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Lack of association between Vitamin D status and free light chains profile with different chronic HCV-related liver and extrahepatic disorders

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Abstract. – OBJECTIVE: A still uncertain association between vitamin D levels and HCV chronic liver diseases has been reported. Increased levels of serum-ree light chains (FLCs) and an altered k/λ FLC ratio correlate with Mixed Cryoglobulinemia (MC) vasculitis and/or B-cell non-Hodgkin's lymphoma in HCV-positive patients. We aimed to investigate the possible role of vitamin D, vitamin D Binding Protein (DBP), and FLCs levels as a tool for discriminating different stages of HCV- related MC and chronic liver diseases.

PATIENTS AND METHODS: Sixty-five untreated patients were retrospectively enrolled and 21 healthy blood donors (HBD) were used as controls. Vitamin D, DBP, FLCs, and cryoglobulins levels were measured. Based on cryoglobulins, patients were divided in three subgroups (without cryoglobulins, type II, and type III).

RESULTS: We didn't find any significant differences in vitamin D and DBP levels between HCV patients' main groups and HBD. Serum FLCs levels were significantly higher in HCV patients than in HBD. FLCs ratio among patients' subgroups did not reveal differences.

CONCLUSIONS: Our results confirm the presence of an increased serum level of FLCs in HCV patients and suggest that nor vitamin D and DBP or FLC levels can be considered reliable biomarkers for discriminating different stages of HCV-associated chronic liver diseases and/or HCV-associated extrahepatic manifestation. We confirm that serological FLCs levels are significantly higher in patients than in HBD as a signature of B cell activation in course of HCV infection.

Key Words:
Vitamin D, Free light chains, Vitamin D binding protein, HCV disorders.

Abbreviations

HCV: Hepatitis C virus; MC: Mixed Cryoglobulinemia; B-NHL: B-cell non-Hodgkin's lymphoma; CGs: Cryoglobulins; RF: rheumatoid factor; DBP: Vitamin D binding protein; FLCs: free light chains; HBD: healthy blood donors.

Introduction

Hepatitis C virus (HCV) infection is characterized by a chronic clinical course with different multi-organ involvements. The current

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standard of care rapidly changed over the years and new Direct Acting Antivirals made HCV treatment more effective[1]. HCV chronic infection is associated with serious morbidity and mortality due to liver complications, i.e., cirrhosis and liver cancer. The possible development of extrahepatic manifestations may occur in up to 74% of patients linked to clonal B-cell expansion leading to monoclonal gammopathy of undetermined significance (MGUS), mixed cryoglobulinemia (MC), and overt B-cell non-Hodgkin's lymphoma (B-NHL)[2,3]. MC represents the most frequent, best known, and strictly HCV-associated extrahepatic manifestation. As reported by different studies[4,5], more than 90% of MC patients are HCV positive. MC is considered a crosslink between an autoimmune and B-lymphoproliferative disorder, clinically benign but evolving to a frank malignancy in about 8-10% of cases[5,6]. MC is systemic vasculitis characterized by an intravascular precipitate of cryoglobulins (CGs), consisting in immune complexes from the patient's serum (IgGs and IgMs) that precipitate at a temperature less than 37°C and re-dissolve when rewarmed *in vitro*.

Meltzer et al[7,8] firstly described MC as a triad of purpura, fatigue, and arthralgia. The IgM component has rheumatoid factor (RF) activity and is produced by clonally expanded autoreactive B cells[7].

Vitamin D is a micronutrient that is considered essential for human health with beneficial effects on several body systems, including the innate immune system, other than in bone metabolism. Most of its requirements can be generated by an individual's exposure to summer-like sunlight, with dietary sources playing a supporting role when sunlight exposure is limited. In the hepatocytes, vitamin D can be hydroxylated by cytochrome P450 enzymes into 25(OH)D[9] that, thanks to the half-life of 2-3 weeks, represent the main circulating form.

The active form of vitamin D, the 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] (calcitriol), generated mostly in renal tubular cells, mediates its beneficial effects through the specific receptor (VDR). Calcitriol displays a very short half-life (6-8 h) and its circulating levels are modulated not only by parathyroid hormone (PTH) and serum ionized calcium levels but also the serum 25(OH)D availability represents a key regulator. For these reasons the 25(OH)D form is considered the best indicator of Vitamin D. The deficiency status is defined by a serum 25(OH)D level below 10 ng/mL that is very common among the elderly, mainly for the low dietary vitamin D intake and the lack of sunlight exposure[10]. Vitamin D deficiency is involved in the pathogenesis of chronic liver diseases caused by hepatitis B and C viruses; a close association among vitamin D deficiency and HBV and HCV infections is found worldwide[11-16].

In course of advanced liver disease, total vitamin D levels are frequently low, due to malabsorption, to the failure of hepatic hydroxylation of 25-calciferol into 25(OH)D, and to the decreased hepatic synthesis of the two main transporters: albumin and Vitamin D binding protein (DBP)[16]. DBP is an alpha2-globulin with a molecular weight of 52 and 59 kDa member of the albuminoid superfamily; it displays three major phenotypes (DBP1F, DBP1S, and DBP2) and more than 120 unique variants have been described until now[17]. DBP has been detected in different body fluids (serum, urine, breast milk, ascitic fluid, cerebrospinal fluid, saliva, and seminal fluid) and organs (brain, heart, lungs, kidneys, placenta, spleen, testes, and uterus). Although the most relevant functions are the binding, the solubilization, and the transport of vitamin D and its metabolites, DBP low levels have been correlated with autoimmune and inflammatory diseases, aspirin resistance (i.e., failure of aspirin to inhibit platelet function)[18] and arthritis[19]. Decreased production of DBP and albumin might play a critical role in chronic liver disease. However, patients with cirrhosis and low albumin concentrations have higher free 25(OH)D levels as a probable index of chronic inflammation. Low circulating levels of vitamin D have been also associated with a higher risk of B-NHL; on the contrary, excessive sun exposure, followed by an increased vitamin D level, has been associated with a lower risk of B-NHL[20].

Serological levels of free light chains (FLCs) are produced in excess of heavy chains during the synthesis of immunoglobulins by plasma cells. In subjects with normal kidney function their serum levels can be considered as a direct marker of B cell activity/dysfunction, which is otherwise difficult to measure. Their employment in routine clinical practice proved to be particularly useful for autoantibody-mediated diseases like systemic autoimmune disorders[21]. FLCs represent a mixture of molecules that share only the molecular weight and some typical properties, but the variable structure of both monoclonal and polyclonal FLCs may influence an individual FLC's antigen-binding ability, its interaction with immune cells, and the pathogenic potential. The specific structural features distinguishing the pathological and non-pathological FLCs have not been well described[22]. Serological FLCs levels are altered in most of HCV-positive patients: an abnormally elevated k/λ ratio seems to be a very interesting marker as it is consistently associated with the presence of MC vasculitis and/or B-NHL even in patients without any symptoms, in the early-stage of disease[23,24].

In this research, we assessed serological 25(OH)D and DPB levels in HCV-positive naïve and MC patients with different types of CGs. FLCs levels were measured in all patients' sera for the evaluation of immune activation and inflammation degree. Our aim was to evaluate for the first time the possible role of this peculiar biomarkers signature as a tool for discriminating different stages of HCV-associated chronic liver diseases and/or HCV-associated extrahepatic manifestation.

Patients and Methods

Patients

Sixty-five untreated patients with chronic HCV infection were retrospectively enrolled, including 42 HCV-naïve (34 with the asymptomatic presence of CGs) and 23 HCV-positive patients with symptomatic MC. Based on CGs, patients were divided into three subgroups (without CGs, type II, and type III).

Serum samples from 21 healthy blood donors (HBD) age- and sex-matched were used as negative controls. Patients were recruited at the Liver Diseases Clinic, Institute of Internal Medicine, Fondazione Policlinico Universitario "A. Gemelli" – IRCCS (Rome, Italy). Quantitative HCV-RNA detection was determined by a routine method and the virus genotype was determined for each sample (Siemens Healthcare, Erlangen, Germany).

All patients had an estimated glomerular filtration rate of ≥60 mL/min/1.73 m^2. Histological features of liver specimens were analyzed with METAVIR group scoring, assessed by transient elastography Fibroscan[â], ranged from F0 to F4[25].

Patients who received vitamin D supplementation in their diet (during the last 2 years) were excluded from the study.

For HCV naïve patients the inclusion criteria were: no previous antiviral treatments, absence of symptoms of hepatic disease and autoimmune and/or B-cell lymphoproliferative disorders, normal levels of C4 and C3, METAVIR score F0, presence/absence of asymptomatic CGs. Exclusion criteria were: abnormal ALT values, renal involvement or other symptoms related to MC vasculitis, inflammatory diseases with elevated levels of C-reactive protein or co-infections (HIV and HBV).

For HCV-related MC patients, the inclusion criteria were: free antiviral therapy period of at least 6 months, advanced chronic liver fibrosis assessed with METAVIR score F2 and MC syndrome according to the classification criteria for MC proposed by the Italian Group for the Study of Cryoglobulinemia in 1989 and revised in 2002[26]. Exclusion criteria were: presence of chronic liver disease due to other non-HCV causes positivity for HBV surface antigen and anti-HIV antibodies.

Since exposure to sunlight is the main source of vitamin D production and as it changes during seasons, the status of serum vitamin D was evaluated during spring.

This research was approved by the Ethical Committee of Fondazione Policlinico Universitario "A. Gemelli" – IRCCS. Written informed consent was obtained from all individuals included in the study. The study protocol is conformed to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008).

Laboratory Methods

Sera were obtained by standard centrifugation, divided into aliquots, and stored at –80°C until analysis.

The 25(OH)D was assayed in a blinded fashion and in a single batch by a standardized clinical assay, the Roche 25-OH Vitamin D Total (Roche Diagnostics, Mannheim, Germany). It consists of a fully automated sensitive immunoassay that employs a recombinant fusion construct of the vitamin D receptor ligand-binding domain for specific capture of 25(OH)D. The dynamic range of the assay is 4.0-150 ng/mL. The Vitamin D status was defined according to 25(OH)D levels: normal (≥30 ng/mL), deficient (<10 ng/mL), and insufficient (10-29 ng/mL)[27].

The DBP was assessed using a solid phase enzyme-linked immunosorbent assay (ELISA), the Quantikine Human Vitamin D Binding Protein immunoassay (R&D Systems, Minneapolis, MN, USA), according to manufacturer's instructions (normal range: 30-60 ng/mL according to Clinical and Laboratory Standards Institute guidelines[28]).

FLCs were measured by means of the Optilite analyzer (Freelite™ Human Kappa and Lambda Free Kits, The Binding Site, Birmingham, UK) according to the manufacturer's recommendations. Free k normal range: 3.3-19.4 mg/L; free λ normal range: 5.7-26.3 mg/L). A ratio of k/λ <0.26 or >1.65 is abnormal. For CGs detection, 20 mL of peripheral blood were collected and immediately stored at 37°C in pre-warmed tubes without anticoagulant for 30 min to enable complete blood clotting.

Serum samples were transferred to Wintrobe tubes and stored for at least 7 days at 4°C to evaluate the presence of precipitates. The cryocrit percentage was assessed and the remaining cryoprecipitate was recovered and washed three times. The cryoprecipitate was re-suspended with an appropriate volume of 3% PEG 6000 solution and re-solubilized for 30 min at 37°C. CGs were characterized by immunofixation electrophoresis using a G26 Fully automated system (Interlab, Rome, Italy).

Statistical Analysis

Data were analyzed using the Statistical Package for Social Science version 15.0 (SPSS, Chicago, IL-USA). Continuous variables are reported as mean±standard deviation (SD) or median (range) according to the distribution of the data, while categorical variables are reported as number and percentage. Analysis of categorical variables was performed by the Chi-square test or Fisher's exact when appropriate. Comparisons between groups of continuous variables were performed by the Mann-Whitney U-test or *t*-test, according to the data distribution. A two-sided *p*-value <0.05 was considered associated with statistical significance.

Results

Serological Vitamin D Profile in HCV Patients and Controls

When we analyzed the mean levels of 25(OH) D in serum samples from all patients and controls, we did not observe any statistically significant difference: 33.3±19.2 ng/mL *vs.* 31±13.1 ng/mL for 25(OH)D. More in details, among healthy controls, levels of 25(OH)D were normal in 9/21 (42.86%), insufficient in 11/21 (52.4%), and deficient in 1/21 (4.8%); in patients, the 25(OH)D levels were normal in 34/65 (52.3%), insufficient in 26/65 (40%), and deficient in 5/65 (7.7%) (Figure 1, panel A). The analysis of 25(OH)D levels in subgroups of patients with different CGs did not show any significant difference. In the subgroup without CGs, 3/8 (37.5%) were normal while 5/8 (62.5%) displayed insufficient levels of 25(OH)D; no one with deficiency. In the subgroup of type II CGs, levels were normal in 23/37 (62.17%), insufficient in 11/37 (29.72%), and deficient in 3/37 (81.08%). In the subgroup of type III CGs, 8/20 (40%) displayed normal levels of 25(OH)D, 10/20 (50%) insufficient, and 2/20 (10%) deficient levels (Figure 1, panel B).

When we analyzed the mean levels of DBP we did not observe any statistically significant difference among patients and controls: 26.6±16.4 ng/mL *vs.* 26.5±10.2 ng/mL for DBP (Figure 1, panel C). Analyzing the mean levels of 25(OH)D and DBP between HCV naïve and symptomatic patients with MC, we did not find any significant difference (data not shown).

The analysis of DBP mean levels among different patients' subgroups did not reveal any significant difference (Figure 1, panel D).

Free Light Chains Assessments in HCV Patients and Controls

As we reported in Table I, serological FLC mean levels were significantly higher all HCV patients (naïve + symptomatic MC) then in HBD (k: 42.2±44.7 *vs.* 9.2±4.2 mg/L, $p<0.001$; λ: 25.3±22.3 *vs.* 11.7±6.1 mg/L, $p<0.001$; k/λ: 1.8±1.2 *vs.* 0.8±0.2, $p<0.001$).

The statistical analysis of the free k and λ chains and their ratio among patients' subgroups (no CGs, type II, and type III CGs) did not reveal significant difference (k: 36.4±17.3 45.3±56.9 and 38.6±20.7 mg/L respectively; λ: 21.3±11.2, 24.0±27.4, and 29.2±13.2 mg/L, respectively; k/λ: 2.0±1.5, 2.0±1.4 and 1.3±0.5, respectively) (Figure 2, panel A, B, C).

Discussion

It is well established that vitamin D plays not only a crucial role in the regulation of calcium homeostasis and bone rearrangement but also participates to the adaptive immune response, promoting the switch from Th1 (pro-inflammatory) to Th2 (anti-inflammatory)-mediated immune

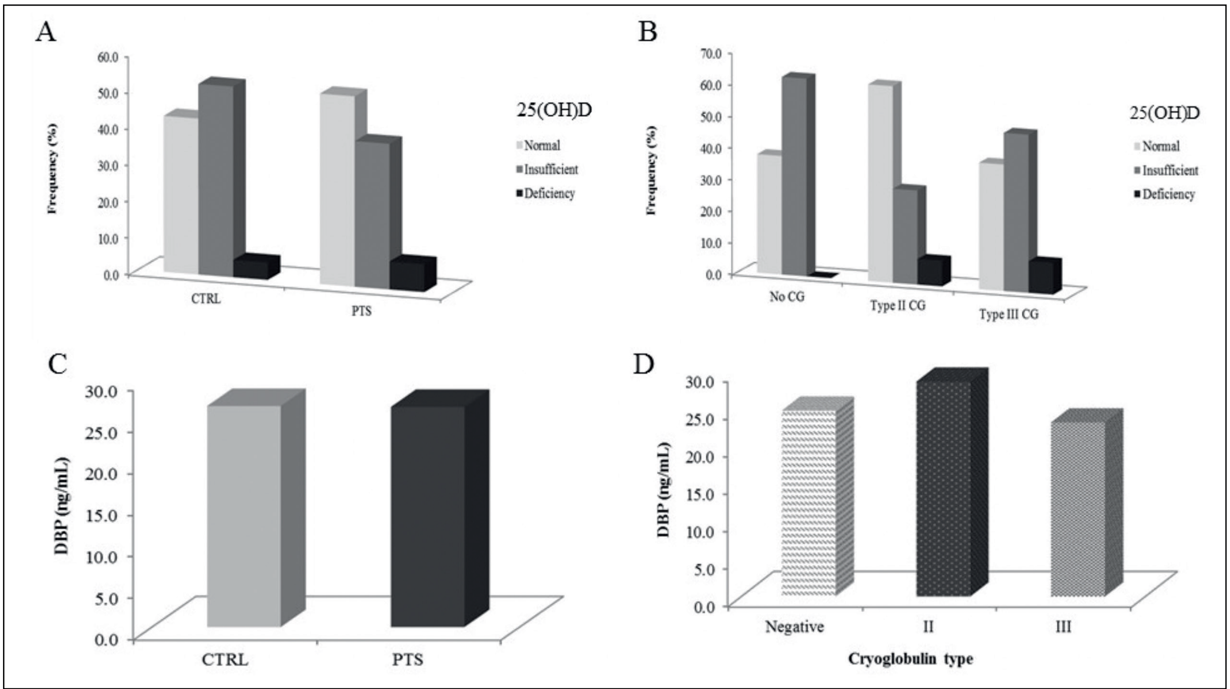

Figure 1. Frequency and distribution of Vitamin D and Vitamin D binding protein among study populations. **A,** Frequency of 25(OH)D levels between patients and controls; **B,** Frequency of 25(OH) D levels between different Cryoglobulin subgroups; **C,** Distribution of DBP concentration between patients and controls; **D,** Distribution of DBP concentration between different Cryoglobulin subgroups.

responses and modulating immune cells such as monocytes, macrophages, and B and T lymphocytes[29]. For this reason, vitamin D is considered an immuno-regulator of autoimmune disorders. According to the evidence that the 25(OH)D reduced the expression of collagen and other pro-fibrotic factors, leading to decreased fibrosis, its dietary supplementation appeared as a good strategy for the prevention and management of HCV related fibrotic disorders patients[30,31]. Therefore, the evaluation of serological vitamin D profile in HCV patients with extrahepatic manifestations such as MC, could be particularly interesting[31-33].

While it is well accepted that patients with chronic HCV infection display low levels of 25(OH)D, its role as a prognostic biomarker is still an unresolved question. Indeed, the association between low levels of 25(OH)D and the

Table I. Main characteristics of the study population.

	HCV patients	Healthy controls	*p*-value
N°	65 (42 HCV Naïve; 23 HCV symptomatic MC)	21	–
Sex	31M/34F	11M/10F	–
Age (years)	63 ± 9.8	42 ± 5	–
Vitamin D (ng/mL)	33.3 ± 19.2	31 ± 13.1	NS
DBP (ng/mL)	26.6 ± 16.4	26.5 ± 10.2	NS
FLCκ (mg/L)	42.2 ± 44.7	9.2 ± 4.2	< 0.001
FLCλ (mg/L)	25.3 ± 22.3	11.7 ± 6.1	< 0.001
κ/λ	1.8 ± 1.2	0.8 ± 0.2	< 0.001
METAVIR score	HCV naïve F0-F1		
HCV MC F2-F4	–	–	
CG Type II (no.)	37	–	–
CG Type III (no.)	20	–	–
CG Negativem (no.)	8		

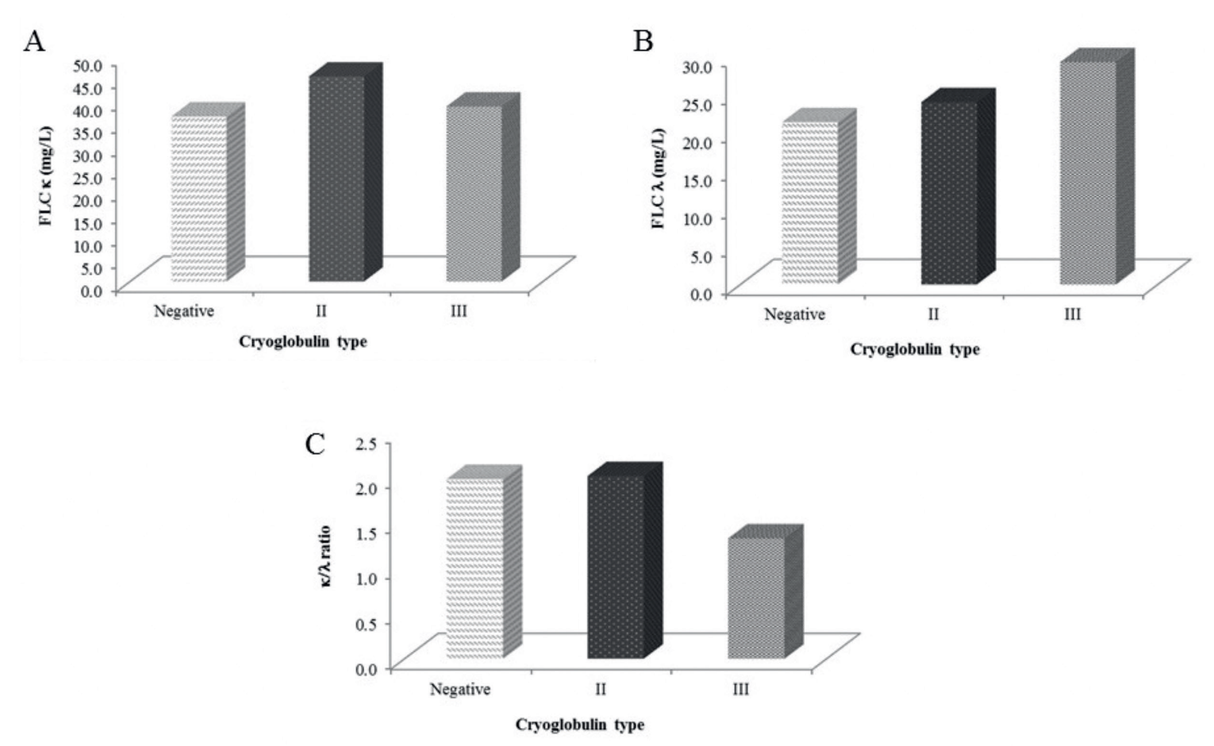

Figure 2. FLCs distribution between different Cryoglobulin subgroups.

progression/degree of liver fibrosis and response to antiviral therapy have been reported with conflicting results in HCV patients[34-37]. We aimed to analyze the vitamin D profile in HCV naïve patients and in patients with extrahepatic manifestations (HCV-related MC). Notably, for the first time, we correlated the 25(OH)D serological levels to different types of CGs (II and III), evaluating its role as a prognostic biomarker.

Considering that vitamin D deficiency represents the major public health problem in the world, it is not surprising that we found similar low levels in patients and in healthy controls[38,39]. Indeed, we did not find any significant difference in 25(OH)D vitamin D and DBP levels between patients and healthy controls (Figures 1A and B), between the two HCV patients' main groups (naïve *vs.* MC) (data not shown) and among patients' subgroups (Figures 1C and D). Of note, 25(OH)D was normal in 34/65 HCV patients (52.3%) and the mean level was >10 ng/mL in the great majority of patients for each subgroup with a very low occurrence of deficiency status (<10 ng/mL). Our results are in contrast with literature data that described a prevalence of insufficient and/or deficient 25(OH)D levels in correlation with the severity of the extra-hepatic manifestations as in MC[40,41]. On the contrary, our findings depict a scenario characterized by a lack of correlation between 25(OH)D serological levels and the severity of extrahepatic complications. Terrier et al[41] showed that low 25(OH) D levels correlate with the presence of MC and systemic vasculitis in chronic HCV infection. This is probably due to the different patients' population and selection criteria and to another method for 25(OH)D measurement. Although the 5 patients with 25(OH)D deficient levels (<10 ng/mL) displayed severe extrahepatic manifestations (METAVIR score F4).

In addition to 25(OH)D, for the first time, we assessed serological DBP levels, the binding protein that carries up to 88% of 25(OH)D and up to 85% of 1,25$(OH)_2$D (the active hormonal form of vitamin D[42]. Moreover, DBP can be converted into a macrophage-activator factor and actin-free DBP has been correlated with organ dysfunction in acute liver failure in association with different stages of fibrosis. Furthermore, DBP is a serologic biomarker decreasing in liver fibrosis (F2-F4). In combination with alpha 2 macroglobulin and apolipoprotein AI, it may predict different stages of liver fibrosis and its employment, if validated, could reduce the more invasive liver biopsy[43].

Here, we did not observe any significant difference in DBP levels between healthy donors and HCV patients, and among the different subgroups of HCV patients, suggesting that a reduction of risk for liver fibrosis is independent to serological DBP levels.

A common feature of diseases characterized by immune stimulation and B-cell activation, such as infection, inflammation, and autoimmune disease, is the frequent occurrence of polyclonal immunoglobulins. FLCs may play a crucial role in the pathogenesis of immune-inflammatory diseases and their overproduction usually is a result of different degree of chronic immune stimulation (Figure 2), with a ratio that remains unchanged. FLCs are involved in several diseases related to abnormalities in the pathways of natural and acquired immunity, interacting with specific immune mediators, surface membrane receptors, and various cells of the immune system[21]. Polyclonal FLCs have been measured as possible interesting markers in chronic HCV infection and as probable predictive immune-biomarker associated with more severe and active disease, comorbidity and mortality[44-46]. These findings could provide valuable insights into the role of inflammatory mechanisms in HCV pathophysiology. In analogy to previous reports, our patients showed higher FLCs levels if compared to HBD, confirming their role as a marker of B-cell dysfunction/activity that occurs in the course of HCV infection[44,45,47]. On the contrary, the analysis of free k and λ chains and k/λ among patients' subgroups did not reveal significant differences (Figure 2). This finding could be suggestive of a lack of clonal evolution of FLCs[23].

Conclusions

Our results demonstrate the presence of an increased serum level of FLCs in HCV patients as a signature of B cell activation in HCV related MC. However, we suggest that nor 25(OH)D and DBP levels or FLCs can be considered reliable biomarkers for discriminating different stages of HCV-associated chronic liver diseases and/or HCV-associated extrahepatic manifestation. The major limitation of our study is the small sample size due to the relatively slow progression of these diseases.

Assessments on a larger number of subjects will be necessary to better define the role of vitamin D in liver pathology.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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