least other 52 families of AMP** (20 expressed by the GR, 17 by the AR and 15 by the T3Ra).[47,54] In this respect, it is worrisome to imagine the effects that the inhibition of these nuclear receptors (i.e., VDR, GR, AR, T3Ra) can have on the endocrine and immune system. The results published by Brahmachary et al.[54] give an idea of the extent of this matter. They performed an *in silico* analysis, showing how these nuclear receptors cooperate to express numerous families of AMPs. For example, **the alpha defensins, beta defensins and cathelicidins families of AMPs are expressed by activation of, not only the VDR, but also the GR, AR, and T3Ra nuclear receptors**. Furthermore, these receptors affect the immune system in other ways: GR regulates lymphocyte apoptosis, T cell development and inflammatory responses, among many other physiological processes; AR is involved on the maturation of T and B lymphocytes, while T3Ra regulates B-cell production levels (Brahmachary et al, 2006). Therefore, it is reasonable to infer that the resulting **high levels of VD1,25, stop being efficient in killing pathogens through the induced-production of AMPs, becoming immune-suppressive instead**, **because VD1,25 and other vitamin D metabolites will be actually impeding the formation of AMPs**.

It is important to note that **vitamin D supplementation has been considered to be immune-suppressive when reaches serum levels of 20ng/ml** or higher.[55] However, other authors estimate that levels of VD25 within the range of 20–30 ng/ml are not likely to suppress the immune system nor inhibit bacterial elimination.[6]

Accordingly, **vitamin D supplementation possesses multiple immunosuppressant properties**, as described by Arnson et al,[56] and consequently, Vitamin D supplementation has shown to improve clinical symptoms in various animal models, such as autoimmune encephalomyelitis, collagen-induced arthritis,

type 1 diabetes mellitus, inflammatory bowel disease, autoimmune thyroiditis and systemic lupus erythematosus. (Arnson et al, 2007).

Furthermore, the persistent intracellular bacteria compete and **displace commensal organisms** from their niche,[6] what can lead to dysbiosis and leaky gut.

Since VD1,25 is known to antagonize the VDR itself,[44] as the active hormone and bacterial ligands accumulate, **the innate immune system is less able to effectively target pathogens,** creating a snowball effect, and becoming gradually easier for the body to get invaded by pathogens as the immune system becomes weaker, and symptoms get chronic, worse and broader.[43]

This whole picture might be different for healthy people as stated by Marshall[57]. Thus, healthy individuals convert the VD25 into VD1,25 in order to form AMPs and kill the invading pathogens. On the contrary, in people with chronic diseases who have the VDR blocked by bacterial ligands and through other means, levels of **VD1,25 increases to the point where other receptors are blocked and therefore AMPs can't be expressed**. Marshall also notes that temporarily symptomatic relief can occur in patients taking vitamin D supplementation or in those who sunbathe frequently, because their vitamin D gets higher and the immune-suppression gets worse accordingly.

Further validating the alternate theory, the VDR protein has been found to be significantly lower in IBD and colitis-associated colon cancer patients.[17] Likewise, as Mangin et al. describe, VD1,25 was elevated in the synovial fluid of patients with rheumatoid arthritis. Also, **cathelicidin was decreased in patients with Sarcoidosis**, regardless of normal vitamin D levels. In the same vein, 42% of patients with Crohn's disease had levels of VD1,25 above >60 pg/ml, while the authors speculated that the probable source of the high VD1,25 was the inflamed intestine; moreover, **VD1,25 levels correlated with disease activity**. Furthermore, as mentioned above, Blaney et al.[48] showed that **most of the 100 Canadian participants in their study, with numerous chronic diseases, had very high VD1,25 levels**, while **any patient had below-normal levels of VD1,25**. Finally, as reviewed above, low levels of VD25 have been reported in numerous chronic inflammatory and autoimmune diseases.

4.3. <u>THE ALTERNATE HYPOTHESIS AND CHRONIC DISEASES PATHOGENESIS</u>:

In the recently published Proal et al.[58] study, the authors perform a comprehensive review on the role of the microbiome in chronic inflammatory and autoimmune diseases, and offer a plausible explanation that ties the alternate theory reviewed above (i.e., intracellular wall-deficient-bacteria persist in nucleated cells, causing chronic inflammation, which leads to sustained low VD25 and high VD1,25 levels) with the probable origin of symptoms shown in these chronic conditions. The authors explain how **most bacteria and viruses** known to form **part of the human microbiome are capable, under conditions of imbalance and immunosuppression, of behaving as pathogens**. For instance, the authors point out how S. pneumoniae can persist either as a commensal microbe, or as a virulent pathogen, depending on its ability to evade the body's immune system response. Thus, many **pathogens associated with microbiome dysbiosis can persist inside macrophages, astrocytes**, and other cells of the immune system (Proal et al, 2018).

In addition, Proal et al. highlight the **link of many autoimmune conditions with microbiome dysbiosis**, as shown in type 1 and 2 diabetes, MS, RA, psoriasis, psoriatic arthritis, sarcoidosis, and system lupus erythematosus. In this disease-model, as described by the authors, the **inflammation is generated in response to microbiome-associated antibodies**. These antibodies should be viewed as normal antibodies created in response to microbiome pathogens in the context of **cross-reactivity between microbes and the host proteins**, in contrast to the classical concept of "auto-antibodies" (Proal et al, 2018).

Finally, Proal et al. point out that chronic inflammatory diseases are polymicrobial in nature. Thus, **microbiome pathogens interact to drive inflammation** and other disease processes, what results in **"secondary" damage to the human tissues by antibodies directed towards these microbes** (Proal et al,

2018). This process would therefore constitute the direct cause of symptoms in chronic inflammatory and autoimmune diseases.

Bibliography:

[1] Shoenfeld Y., Agmon-Levin, N., &Rose, N. (2015). Infection and autoimmunity (2nd ed., pp. 163-182). Elsevier BV.

[2] Kimball S, Vieth R, Dosch H-M, et al. Cholecalciferol Plus Calcium Suppresses Abnormal PBMC Reactivity in Patients with Multiple Sclerosis. The Journal of Clinical Endocrinology and Metabolism. 2011;96(9):2826-2834.

[3] Dall'Ara et al. Vitamin D and systemic lupus erythematous: a review of immunological and clinical aspects. Clin Exp Rheumatol. 2018 Jan-Feb;36(1):153-162. Epub 2017 Nov 14.

[4] Rosen, Y., Daich, J., Soliman, I., Brathwaite, E., & Shoenfeld, Y. (2016). Vitamin D and autoimmunity. Scandinavian Journal Of Rheumatology, 45(6), 439-447.

[5] Dickie LJ, Church LD, Coulthard LR, et al. Vitamin D3 down-regulates intracellular Toll-like receptor 9 expression and Toll-like receptor 9-induced IL-6 production in human monocytes. Rheumatology (Oxford). 2010 Aug;49(8):1466-71.

[6] Mangin M, Sinha R, Fincher K. Inflammation and vitamin D: the infection connection. Inflammation Research. 2014;63(10):803-819.

[7] Sousa FH, Casanova V, Findlay F, et al. Cathelicidins display conserved direct antiviral activity towards rhinovirus. Peptides. 2017;95:76-83.

[8] Kościuczuk EM, Lisowski P, Jarczak J, et al. Cathelicidins: family of antimicrobial peptides. A review. Molecular Biology Reports. 2012;39(12):10957-10970.

[9] Kumar NP, Moideen K, Viswanathan V, et al. Heightened circulating levels of antimicrobial peptides in tuberculosis—Diabetes co-morbidity and reversal upon treatment. Basu J, ed. PLoS ONE. 2017;12(9):e0184753.

[10] Gombart AF. The vitamin D–antimicrobial peptide pathway and its role in protection against infection. Future microbiology. 2009;4:1151.

[11] Rode AKO, Kongsbak M, Hansen MM, et al. Vitamin D Counteracts Mycobacterium tuberculosis-Induced Cathelicidin Downregulation in Dendritic Cells and Allows Th1 Differentiation and IFNγ Secretion. Frontiers in Immunology. 2017;8:656.

[12] Ota, K., Dambaeva, S., Kim, M., et al. 1,25-Dihydroxy-vitamin D3 regulates NK-cell cytotoxicity, cytokine secretion, and degranulation in women with recurrent pregnancy losses. European Journal Of Immunology. 2015; 45(11), 3188-3199.

[13] Lorenzo Piemonti, et al. Vitamin D3 Affects Differentiation, Maturation, and Function of Human Monocyte-Derived Dendritic Cells. The Journal of Immunology May 1, 2000, 164 (9) 4443-4451.

[14] Hewison M. Vitamin D and the immune system. Journal of endocrinology.1992;132,173-175.

[15] Wagner, M., Poeck, H., Jahrsdoerfer, B., et al. IL-12p70-Dependent Th1 Induction by Human B Cells Requires Combined Activation with CD40 Ligand and CpG DNA. The Journal Of Immunology. 2004;172(2), 954-963.

[16] Kimball S, Vieth R, Dosch H-M, et al. Cholecalciferol Plus Calcium Suppresses Abnormal PBMC Reactivity in Patients with Multiple Sclerosis. The Journal of Clinical Endocrinology and Metabolism. 2011;96(9):2826-2834.

[17] Sun J. Vitamin D and mucosal immune function. Current opinion in gastroenterology. 2010;26(6):591-595.

[18] Sharon P, Stenson W. Enhanced Synthesis of Leukotriene B4 by Colonic Mucosa in Inflammatory Bowel Disease. Gastroenterology. 1984;86:453-6

[19] Agborsangaya, C., Lehtinen, T., Toriola, et al. Association between Epstein–Barr virus infection and risk for development of pregnancy-associated breast cancer: Joint effect with vitamin D?. European Journal Of Cancer. 2011;47(1), 116-120.

[20] Agborsangaya, C., Surcel, H., Toriola, A., et al. Serum 25-hydroxyvitamin D at pregnancy and risk of breast cancer in a prospective study. European Journal Of Cancer. 2010;46(3), 467-470.

[21] Wei H., Jing H., Wei Q., et al. Associations of the risk of lung cancer with serum 25-hydroxyvitamin D level and dietary vitamin D intake: A dose-response PRISMA meta-analysis.Medicine (Baltimore). 2018;97(37):e12282.

[22] Elhusseini H, Elkafas H, Abdelaziz M, et al. Diet-induced vitamin D deficiency triggers inflammation and DNA damage profile in murine myometrium. International Journal of Women's Health. 2018;10:503-514.

[23] Tuohimaa P, Tenkanen L, Ahonen M, et al. Both high and low levels of blood vitamin D are associated with a higher prostate cancer risk: A longitudinal, nested case-control study in the Nordic countries. International Journal Of Cancer. 2003; 108(1), 104-108.

[24] Unger, W., Laban, S., Kleijwegt, F., van der Slik, A., & Roep, B. Induction of Treg by monocyte-derived DC modulated by vitamin D3or dexamethasone: Differential role for PD-L1. European Journal Of Immunology. 2009;39(11), 3147-3159.

[25] Waterhouse J, Perez T, Albert P, et al. Reversing Bacteria-induced Vitamin D Receptor Dysfunction Is Key to Autoimmune Disease. Annals Of The New York Academy Of Sciences. 2009;1173(1), 757-765.

[26] Sadeghi K, Wessner B, Laggner U. Vitamin D3 down-regulates monocyte TLR expression and triggers hyporesponsiveness to pathogen-associated molecular patterns. Eur J Immunol. 2006 Feb;36(2):361-70.

[27] Mahla RS, Reddy MC, Prasad DVR, et al. Sweeten PAMPs: Role of Sugar Complexed PAMPs in Innate Immunity and Vaccine Biology. Frontiers in Immunology. 2013;4:248.

[28] Hausmann M, Kiessling S, Mestermann S, et al. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology. 2002 Jun;122(7):1987-2000.

[29] Meyer-Bahlburg A, Rawlings DJ. B Cell Autonomous TLR Signaling and Autoimmunity. Autoimmunity reviews. 2008;7(4):313-316. doi:10.1016/j.autrev.2007.11.027.

[30] Jelena R, Danica M, Veličkov A, et al. Vitamin D immunomodulatory effect. Acta Medica Medianae. 2012;51:58-64.

[31] Lucas K, Maes M. Role of the Toll Like receptor (TLR) radical cycle in chronic inflammation: possible treatments targeting the TLR4 pathway. Mol Neurobiol. 2013 Aug;48(1):190-204.

[32] Kim EW, Teles RMB, Haile S, et al. Vitamin D status contributes to the antimicrobial activity of macrophages against Mycobacterium leprae. Small PLC, ed. PLoS Neglected Tropical Diseases. 2018;12(7):e0006608.

[33] Wasnik, S., Rundle, C., Baylink, D., et al. 1,25-Dihydroxyvitamin D suppresses M1 macrophages and promotes M2 differentiation at bone injury sites. JCI Insight. 2018;3(17).

[34] Chatenoud, L. Immune therapies of autoimmune diseases: are we approaching a real cure?. Current Opinion In Immunology. 2006;18(6), 710-717.

[35] Grossmann R, Zughaier S, Liu S, et al. Impact of vitamin D supplementation on markers of inflammation in adults with cystic fibrosis hospitalized for a pulmonary exacerbation. European journal of clinical nutrition. 2012;66(9):1072-1074.

[36] Pincikova T, Paquin-Proulx D, Sandberg JK, et al. Vitamin D treatment modulates immune activation in cystic fibrosis. Clinical and Experimental Immunology. 2017;189(3):359-371.

[37] Vivier E, Raulet DH, Moretta A, et al. Innate or Adaptive Immunity? The Example of Natural Killer Cells. Science (New York, NY). 2011;331(6013):44-49.

[38] Leung KH. Inhibition of human natural killer cell and lymphokine-activated killer cell cytotoxicity and differentiation by vitamin D3. Scand J Immunol. 1989 Aug;30(2):199-208.

[39] National Center for Biotechnology Information. PubChem Compound Database; CID=9953732, https://pubchem.ncbi.nlm.nih.gov/compound/9953732 (accessed Sept. 27, 2018)

[40] Zehnder D1, Bland R, Chana RS, et al.Synthesis of 1,25-dihydroxyvitamin D(3) by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion. J Am Soc Nephrol. 2002 Mar;13(3):621-9.

[41] van Etten E, Mathieu C.Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts. J Steroid Biochem Mol Biol. 2005 Oct;97(1-2):93-101.

[42] Bolland MJ, Grey A, Gamble GD, et al. The effect of vitamin D supplementation on skeletal, vascular, or cancer outcomes: a trial sequential meta-analysis. Lancet Diabetes Endocrinol. 2014 Apr;2(4):307-20.

[43] Albert, P., Proal, A., Marshall, T. Vitamin D: The alternative hypothesis. Autoimmunity Reviews. 2009;8(8), 639-644.

[44] Lechner J, Aschoff J, Rudi T. The vitamin D receptor and the etiology of RANTES/CCL-expressive fatty-degenerative osteolysis of the jawbone: an interface between osteoimmunology and bone metabolism. International Journal of General Medicine. 2018;11:155-166.

[45] Edfeldt K, Liu PT, Chun R, et al. T-cell cytokines differentially control human monocyte antimicrobial responses by regulating vitamin D metabolism. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(52):22593-22598.

[46] Cervantes JL, Dunham-Ems SM, La Vake CJ, et al. Phagosomal signaling by Borrelia burgdorferi in human monocytes involves Toll-like receptor (TLR) 2 and TLR8 cooperativity and TLR8-mediated induction of IFN-β. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(9):3683-3688.

[47] Marshall, T. Vitamin D discovery outpaces FDA decision making. Bioessays. 2008;30(2), 173-182.

[48] Blaney, G, Albert, P, Proal, A. Vitamin D Metabolites as Clinical Markers in Autoimmune and Chronic Disease. Annals Of The New York Academy Of Sciences. 2009;1173(1), 384-390.

[49] Yoshizawa T, Handa Y, Uematsu Y, et al. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat Genet. 1997 Aug;16(4):391-6.

[50] Bell NH, Shaw S, Turner RT. Evidence that 1,25-dihydroxyvitamin D3 inhibits the hepatic production of 25-hydroxyvitamin D in man. Journal of Clinical Investigation. 1984;74(4):1540-1544.

[51] Waldron JL, Ashby HL, Cornes MP, et al .Vitamin D: a negative acute phase reactant. J Clin Pathol. 2013 Jul;66(7):620-2.

[52] Waterhouse JC, Marshall TG, Fenter B, et al. High levels of active 1,25-dihydroxyvitamin D despite low levels of the 25-hydroxyvitamin D precursor-implications of dysregulated vitamin D for diagnosis and treatment of chronic disease. In: Stolzt VD, editor. Vitamin D: New Research. New York: Nova Science Publishers; 2006.

[53] Chen Y, Liu W, Sun T, et al. 1,25-Dihydroxyvitamin D Promotes Negative Feedback Regulation of Toll-Like Receptor Signaling via Targeting MicroRNA-155-SOCS1 in Macrophages. Journal of immunology (Baltimore, Md : 1950). 2013;190(7):3687-3695.

[54] Brahmachary M, Schönbach C, Yang L, et al. Computational promoter analysis of mouse, rat and human antimicrobial peptide-coding genes. BMC Bioinformatics. 2006;7(Suppl 5):S8.

[55] Marshall, T.G. 2006. VDR nuclear receptor competence is the key to recovery from chronic inflammatory and autoimmune disease. Days of Molecular Medicine. Stockholm, Sweden, May 24–2

[56] Arnson Y, Amital H, Shoenfeld Y. Vitamin D and autoimmunity: new aetiological and therapeutic considerations. Annals of the Rheumatic Diseases. 2007;66(9):1137-1142.

[57] Marshall, T. (2017, March 29). Sunshine/light exposure as therapy [scientific article]. Retrieved from: https://mpkb.org/home/othertreatments/sunshine#sunlight_can_be_immunosuppressive

[58] Proal A, Marshall TG. Re-framing the theory of autoimmunity in the era of the microbiome: persistent pathogens, autoantibodies, and molecular mimicry.Discov Med. 2018 Jun;25(140):299-308.