The serum level of 25-hydroxyvitamin D and the hepatic necroinflammatory activity in hepatitis C virus infection

Simona Alexandra Iacob, Dorina Banica, Eugenia Panaitescu, Manole Cojocaru and Diana Iacob

Abstract— Low levels of serum 25-hydroxyvitamin D (25(OH)D) related to severe fibrosis have been recently reported in hepatitis C virus (HCV) infection. The objective of our study was to assess the plasma levels of 25(OH)D in HCV infection and its correlation with the immune status and necroinflammatory activity. The level of serum calcium (Ca), phosphorus (Ph), 25(OH)D, alanine aminotransferase , aspartate aminotransferase, and the count of CD4+T cells and CD8+T cells were measured in 25 patients with HCV infection and 21 controls and correlated with the Actitest/Fibrotest results. All the HCV patients and healthy controls displayed a significantly lower plasma level of 25(OH)D (29.5714 nmol/l, respectively 29.2701 nmol/l). Despite the critically low 25(OH)D concentration, the phosphocalcium serum values were in the normal range. The patients with a high necroinflammation score (A2-A3) displayed a strong positive correlation between 25(OH)D and ALT plasma levels (R=0.530) as well as between the 25(OH)D and the CD4+T cells number (R=0.703). In these patients, the CD4+T cells count was correlated not only with the 25(OH)D serum level but also with the serum calcium concentration (R=0.841). Patients with a lower inflammation score (A0-A1) presented a negative correlation between 25(OH)D and TGO (R= - 0.548). In conclusion, strong positive correlations were found between the 25(OH)D plasma levels, the immune status and calcium levels in patients with HCV and high necroinflammatory activity.

Keywords—25-hydroxyvitamin D, Hepatitis C virus, Hepatic necroinflammation

I. INTRODUCTION

The evolution towards cirrhosis of HCV patients is determined by multiple factors related to both HCV strain and the biological and genetic parameters of the host [1]. As such, polymorphisms of vitamin D receptors (VDR) and vitamin D deficiency were reported in HBV patients [2], [3] and recently in HCV patients [4], [5]. The explanation resides in the excruciating importance of the liver function concerning vitamin D metabolism by 25-hydroxylation of vitamin D, bile removal of vitamin D metabolites and the synthesis of vitamin D binding protein). Thus the vitamin D deficiency registered in patients with chronic hepatitis and cirrhosis could lead to osteoporosis [6]; the treatment with vitamin D is presently indicated for these high risk categories. Recent studies suggests a relation of vitamin D status with fibrosis progression and response to the interferon-based therapy [4], [5]. However the actual role of vitamin D in the hepatic inflammatory process was only sporadically studied. Taking into account the anti-inflammatory actions displayed by vitamin D in several in vitro studies [7], [8], it is questionable whether vitamin D could also influence hepatic inflammation in HCV patients and if so, which are the stages of the HCV infection requiring higher vitamin D levels.

II. METHODS AND MATERIALS

Patients. A number of 46.Caucasian subjects aged 43.6years (SD=16.14), 17 males and 29 women, HIV negative, were selected. Out of these, 25 subjects were diagnosed with HCV infection and 21 were healthy controls. The patients presented a body mass index < 30kg/m2, no underlying renal disease, cardiovascular disease, diabetes, autoimmunity or malignancy, not taking any medications or vitamin supplements.

Samples. Samples were collected between January and September 2009. Samples were taken after the informed consent was obtained. Serum collection for 25-hydroxyvitamin D (25(OH)D) , were obtained in EDTA tubes and centrifuged for 20 minutes at 1100 – 1300 rpm. then stored at -80°C prior to analysis.

Tests. Subjects had baseline laboratory tests performed by standard hospital laboratory methods including: hemogram, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total and ionized serum calcium, serum phosphorous, hepatitis viral markers (Anti-HCV, HBsAg, Anti-HBs, HBcAg, Anti-HBc IgG/IgM), Anti-HIV, CD4+T cell and CD8+T cells (flow cytometric detection). The RNA HCV viral load was detected with Real-time PCR assay (Roche Cobas TaqMan, limit of detection 45ui/ml). The Fibrotest and Actitest score (BioPredictive) was used to assess the fibrosis and necroinflammatory liver activity.
Plasma levels of 25(OH)D, expressed as nmol/L were assessed using Elisa (IDS 25-Hydroxy Vitamin D EIA kit, Immunodiagnostic Systems Ltd, UK - detection range 6–360 nmol/L).

All protocols followed the manufacturer’s Instructions. Each Elisa test was run in duplicate, with mean absorbance computed from the average for 2 wells normalized to a zero calibrator well. Levels of vitamin D in test samples were derived by fitting a 2-parameter logistic curve to 6 standard levels. The intra-assay CV for 25 (OH)D and LL37 were <8% and <10% respectively. The inter-assay CV for 25 (OH)D and LL37 were <10% for both.

Diagnosis. Diagnosis of HCV infections followed CDC criteria (Guidelines For Viral Hepatitis Surveillance And Case Management, 2009 http://www.cdc.gov/hepatitis/SurveillanceGuidelines.htm). The scores for necro-inflammatory activity range were considered from A0 to A3 (A0= no activity, A1 = minimal activity, A2 = moderate activity, A3 = severe activity). Fibrosis was scored from F0-F4 Cirrhosis was defined by Fibrotest score (F≥4), abdominal ultrasonography and liver function tests. [9], [10], [11].

Vitamin D status was defined according to 25(OH)D serum levels. Vitamin D deficiency/ insufficiency was defined as a 25(OH)D concentration <80nmol/L and <50 nmol/L respectively.

The HCV status was classified according to the Actitest (A0/A3) and Fibrotest (F0-F4). 13 patients presented low necroinflammatory activity (A0-A1) and 12 patients presented a high necroinflammatory activity (A2-A3). 5 patients were classified as cirrhosis (F4) and 20 as chronic hepatitis.

The immune status was evaluated by CD4+T cell and CD8+T cell count and CD4/CD8 index.

Statistical analysis: Results were given as means or median. When Bartlett’s test indicated that the group comparisons had equal variances Student T or one-way ANOVA and Tukey’s multiple comparison post hoc tests were performed. When the group data showed unequal variances, nonparametric Mann-Whitney or Wilcoxon/Kruskal-Wallis and Dunn’s multiple comparison post hoc tests were used. Correlations were evaluated for statistical significance with Pearson’s test.

P < 0.05 was considered significant. Statistical tests were performed using SPSS software (version 15).

The study was performed in accordance with the principles of the Declaration of Helsinki. Approval was obtained from the hospital’s Institutional Review Board and Ethics Committee and written informed consent was obtained from all patients and controls.

III. RESULTS

The study evaluates 25 patients with HCV infection, genotype 1 (RNA HCV between 22808 and 2770x10^6 ui/ml) and 21 healthy subjects.

Subjects were classified according to the underlying necrotic inflammatory activity into patients with active HCV hepatitis (A2-A3) and patients with inactive HCV hepatitis (A0-A1).

Relevant demographic and biological data collected from HCV patients are presented in table 1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HCV patients</th>
<th>controls</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>50.58±14.87</td>
<td>35.61±13.90</td>
<td>0.0012</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>8/17</td>
<td>9/12</td>
<td>0.4473</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>70</td>
<td>25</td>
<td>0.0000</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>59</td>
<td>23</td>
<td>0.0000</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>6.32 x 10^3 ±2.1248 x 10^3</td>
<td>7.5143 x 10^3 ±1.7746 x 10^3</td>
<td>0.0483</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.2560 x 10^3 ±0.8073 x 10^3</td>
<td>2.2857 x 10^3 ±0.7023 x 10^3</td>
<td>0.8957</td>
</tr>
<tr>
<td>CD4 number cells</td>
<td>836.13 ±310.5243</td>
<td>899.615 ±260.1405</td>
<td>0.5402</td>
</tr>
<tr>
<td>CD8 number cells</td>
<td>429.363 ±183.9603</td>
<td>590.416 ±198.8295</td>
<td>0.0239</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>2.1605 ±0.6911</td>
<td>1.7658 ±0.8659</td>
<td>0.2263</td>
</tr>
<tr>
<td>Serum 25(OH)D nmol/l</td>
<td>29.5714 ±7.5514</td>
<td>29.2701 nmol/l ±9.4078</td>
<td>0.9046</td>
</tr>
<tr>
<td>Serum calcium (total) mg/dl</td>
<td>9.6000 ±0.4651</td>
<td>9.800 ±0.3225</td>
<td>0.1081</td>
</tr>
<tr>
<td>Serum calcium (ionized) mg/dl</td>
<td>4.1400 ±0.2062</td>
<td>4.1750 ±0.1711</td>
<td>0.5628</td>
</tr>
<tr>
<td>Serum phosphorus mg/dl</td>
<td>3.7304 ±0.6034</td>
<td>3.8524 ±0.6750</td>
<td>0.5303</td>
</tr>
<tr>
<td>Grade of inflammation</td>
<td>A0 (3), A1 (10), A2 (1), A3 (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage of fibrosis</td>
<td>F1 (1), F2 (8), F3 (11), F4 (5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Serum level of 25(OH)D in HCV infections and healthy subjects. 25(OH)D status reveals vitamin D insufficiency in all patient categories (<50 nmol/L). No differences were recorded on patients according to age or sex criteria. Serum levels of 25(OH)D did not differ between the two groups of subjects : 29.5714 nmol/l in patients and 29.2701 nmol/l in healthy controls (p = 0.9046, Student T). (Fig 1a).

Fig 1a. Serum level of 25(OH)D in HCV infections and healthy subjects.

The lowest 25(OH)D plasma concentration was found in patients with cirrhosis but no statistic significant difference were found towards other patient groups (p=0.02419 StudentT). (Fig 1b).
Fig 1b. Serum level of 25(OH)D in HCV cirrhosis, chronic hepatitis and controls

Correlations between the 25(OH)D level and CD4+T cell count. A positive correlation was found for the total number of HCV patients (R=0.507, Pearson correlation) and furthermore on the active HCV hepatitis (R=0.703 Pearson correlation). This correlation was not consistent with data recorded from inactive HCV patients (R=0.094). (Fig. 2)

Fig. 2. Correlations between the 25(OH)D level and CD4+T cell count in all the HCV patients and the group of active versus inactive HCV patients

Correlations between the 25(OH)D level and CD8+T cell count. No significant positive correlation was obtained for neither the total number of HCV patients (R=0.153 Pearson correlation) or the active or inactive HCV patients (R=0.202, respectively, R=0.094, Pearson correlation). (Fig3)

Fig 3. Correlations between the 25(OH)D level and CD8+T cell count in all the HCV patients and the group of active versus inactive HCV patients

Correlations between 25(OH)D level and ALT and AST level. We did not find significant correlations correlation between 25(OH)D and serum aminotransferases in the total group of HCV hepatitis. Active HCV patients displayed a positive correlation between 25(OH)D and ALT (R=0.530 Pearson correlation) and a weak correlation between 25(OH)D and AST (R=0.401 Pearson correlation). Inactive HCV patients displayed a strong negative correlation with AST (R= -0.553) and only a weak negative correlation with ALT (R= -0.412). No such correlations were found in healthy controls. (Fig.4a and Fig. 4b)

Fig. 4a. Correlations between 25(OH)D level and ALT

Fig. 4b. Correlations between 25(OH)D level and AST

Correlation between CD4+T cell count and calcium levels. Active HCV patients exhibited a strong positive correlation between CD4+T cell count and total calcium values (R=0.841 Pearson correlation) and a positive correlation between CD8+T cell and ionized calcium (R=0.652 Pearson correlation) (Fig 5a and Fig 5b). No correlations were found in what regards inactive HCV patients.

Fig 5a. Correlation between CD4+T cell count and calcium level
Vitamin D acts at a cellular level by interacting with VDR found in cells of the immune system and numerous tissues, including hepatocytes and cells of the biliary epithelium [12]-[15]. The antigenic activation of Th lymphocytes lead to a massive increase in the VDR expression [16]. The implications of VDR in the immune response modulation remains unclear and data obtained in vitro are still contradictory.

The importance of vitamin D in the inflammatory and immune response was already proven in various studies [17]-[20]. Certain immune modulating activities controled by vitamin D and currently observed in vitro could interfere with the HCV action:

- Vitamin D maintains self tolerance through a decrease in the inflammatory activity of Th1 cytokines promoted by CD4+ Th1 cells, while Th 2 cytokines are stimulated instead [21]. On the other side, the Th1 cytokines activity is essential in HCV clearance [22] but the prolonged Th1 response in HCV chronic infections is linked with hepatic lesions [23], [24].
- VD limits the inflammation through its effects on dendritic cells (DCs) [25]. Functional inhibition of DCs promotes the HCV persistence [26]. HCV also, by limiting antigenic recognition induce a similar effect on DCs.
- VD stimulates T cell activation, promoting hepatocyte apoptosis. [27]. HCV also upregulates hepatic apoptosis [28], [29].

To conclude with, in vitro data suggests that VD action could posses a favorable effect on HCV replication by maintaining hepatic tolerance and decreasing the inflammatory response in the initial stages of the infection. Thus the antiinflammatory effect of vitamin D is not protective within the first stages of the diseases when the action of CD4+Th1 cells is essential for removing the virus. In the later stages of the HCV infection, vitamin D could prevent the inflammatory response initiated by endogenous molecules or other associated infections. Unfortunately 25(OH)D level declines simultaneously with hepatic fibrosis following the inability of the liver to further hydroxylate vitamin D.

The present study recorded an extremely low value of 25(OH)D in all patients with HCV infection. The similar level observed in controls could indicate a populational deficiency. However no such correlation was found for any acknowledged factor such as nutrition, age, geography or body index mass. On the other hand, the normal values of calcium and phosphorus and the absence of any underlying diseases in studied controls proves that the 25(OH)D deficiency is well tolerated. Unfortunately bone metabolism was not studied in these patients.

Patients with cirrhosis expressed a slightly lower 25(OH)D value but no statistic significant difference was found compared to patients with low fibrosis (25.9760 nmol/l versus 30.4703 nmol/ml)

Patients with active HCV infection (A2-A3 inflammation score) presented positive correlations between plasma 25(OH)D and CD4+ T cells count (R=0.703), as well as between plasma 25(OH)D and ALT level (R=0.530). The active HCV patients exhibited a strong positive correlation between the CD4+ T-cell count and the total or ionized calcium level (R=0.841 respectively R=0.621). Patients with lower inflammation grades (A0-A1) exhibited negative correlations between 25(OH)D and AST (R=-0.548).

These data underline the pro-inflammatory action of 25(OH)D in patients with significant necrotic inflammatory activity and are contradictory to in vitro data until present. A larger number of patients with HCV infection in different stages of the disease should be investigated to analyze the differences noted. Moreover, the cellular action of HCV on the VDR could be taken in discussion as long as HCV replicates in DCs, CD4+ T cells or CD8+ T cells. In vivo analysis of the correlations between vitamin D and the inflammatory response in HCV is also required owing to the wide spread of VDRs in almost all tissues and to their implications in inflammatory, auto-immune and malignant processes.

V. CONCLUSION

Data collected from HCV patients with low grade of inflammation (A0-A1) revealed negative correlations between the plasma 25(OH)D level and the necroinflammation.

Patients with HCV infections and a high scores of inflammation (A2/A3) presented a strong positive correlation between 25(OH)D and both hepatocytolysis and CD4+T cells count and also between CD4+T cell and calcium level. Therefore, including vitamin D and calcium in the diet of these patients could promote CD4+ T cells multiplication and support a chronic inflammatory response inducing fibrosis.

REFERENCES


