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Vitamin D deficiency and vitamin D therapy in chronic hepatitis C

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ABSTRACT

Background. Vitamin D has immunomodulatory properties, exerts an anti-hepatitis C virus (HCV) effect *in vitro* and improves response to interferon-based therapy in patients with chronic hepatitis C (CHC). Low serum levels of 25(OH) vitamin D [25(OH)D] are frequently found in CHC patients and seem to be related to more advanced stages of liver fibrosis. The study aims to establish the incidence of vitamin D deficiency in Spanish patients with CHC, its possible relation with features of liver damage and with the IL28B gene polymorphism, and the immediate effect of vitamin D therapy on CHC-related analytical variables. **Materials and methods.** Baseline serum 25(OH)D levels were measured in 108 consecutive CHC patients (60 men, age 54.3 ± 10.5 yrs). Results of transient elastography and of IL28B rs12979860C/T genotype were available in 89 and 95 patients, respectively. Forty one patients with insufficient levels of 25(OH)D received vitamin D supplements and were re-evaluated thereafter. **Results.** Deficiency of vitamin D (< 20 µg/dL) and suboptimal levels (20-30 µg/mL) were detected in 36.1% and 40.9% of patients, respectively. No relationships were found between 25(OH)D levels and biochemical liver tests, fibrosis stage and IL28B genotype. Vitamin D therapy normalized 25(OH)D levels in all treated patients, but did not modify significantly HCV-RNA serum levels or biochemical tests. **Conclusions.** Vitamin D deficiency is common in Spanish patients with CHC but it is related neither to biochemical and virological variables nor with the fibrosis stage and IL28B polymorphism. Vitamin D therapy has no immediate effect on HCV-RNA serum levels.

Key words. Hepatitis C virus. Calcitriol. Fibrosis. IL28B.

INTRODUCTION

Calcitriol [1,25(OH)2 Vitamin D], the active form of vitamin D, results from two successive hydroxylations of vitamin D that take place in the liver (25-hydroxylation) and in the kidney (1-hydroxylation).[1,2] Calcitriol is a component of the hormonal system that maintains calcium and phosphorus homeostasis. In addition, vitamin D has immunomodulatory effects as it reduces the levels of proinflammatory cytokines and promotes innate immune response.[3-5] As extrarenal synthesis of calcitriol has been demonstrated in many other tissues, it allows local availability of the active vitamin as an autocrine/paracrine factor in the exact site and moment where it is needed.[1]

Serum 25-hydroxy vitamin D [cholecalciferol, 25(OH)D] is the main circulating form of vitamin D and the most appropriate indicator of vitamin D status. The optimal level of serum 25(OH)D has been established at ≥ 30 ng/mL[6] although the limit for

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clinical deficiency has been proposed at a level < 20 ng/mL. The intermediate range between 20 and 30 ng/mL is considered as insufficiency of vitamin D that should be corrected.[1] Several studies have unanimously shown that vitamin D deficiency is very common in patients with chronic hepatitis C virus infection and directly correlates with the stage of fibrosis both in monoinfected[7-10] and in HCV-HIV coinfected[11] patients.

In addition, vitamin D deficiency is associated with poor response to interferon-based therapy in patients with chronic hepatitis C[10,12,13] and vitamin D supplementation may improve the rate of sustained viral response (SVR: non detectable HCV-RNA in serum 6 months after the end of antiviral therapy).[14,15]

Three different *in vitro* assays have shown that vitamin D suppresses HCV production suggesting a direct antiviral effect of the vitamin[16-18] but, to the best of our knowledge, no previous studies have been published on the possible beneficial effect of isolated vitamin D therapy on the natural history of chronic HCV-induced liver disease.

The aim of the present study has been to establish the incidence and severity of vitamin D deficiency in a cohort of Spanish patients with chronic hepatitis C, its possible relation with the clinical features of the disease and the effect of vitamin D supplementation on biochemical and virological parameters in a subset of patients with deficiency or suboptimal levels of serum vitamin D.

MATERIAL AND METHODS

Patients

During the 5 first months of 2012 (from January 18 to May 31) we systematically determined the serum levels of 25(OH)D in all the HCV-infected outpatients attending to our Liver Unit excluding those under current antiviral therapy or those who had been treated in the previous six months (regardless of the results of therapy), and those who had obtained sustained viral response (SVR) to former therapy. A total of 182 patients were screened. For the aims of the present study, patients were excluded if they had evidence of decompensated liver cirrhosis and/or hepatocellular carcinoma (3 cases), a positive HIV antibody test result, a positive result for HBsAg (surface antigen of the hepatitis B virus) (1 case), a serum creatinine level ≥ 1.25 mg/dL (2 cases) or had used calcium and/or vitamin D supplements in the previous 3 months (72 cases). The remaining 108 patients (67 men, mean age 51.1 ± 9.3, and 41 women, mean age 59.6 ± 10.3 years, $p < 0.001$) fulfilled the requisites to be included in the study. All patients provided informed consent in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of the Hospital Clínico San Carlos, Madrid, Spain.

Data on the stage of liver fibrosis measured with transient elastography performed in the previous 6 months were available on 89 patients. The IL28B rs12979860C/T polymorphism was determined in 95 patients. Methods to determine HCV load in plasma, HCV genotype and subtype and IL28B rs12979860C/T genotype have been described elsewhere.[19] Haematological and biochemical analysis were performed by standard tests. Sixty patients were naïve for antiviral therapy whereas the remaining 48 had received unsuccessful interferon-based therapy more than a year before their inclusion in the study.

Determination of 25 (OH)Vitamin D in serum

A venous blood sample was collected after overnight fast with a Vacutainer® System (Becton Dickinson, Frankin Lakes, USA). After 30 minutes, samples were centrifuged at 4 ºC and refrigerated until analysis.

Total 25-OH Vitamin D was measured by a chemiluminescent microparticle immunoassay in an Architect® Analyzer (Abbott Diagnostics, Wiesbaden, Germany). An aliquot of the pre-treated sample is combined with paramagnetic anti-vitamin D coated microparticles. A biotinylated vitamin D anti-Biotin acridinium-labeled conjugate complex is added to the reaction mixture and binds to unoccupied binding sites of the anti-vitamin D coated microparticles. After washing, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as Relative Light Units (RLUs). An indirect relationship exists between the amount of vitamin D in the sample and the RLUs detected by the ARCHITECT System optics.

The method has a functional sensitivity below 4 ng/mL, analytic range to 160 ng/mL and the interassay coefficient of variation was 6.9%, 5.2%, and 3.9% for levels of 20, 40 and 75 ng/mL of 25-OH Vitamin D respectively. The quality of method is evaluated by Vitamin D External Quality Assessment Scheme (DEQAS).

Vitamin D deficiency is defined as a 25(OH)D < 20 ng/mL and vitamin D insufficiency as a 25(OH)D of 20-29,9 ng/mL, in accordance with current guidelines.[6]

Vitamin D therapy

Most patients with deficiency or insufficiency of vitamin D were recommended to correct this status with vitamin D therapy. Those patients that accepted were scheduled to receive oral supplements of 25(OH)D at a dose of 4,000 IU/day for patients with 25(OH)D < 10 ng/mL; 2,000 IU/day for patients with 25(OH)D between 10 and 20 ng/mL; and 1,000 IU/day for patients with 25(OH)D between 20 and 30 ng/mL, in accordance with recent recommendations.[1] After 5-7 weeks of therapy, a blood sample was obtained to measure haematological, biochemical and virological parameters and 25(OH)D serum levels in order to evaluate the possible effects of vit D suplementation on these parameters and to schedule a maintenance dose of vitamin D.

Statistical analysis

Continuous variables, expressed as mean (SD), were compared with the Student' t test or the Mann-Whitney U test, each when adequate, depending on their Gaussian distribution. The Spearman correlation test was used to express correlations between variables. A p value < 0.05 was considered significant. Categorical variables were compared with the χ^2 or the Fisher exact tests, each when appropriate, and the effect of differences was established by calculating the odds ratio with the 95% confidence interval. The statistical analysis was carried out using SPSS software (version 17) (Centers for Disease Control, Atlanta) and with Epi-Data software for specific tests.

RESULTS

Baseline analysis

Table 1 reflects the demographical, clinical, biochemical and virological characteristics of the patients. At baseline 39 patients (36.1%) had vitamin D deficiency [25(OH)D < 20 ng/mL] that was severe [25(OH)D < 10 ng/mL] in 6 (5.6%) of them. Fifty five patients (50.9%) had suboptimal levels [25(OH)D = 20-30 ng/mL] and 14 patients (13%) had normal levels [25(OH)D ≥ 30 ng/mL].

No significant correlationships were found between 25(OH)D serum levels and baseline variables, including viral load, with the exceptions of serum calcium (rho = 0.207, p = 0.032) and phosphorus (rho = -0.195, p = 0.044). Serum 25(OH)D levels were significantly higher in men than in women (23.02 ± 6.85 ng/mL *vs.* 20.21 ± 7.45 ng/mL, p = 0.041).

The IL28B rs12979860 gene polymorphism was determined in 95 patients. No differences were found in 25(OH)D serum levels among the three possible genotypes (CC: 27 cases; CT: 54 cases; TT: 14 cases) (Figure 1).

The fibrosis stage of the liver was indirectly measured by transient elastography (Fibroscan®) in 89 patients. Again, no differences in 25(OH)D serum levels were found among the 4 fibrosis stages defined with this method (0-1: 33 cases; 2: 16 cases; 3: 13 cases; 4: 27 cases) (Figure 2).

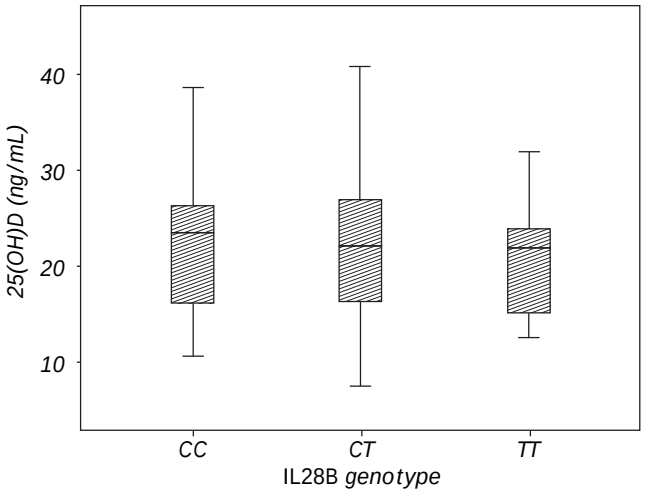

Figure 1. *Distribution of 25(OH)D serum levels according to the IL28B genotype in 95 patients with chronic hepatitis C.*

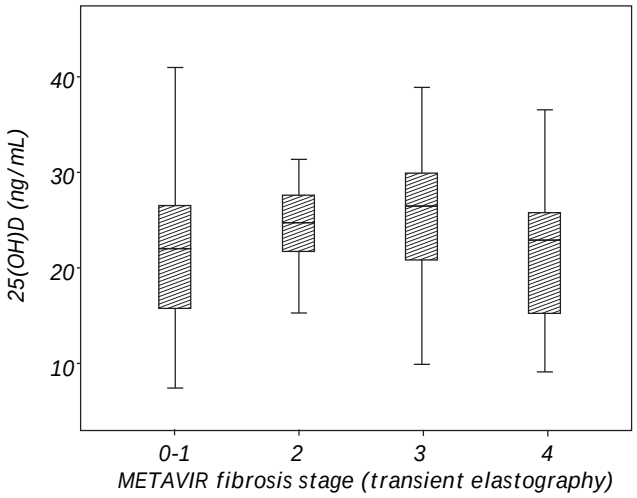

Figure 2. *Distribution of 25(OH)D serum levels according to the METAVIR fibrosis stage determined by transient elastography in 89 patients with chronic hepatitis C.*

Vitamin D supplementation

Forty one patients (24 men) with deficiency or suboptimal levels of serum 25 (OH) Vit D (range 6.4-28.8 ng/mL) completed therapy with vitamin D supplements and the laboratory tests were repeated as described in methods. As in the whole cohort, women were older than men (61.7 ± 9.4 *vs.* 52.2 ± 9.0 years, $p < 0.001$).

Results are shown in table 2. Thirty seven patients reached optimal serum levels of 25(OH)D (range 30.6-93.6 ng/mL), whereas 4 patients showed values considered as potentially toxic (from 103 to 150 ng/mL). In these 4 patients the determination was repeated two weeks after the end of vitamin D therapy and in all cases the values decreased to the optimal interval.

No significant changes were observed when comparing baseline and end-of-therapy values of haematological and biochemical parameters. HCV viral load (expressed as log 10) experienced a non-significant increase at the end of therapy ($p = 0.121$).

DISCUSSION

Our study confirms that vitamin D deficiency is very common in Spanish patients with chronic hepatitis C, as only 13% of patients had 25(OH)D serum levels considered as optimal. Indeed, if the lower level of optimal 25(OH)D status were fixed at 32 ng/mL, as suggested by Hochwald, *et al.*,[20] in Israel (a country with a climate similar to that found of Madrid, Spain), only seven patients (6.5%) in our study would have been classified in this group. The higher value found in our patients was 40.8 ng/mL. Our study was carried out during the first 5 months of 2012, and it is known that vit D levels are lower in the winter.[21] However, it should be noted that the first three months of 2012 were unexpectedly warm and sunny in the Madrid area,

Table 1. Analysis of biochemical and virological variables in relation with the serum level of 25(OH)D in 108 patients with chronic hepatitis C.

Variable		Serum 25(OH)D		Significance
	All patients (108 cases)	< 20 ng/mL (39 patients)	≥ 20 ng/mL (69 patients)	
Age (yrs)	54.3 (10.5)	56.4 (10.0)	53.1 (10.7)	0.122
Gender (M/F)	67/41	20/19	47/22	O.R. = 0.493. 95 % C.I. = 0.220-1.104
Platelets (10^9/L)	177 (67)	168 (70)	183 (65)	0.272
Bilirubin (mg/dL)	0.96 (0.45)	0.96 (0.49)	0.97 (0.43)	0.686
AST (IU/L)	74 (50)	68 (43)	78 (54)	0.613
ALT (IU/L)	92 (72)	76 (42)	101 (83)	0.217
GGT (IU/L)	82 (76)	88 (68)	79 (81)	0.213
Alkaline phosphatase (IU/L	91 (33)	90 (31)	92 (35)	0.883
Cholesterol (mg/dL)	166 (34)	162 (39)	168 (31)	0.332
Triglycerides (mg/dL)	109 (55)	118 (62)	104 (51)	0.107
Blood glucose (mg/dL)	104 (25)	97 (12)	107 (29)	**0.022**
Uric acid (mg/dL)	5.5 (1.5)	5.2 (1.5)	5.6 (1.5)	0.174
Serum calcium (mg/dL)	9.51 (0.42)	9.40 (0.33)	9.57 (0.45)	**0.041**
Serum phosphorus (mg/dL)	3.25 (0.53)	3.32 (0.37)	3.22 (0.60)	0.255
Ferritin (ng/mL)	273 (274)	344 (342)	233 (219)	0.105
HCV-RNA (log. IU/mL) 10)	6.36 (0.70)	6.36 (0.71)	6.35 (0.70)	0.926
HCV viral load ≤/> 400,000 IU/mL	13/95	Abr-35	Sep-60	O.R. = 0.762. 95 % C.I. = 0.218 – 2.658
Viral genotype 1/non 1.	87/21	30-Sep	57/12	O.R. = 0.702. 95 % C.I. = 0.266 – 1.853
Il28B rs12979860 T allele carriers/non carriers [1]	*68/27*	*25-Sep*	*43/18*	*O.R. = 1.163. 95 % C.I. = 0.454 – 2.976*
Transient elastography fibrosis stage (≤ 2 / > 2) [2]	49/40	17-Dic	32/28	O.R. = 1.240. *95 %* C.I. = 0.506 – 3.036
Naïve/former therapeutic failure	60/48	23/16	37/32	O.R. = 1.243. *95 %* C.I. = 0.562 – 2.571

[1]Available in 95 patients. [2]Available in 89 patients.

Table 2. Effect of vitamin D supplementation on biochemical and virological variables in 41 patients with chronic hepatitis C and deficiency or insufficiency of vitamin D.*

Variable	Baseline	End of vit. D therapy	P value
Serum 25(OH)D (ng/mL)	18.39 (5.68)	59.66 (25.93)	< 0.001
HCV-RNA (log. 10 IU/mL)	6.37 (0.68)	6.48 (0.55)	0.121
ALT (IU/L)	88 (71)	90 (80)	0.783
AST (IU/L)	74 (53)	77 (52)	0.634
GGT (IU/L)	76 (52)	81 (65)	0.653
Cholesterol (mg/dL)	162 (40)	161 (42)	0.699
Triglycerides (mg/dL)	108 (53)	101 (53)	0.457
Blood glucose (mg/dL)	98 (14)	100 (11)	0.530
Uric acid (mg/dL)	5.4 (1.4)	5.8 (1.4)	0.042
Serum calcium (mg/dL)	9.5 (0.5)	9.6 (0.4)	0.118
Serum phosphorus (mg/dL)	3.3 (0.4)	3.3 (0.5)	0.862

*Values are shown as mean (SD).

whereas April and May were very cloudy and rainy. Hence, the exposition to the sunlight was greater in winter than in spring (data from the Spanish Agencia Estatal de Meteorología, available at www.aemet.es). These factors probably explain why the proportion of patients with severe vitamin deficiency (< 10 ng/dL) is lower than that found in other studies performed in northern latitudes.[10]

Baseline serum vitamin D levels were significantly lower in women, as previously shown,[12] but this difference may be explained, at least in part, by the older age of the women included in the study as age is inversely correlated with 25(OH)D serum levels.[21,22] The significant correlations found between serum vitamin D and serum calcium and phosphorus may be explained by the role of vitamin D in calcium/phosphorus homeostasis. Glycemia was slightly although significantly lower in patients with low vitamin D levels (< 20 ng/dL) but there is no significant correlation between these two variables (rho = 0.194). No relationships were found with the remaining routine biochemical tests, or with HCV viral load or genotype.

Previous reports[10-12] had pointed out that the deficiency of vitamin D is more severe in patients with moderate-advanced fibrosis (METAVIR F2-F4) than in patients with null-low fibrosis (METAVIR F0-F1). We estimated liver fibrosis with transient elastography in 89 patients and our results do not confirm this relationship.

Polymorphism in the vicinity of the IL28B gene is considered as the most powerful predictor of viral response to interferon-based HCV therapy.[23] It has been shown that the association of low vitamin D serum levels and an unfavourable IL28B genotype increases the risk of therapy failure,[13] but these two predictors were completely independent of each other as the IL28B genotype is not related to 25(OH)D serum level. In this study, we determined the IL28B rs12979860C/T polymorphism in 95 patients and confirmed the absence of any relationship between these two variables.

This is a cross-sectional study that does not permit evaluation of variability of vitamin D serum levels related with seasonal climate changes. Although we not include a control group composed of healthy sex- and age-matched subjects living in the same area, results from the most representative study on serum vitamin D values performed on the Spanish population[21] show very similar proportions of 25(H)D deficiency (< 20 ng/mL) and suboptimal levels (20-30 ng/mL) to those found in our HCV-infected patients (33.9% *vs.* 36.1% and 56.2% *vs.* 50.9%, respectively). Hence, vitamin D deficiency is not more frequent in our patients with chronic hepatitis C than in the general Spanish population.

As vitamin D supplementation in HCV-infected patients seems to improve the efficacy of antiviral therapy,[15] we have hypothesized that isolate administration of this vitamin in patients with low serum levels of 25(OH)D could have a beneficial effect on virological or biochemical parameters. In support of this hypothesis, three different *in vitro* assays[16-18] have shown that vitamin D suppresses HCV replication, a finding that is in agreement with the inmunomodulatory effect of vitamin D.[2,3] However, in this study we observed no effect on viral load or on liver-related biochemical tests after fully correcting low serum levels of vitamin D in 41 patients with chronic hepatitis C. Accepting as clinically significant a drop of 0.5 log10 in HCV-RNA serum concentration (from 6.12 to 5.62), a sample size of 7 cases

was sufficient to test the hypothesis that vitamin D therapy may have some therapeutic value in chronic hepatitis C ($\alpha < 0.05$; $\beta > 0.80$, bilateral test).

We conclude that vitamin D deficiency is common in Spanish patients with chronic hepatitis C, but it is related neither with the fibrosis stage nor with the *IL28B* gene polymorphism. Short-term therapy with vitamin D fully normalizes serum 25(OH) vitamin D levels in these patients but has no immediate effect on viral load and does not improve biochemical markers of liver necroinflammation.

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