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Diagnostic and therapeutical role of vitamin D in chronic hepatitis C virus infection

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1. ABSTRACT

Although initially identified as a calcium homeostatic hormone, vitamin D is now known to have pleiotropic functions, dealing with both innate and adaptative immunity. Calcitriol mediates its biological effects by binding to the vitamin D receptor (VDR), which is expressed not only by intestine, bone and kidney but also on cell membranes of T lymphocytes, B lymphocytes, dendritic cells and macrophages. Vitamin D plays a role on the degree of liver damage in patients with chronic hepatitis C (CHC): low vitamin D levels have been associated with high hepatic necroinflammatory activity and progression of liver fibrosis. Vitamin D, in CHC patients, could also affect the response to antiviral therapy: in fact, recent studies have shown a relationship between low responsiveness to IFN-based therapy and low vitamin D serum levels. Further studies are required to better assess if vitamin D could work as a reliable noninvasive marker of liver fibrosis and whether vitamin D supplementation could be given to all CHC patients together with standard antiviral treatment, in order to improve the rate of sustained virological response (SVR).

2. INTRODUCTION

The term vitamin D refers collectively to vitamin D2 and vitamin D3: the former is produced in various plant materials, yeast and fungi when they are exposed to UVB radiation, the latter is synthesized in the skin by the exposure to solar UVB irradiation. When the skin is exposed to sunlight, UVB irradiation is absorbed by 7-dehydrocholesterol in the epidermis and dermis and is converted to previtamin D3. Once formed, previtamin D3 rapidly undergoes an isomerization induced by the body's temperature to form vitamin D3, also called cholecalciferol. Humans can obtain both forms in the diet by consumption of either animal or plant products. Biologically active vitamin D is generated via hepatic 25-hydroxylation catalyzed by different isoforms of cytochrome P450 (CYP), namely CYP2R1, CYP27A1 and others, to produce 25-hydroxyvitamin D (25 (OH)D). 25 (OH)D circulates in the blood bound to the vitamin D-binding protein (VDBP) and becomes fully activated when it is converted into 1,25-dihydroxyvitamin D (1,25 (OH)2D) by the mitochondrial 1-alpha-hydroxylase enzyme (CYP27B1). (1-5)

The majority of the body's 1,25 (OH)2D, also called calcitriol, is synthesized in the renal tubules of the kidney, but synthesis also occurs in numerous extrarenal cells that express CYP27B1, such as epithelial cells of the skin, lung, colon, parathyroid glands and immune cells, especially activated macrophages (6). Renal 1-hydroxylation is tightly controlled by calcium homeostatic signals, particularly circulating parathyroid hormone (PTH). PTH induces the production of CYP27B1 by primary renal tubules. As circulating levels of 1,25 (OH)2D rise, it suppresses its own production via a negative feedback loop. Unlike renal tissue, vitamin D production by macrophages is not regulated by Ca2+/PTH, but generates from different immune signals, such as gamma IFN and agonists of the TLR pattern recognition receptors (7-9).

Vitamin D signals are modulated through the vitamin D receptor (VDR) (10), a transcriptional factor belonging to the steroid/hormone receptor family. Target genes contain vitamin D response elements (VDREs) in their promoters, to which heterodimers of VDR and retinoid X receptors (RXRs) can bind and transactivate expression of the target genes (11). VDR activation in the intestine, bone, kidney and parathyroid gland cells leads to the maintenance of calcium and phosphorus levels in the blood and to the maintenance of bone content. The VDR is known to be involved in cell proliferation and differentiation and also in immunomodulatory activities: vitamin D may have a role in cancer, cardiovascular disease, autoimmune disorders and infections.

3. VITAMIN D AND INFECTIOUS DISEASES

Although initially identified as a calcium homeostatic hormone, vitamin D is now known to have pleiotropic functions, dealing with both innate and adaptative immunity, thus strongly influencing the pathophisiology of infectious diseases (12-14).

In fact calcitriol (the active form of vitamin D) mediates its biological effects by binding to the vitamin D receptor (VDR), which is expressed on cell membranes of T lymphocytes, B lymphocytes, neutrophils and antigen-presenting cells, such as dendritic cells and macrophages (15-18). In particular, dendritic cells and macrophages are also able to convert 25 (OH)D to 1,25 (OH)2D, the active form of the hormone (19-20). T- and B-cells are a target for vitamin D, which is able to inhibit T cell proliferation (21) and B cell Ig production (22). Vitamin D signaling represses the transcription of genes encoding TH1 cytokines, such as IL2 and gamma IFN (23, 24). This mechanism polarizes T-helper responses toward a regulatory TH2 phenotype (25). In addition to its direct effects on T cells, vitamin D can also indirectly control adaptative immunity, by modulating antigen presentation: vitamin D promotes the differentiation of myeloid precursors toward the macrophage-dendritic cell phenotype (26), but, at the same time, it is able also to block the more distal maturation of these cells (27-29); resulting in the inhibition of TH1 cells proliferation and a strong polarization towards TH2 response.

One of the most significant developments concerning vitamin D function refers to its influence on the immune response to Mycobacterium tuberculosis.

In 1986 Rook *et al* (30) described the *in vitro* inhibition of mycobacterial growth when vitamin D was added to the medium. Liu *et al* (31) have shown that activation of TLR2 by its ligand, the M.tuberculosis-derived 19-kDa lipoprotein, is able to upregulate the expression of VDR and CYP27B1 in macrophages; this allows the macrophages to synthesize 1,25 (OH)2D, which interacts with the VDR, engages the retinoid X receptor and then induces transcription of some antimicrobial factors, including a peptide of the class of defensins called cathelicidin (32, 33), whose gene product, LL-37, directs the mycobacterial killing. Many genetic and epidemiological studies have linked 25 (OH)D status to both TB disease progression and susceptibility. In 1985 Grange *et al* reported that, among 40 Indonesian patients with active TB treated with anti-TB chemotherapy, those with higher 25 (OH)D serum level at the onset of therapy had a "less active pulmonary disease" (34). In 2000 Wilkinson *et al* (35) found that patients with lower 25 (OH)D serum levels have an increased risk of TB and that some VDR genotypes correlate with overt disease, especially when associated with 25 (OH)D deficiency. Roth *et al* (36) have studied the association between VDR polymorphism and TB treatment outcome, by considering time to microbiological resolution of pulmonary TB, after initiation of a DOTS protocol in a cohort of Peruvian adults. They found that the Fok1 *FF* genotype was associated with faster conversion of mycobacterial cultures if compared with *Ff* and *ff* genotype; the Taq1 *Tt* genotype was associated with a significantly shorter conversion time, compared with *TT* genotype. Genetic variability at the VDR locus can partially explain the influence of ethnicity on individual immune response to TB.

Martineau *et al* (37) found that the blood coming from vitamin D-supplemented subjects was more able to suppress the multiplication of M. tuberculosis surrogate BCG *in vitro* if compared with that of placebo-treated contacts. Considering these results, vitamin D supplementation may prove to be an effective adjuvant therapy for patients with TB.

Vitamin D pathways relate to many infectious agents both bacterial and viral: we can hypothesize that any agent susceptible to vitamin D-induced antimicrobial peptides is a potential target. For instance vitamin D-induced cathelicidin is able to inhibit the growth of urinary pathogens, whereas it is rather ineffective against urogenital commensal bacteria (38)

VDR seems also to play an important role in intestinal homeostasis and host protection from enteric bacterial pathogens. Wu *et al* (39) demonstrated that VDR may modulate inflammatory responses associated with Salmonella typhimurium infection.

Jeng *et al* (40) evaluated the role of vitamin D status and cathelicidin production in patients admitted to

the intensive care unit with sepsis. They found that critically ill patients had lower 25 (OH)D serum levels if compared with healthy controls and also cathelicidin levels were significally lower in patients with sepsis compared with patients without sepsis.

In the first trimester of pregnancy maternal vitamin D deficiency has also been associated with bacterial vaginosis, which is a syndrome characterized by the loss of normal vaginal flora, predominantly Lactobacillus species, and an increased local prevalence of anaerobic bacteria. Bacterial vaginosis is associated with adverse pregnancy outcomes, especially with the risk of preterm birth. Bodnar *et al* (41) have shown an association between serum levels of 25 (OH)D and prevalence of bacterial vaginosis in a cohort of 469 pregnant women.

As refers to respiratory infections, Sabetta *et al* (42) published the results of a prospective cohort study regarding 198 healthy adults, whose serum concentrations of vitamin D were measured over the fall and winter 2009-10. The authors identified a two-fold reduction in the risk of developing viral respiratory infections in the group with 25 (OH)D levels exceeding 38 ng/mL in comparison with that whose 25 (OH)D levels were less than 38 ng/mL.

In general, a number of epidemiological studies well demonstrate a relationship between vitamin D deficiency and increased rate of respiratory infections, including pneumonia. Muhe *et al* (43) have reported that vitamin D deficiency is associated with a 13-fold increased risk of pneumonia in Ethiopian children under 5 years; in Yemen almost 50% of children admitted to hospital because of lower respiratory infections were rachitic (44). Wayse *et al* (45) have described the association between subclinical vitamin D deficiency and acute lower respiratory tract infections in non-rachitic children admitted to a private hospital in India. Karatekin *et al* (46) investigated the link between acute lower respiratory tract infections and vitamin D deficiency in Turkish newborns: they found that serum 25 (OH)D concentrations in patients affected with acute respiratory infections were lower than in healthy controls.

The role of vitamin D in HIV disease progression is still uncertain. Several authors have reported decreased concentrations of 1,25 (OH)2D in HIV patients: in a study in Norway, Haug *et al* (47) showed that 1,25 (OH)2D concentration was significantly lower in 54 HIV-infected patients compared with that of 20 healthy, seronegative donors. HIV-infected patients who were symptomatic had the lowest 1,25 (OH)2D levels. This datum could be due to a defect in the 1-alpha-hydroxylation of 25 (OH)D into 1,25 (OH)2D, probably because of an inhibitory effect of TNF-alpha. In fact a strong correlation between elevated TNF-alpha levels and decreased 1,25 (OH)2D was found. TNF-alpha appears to impair the stimulatory effect of PTH on 25 (OH)D hydroxylation. Furthermore, 1,25 (OH)2D serum concentration was positively correlated with CD4+ cell count.

The risk of HIV infection and disease progression has been examined also in relation to VDR polymorphisms. Barber *et al* (48) studied the prevalence of mutations in the region of the VDR gene corresponding to the BsmI restriction enzyme in relation to HIV status: there was no significant difference in the distribution of genotypes for the BsmI polymorphism between the 185 HIV+ intravenous drug users and the 120 controls which were enrolled, thus suggesting that this polymorphism does not affect susceptibility to HIV infection. Among HIV-infected individuals, those who were homozygous for *B* allele were at higher risk of progression to AIDS or a decline in CD4+ cells to under 200/mm3 after 7 years of follow up, compared with those homozygous for the *b* allele or heterozygous. We can hypothesize the low-risk VDR-*BB* genotype could be associated with reduced response to the immunosuppressive actions of vitamin D, which would normally induce a switch toward activation of TH2 cells and inhibition of TH1 responses. Lack of this inhibition would result in increased proliferation of TH1 cells susceptible to HIV infection.

4. VITAMIN D AND HEPATITIS C VIRUS INFECTION

Studies attempting to measure vitamin D levels in patients affected with non cholestatic chronic hepatitis or cirrhosis of different origins lead to controversial results: some Authors have shown normal levels of serum 25 (OH)D both in chronic hepatitis and cirrhosis (49-51), whereas other Authors reported a significant decrease of 25 (OH)D levels, the best estimate of overall vitamin D status, in comparison with healthy controls (52-54). In addition, low 25 (OH)D levels have been related to poor liver function because of the association between vitamin D status and hepatic function indexes (52) or the stage of cirrhosis (52,55). Accordingly, Arteh *et al* (56) found a high prevalence (92%) of vitamin D deficiency in a cohort of 118 patients with chronic liver disease. Interestingly, this was more common among patients with cirrhosis compared with noncirrhotics. It could be speculated that in advanced liver cirrhosis, the progressive impairment of the hepatic 25-hydroxylation can lead to lower levels of 25 (OH)D. As a consequence, many authors (49, 51-53, 55, 57-59) have focused on the evidence of a reduced mineral bone density in advanced chronic liver disease. Gonzalez-Calvin *et al* (59) described a correlation between low serum levels of 25 (OH)D and reduced bone mass density in cirrhotic individuals. In addition, a recent study (54) found lower 25 (OH)D serum levels in patients with fully compensated biopsy-proven non-alcoholic fatty liver disease, identifying an independent association between the histological characteristics of the disease and low 25 (OH)D levels. Accordingly Nobili *et al* (60) confirmed the inverse relation between vitamin D and severity of NAFLD in a cohort of children.

The status and the significance of vitamin D levels in patients with chronic hepatitis C (CHC) have been recently clarified by Petta *et al* (61). The authors evaluated serum levels of 25 (OH)D in a cohort of 197 patients with biopsy-proven genotype 1 (G1) CHC, at low prevalence of F4 fibrosis. 25 (OH)D levels in these subjects were significantly lower than in controls, also in patients with

minimal liver damage (F1). Low 25 (OH)D levels were independently associated with female sex and high necroinflammatory activity by a multivariate analysis. Tissue expression of CYP27A1 and CYP2R1, liver 25-hydroxylating enzymes, was assessed by immunochemistry in samples from 34 patients with CHC and from 8 subjects of the control group. The degree of expression of CYP27A1, but not CYP2R1, was directly related to vitamin D levels. CYP27A1 expression was also inversely associated with necroinflammation. Finally the authors identified low 25 (OH)D as independently associated to severe fibrosis (62).

All the above cited data therefore suggest a potential relevant role of vitamin D in the mechanisms regulating liver fibrogenesis, a process where hepatic stellate cells exert a central role, and characterized by the excess deposition of extracellular matrix (ECM), which is also altered in structure and profile. Recently, different experimental models have suggested a protective role of vitamin D against the biological mechanisms involved in the production and the progression of liver fibrosis. Vitamin D, by interaction with VDR, may influence the activity of fibroblasts (63, 64) and may reduce the fibrogenic function of liver stellate cells (65, 66). Accordingly, during inflammatory liver injury after endotoxin injection the activation of VDR signaling by vitamin D resulted in attenuation of liver damage *in vivo* (67).

Vitamin D deficiency has been associated with increased circulating levels of tissue matrix metalloproteinase MMP9, whose function has been related to hepatic fibrosis. The gene expression of MMP9 together with its inhibitor TIMP-1, is mediated by activating protein-1 (AP-1), whose activity is elicited *in vitro* by 1,25 (OH)2D. Timms *et al* (64) measured MMP9 and TIMP-1 levels in a cohort of 171 healthy adults and found that MMP9 related inversely to vitamin D status. Vitamin D deficiency was associated with raised MMP9 levels, that could be corrected by vitamin D supplementation. In this study the major independent determinant of TIMP-1 was the VDR polymorphism: in fact, the lowest TIMP-1 levels were associated with the *T* allele of VDR TaqI polymorphism. After one year of vitamin D supplementation there was an increase in molar TIMP-1/ MMP9 ratios. Thus, theoretically, vitamin D may result useful in all those disorders such as virus-induced hepatic fibrosis, where MMP9 upregulation contributes to pathogenesis. This would apply especially for those subjects carrying the *T* allele, since they appear to mount poor TIMP-1 responses to increases in MMP9.

Vitamin D, other than in nonparenchimal liver cells, could contribute to liver damage also by interacting with VDR in intestinal cells. Experimental animal models showed that VDR, together with FXR, another nuclear receptor, induce the expression of mouse fibroblast growth factor (Fgf) 15 (human ortholog FGF19), an intestine-derived hormone that is able to inhibit liver CYP7A1, a key enzyme in regulating lipid and metabolic homeostasis, well known factors contributing to liver damage (68, 69). Accordingly, in human hepatocytes, the Fgf1-mediated phosphorilation of the hepatic FGF receptor, via Erk1/2 pathway, inhibits CYP7A1 (70).

The growing interest to the mechanisms involved in liver fibrogenesis is due to the fact that, in HCV-infected patients, the prognosis and the clinical management of chronic hepatic disease are dependent on the extent of liver fibrosis. Biopsy, even if invasive, painful and with potentially life-threatening complications, remains the gold standard for staging liver disease (71). The diagnostic accuracy of a liver biopsy for assessing liver fibrosis is influenced by many factors: inconsistency in defining pathological features (variety of scoring systems), technical processing of the specimens and variations and quality of biopsy samples. In addition, inter- and/or intra-observer diagnostic discrepancies are estimated to affect 10–20% of assessments of hepatic fibrosis (72-74). The high prevalence of chronic hepatitis C, in addition to the cost and constraints generated by this procedure, has triggered an intensive search for alternative, non-invasive methods for staging the disease. These methods include methodologies derived from elaboration of parameters obtainable with the current liver imaging techniques (ultrasound, computed tomography (CT) scan and magnetic resonance), or from innovative uses of the principles of physics (Fibro-CT, MRI-Elastography, MRI-Spectroscopy, transient elastography (TE)) and acoustic radiation force impulse (ARFI) (75-78). Several alternative biochemical methods have been investigated to predict the stage of liver fibrosis. Fibrotest and Fibrosure, the Forn's index, the Apri test, the Hepascore and the Fib-4 are all indirect biomarkers of liver fibrosis, easy to be used in clinical practice (79-84).

In this complex area of research focused on the identification of noninvasive strategy to diagnose the severity of fibrosis, the determination of serum levels of vitamin D could be useful. In this line Petta *et al*, in CHC, identified low 25 (OH)D as a risk factor for the detection of fibrosis severity, together with other well known noninvasive predictors of fibrosis, such as older age, high grading, high ferritin and low cholesterol levels (62). Interestingly, the overall area under curve (AUC) of the model was good (0.870). In this line, Ho *et al* (85) investigated the serum levels of another vitamin D-related marker, VDBP, in CHC patients, showing that their levels appeared to be down regulated. Moreover the authors demonstrated that serum concentration of VDBP progressively decreased from F0/F1 to F2/F4 stages, suggesting a potential use of VDBP, together with other known biomarkers, to predict the stage of liver fibrosis and, thus, to reduce the extensive use of liver biopsy.

Vitamin D, in CHC patients, could also affect the response to antiviral therapy. The current standard of care for CHC treatment is the combination of pegylated interferon (PEG-IFN) plus ribavirin (RBV) (86, 87). The standard duration of treatment is 48 weeks for G1 and 24 weeks for G2 and G3 (88-90). The introduction of the combination therapy considerably improved the rate of sustained virological response (SVR) in CHC patients, but at the expense of higher cost and poorer tolerability (91).

Moreover, slightly more than half of patients infected with HCV G1 still do not respond to therapy and early treatment withdrawal is necessary to avoid useless side-effects and expense (92). Several virus- and host-related predictors of treatment response are known, such as HCV genotype, baseline viral load, age, body weight, race, gender, liver histology, baseline gamma glutamyltransferase level, baseline alanine aminotransferase level and insulin resistance (93-95). Also, monitoring of early viral kinetics has been shown more useful in predicting long-term treatment outcome (96, 97). Several drugs, such as amantadine (98, 99), silibinin, silymarin (100), cyclophilin inhibitors (101, 102) and nitazoxanide (103, 104) have been attempted alone or in combination with PEG-IFN and RBV in order to increase the SVR rate among G1 HCV-infected individuals and the more promising results will be probably obtained with the use of STAT-C agents (REF), in spite of a great number of side effects. Vitamin D seems to have the potential to favourably influence the virological outcome of PEG-IFN plus RBV treatment in CHC. Again, this datum is supported by the recent findings concerning vitamin D immunomodulatory functions. It has been established that T-cells depend on vitamin D to be activated and to proliferate (105); therefore, it is conceivable that vitamin D deficiency may impair T-cell ability to react against a number of pathogens including HCV. Apart from the immunological activity, some reports indicate vitamin D2 to be able to inhibit HCV RNA replication *in vitro* (106). The mechanisms of this direct antiviral activity are not clearly elucidated though it seems probable that vitamin D2 results in a profound inhibition of RNAs and protein synthesis, by acting on the internal ribosome entry site, NS3-4A serine protease and NS5B polymerase.

In the study of Petta *et al* (61), 167 CHC patients underwent antiviral therapy with PEG-IFN plus RBV. In this group, SVR was achieved by 70/167 patients; low 25 (OH)D levels, low cholesterol and greater steatosis were found to be independent negative risk factors for SVR. The observation of a relationship between low responsiveness to IFN-based therapy and low vitamin D serum levels is confirmed by Bitetto *et al* (107), who studied a cohort of 42 liver transplanted subjects with recurrent hepatitis C (RHC), treated with combination therapy including IFN-alpha and RBV for 48 weeks. 25 (OH)D serum levels were measured in all patients before starting antiviral treatment. In 15 patients oral vitamin D supplementation was administered to avoid bone loss. 16/42 patients achieved SVR and baseline 25 (OH)D levels were identified, together with infection by HCV genotype other than 1, as independent positive predictors of SVR. The association between the outcome of antiviral therapy and the degree of vitamin D deficiency was strict and clear: patients with severe vitamin D deficiency (less than or equal to 10 ng/ml) almost never achieved SVR (only one out of ten), whereas those with higher vitamin D levels (more than 10 and less than or equal to 20 ng/ml) obtained SVR in 30% of the cases and, finally, those with vitamin D concentrations higher than 20 ng/ml, showed a SVR rate of 50%. Furthermore, those patients who were supplemented with cholecalciferol during antiviral therapy achieved SVR more frequently than patients who were not supplemented. In particular, those patients who had serum levels of 25 (OH)D higher than 20 ng/mL and received cholecalciferol supplementation seemed able to achieve the higher rate of SVR in comparison with the other groups. This result was not dependent on HCV genotype, because there was not difference in the prevalence of G1 among the three groups. Having received cholecalciferol supplementation in the presence of a normal or near to normal baseline serum vitamin D concentration and being infected by a HCV genotype other than 1 were the only variables independently associated with SVR. In line with these observations, preliminary data from a small randomized controlled trial on a little cohort of 72 CHC patients randomized to standard of therapy versus standard of therapy plus vitamin D, reported higher rates of early virological response and SVR in CHC patients in the experimental arm (108). All these data highlight the link between vitamin D status and outcome of antiviral treatment of patients affected with CHC and suggest a potential role of vitamin D in modulating viral clearance. Further studies are required to better assess any possible synergism between the standard antiviral therapy against HCV and vitamin D supplementation. In addition, as refers to patients transplanted for HCV related end stage liver disease, they frequently exhibit a remarkable vitamin D deficiency: recent studies (107) detected an inverse association between low pretransplant 25 (OH)D and risk of acute cellular rejection, while post-transplant daily supplementation with cholecalciferol could prevent it.

Finally, it should be interesting also to evaluate the impact of polymorphisms affecting vitamin D serum levels, like those near genes involved in cholesterol synthesis, hydroxylation and vitamin D transport (109), as well as polymorphism affecting VDR expression, like Cdx2 A>G, FokI and the BAT haplotype (110), on the severity of liver fibrosis and the response to antiviral therapy.

In conclusion we are still uncertain whether 25 (OH)D or VDBP could work as reliable noninvasive markers of liver fibrosis. Similarly, it remains to be elucidated whether vitamin D supplementation should be given to vitamin-deficient chronic hepatitis C patients or, as an alternative, extensively to all patients together with standard antiviral treatment. Large, placebo-controlled, randomized trials are needed to find an answer.

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Abbreviations: VDBP: vitamin D binding protein; 25 (OH)D: 25-hydroxyvitamin D; PTH: parathyroid hormone; 1,25 (OH)2D: 1,25-dihydroxyvitamin D; VDR: vitamin D receptor; IFN: interferon; VDRE: vitamin D response element; TLR: Toll-like receptor; TB: tuberculosis; BCG: bacillus Calmette-Guerin; TNF: tumor necrosis factor; HIV: human immunodeficiency virus; NAFLD: non-alcoholic fatty liver disease; CHC: chronic hepatitis C; G1: genotype 1; ECM: extracellular matrix; MMP: matrix metalloproteinase; TIMP-1: tissue inhibitor of metalloproteinases-1; AP-1: activating protein-1; SVR: sustained virological response; RBV: ribavirin; RHC: recurrent hepatitis C

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