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Low Vitamin D Levels Are Associated with the Presence of Serum Cryoglobulins in Patients with Chronic HCV Infection

IVAN GENTILE[1], FILOMENA SCARANO[1], ANNA CELOTTI[1], ENRICA DE IULIIS[2], ROSANNA SCARANO[2], VINCENZA GRANATA[2], BIAGIO PINCHERA[1], MARIANGELA MEOLA[3], ANTONIETTA D'AMBRA[3], MAURO PICCIRILLO[2], FLAVIANO DI PAOLA[2], ERNESTA CAVALCANTI[2], FRANCESCO IZZO[2], NICOLA SCARPATO[3] and GUGLIELMO BORGIA[1]

Departments of [1]Clinical Medicine and Surgery – Section of Infectious Diseases and [3]Molecular Medicine and Medical Biotechnology, Federico II, University of Naples, Naples, Italy; [2]National Tumor Institute "Fondazione G. Pascale", Naples, Italy

Abstract. *Background/Aim: Mixed Cryoglobulinemia (MC) represents the most frequent extrahepatic manifestation of chronic Hepatitis C Virus (HCV) infection. Its pathogenic mechanisms involve HCV-induced chronic stimulation of B-lymphocytes. We aimed to investigate the relationship between serum levels of vitamin D (a regulator of immune response) and the presence of serum cryoglobulins in the setting of HCV infection. Patients and Methods: We evaluated the serum concentration of 25(OH)vitamin D and cryoglobulins in 106 patients with chronic HCV infection. Results: Thirty patients (28.3%) showed the presence of serum cryoglobulins. For the cohort overall, the median serum 25(OH)vitamin D level was 10.95 ng/ml. Patients with serum cryoglobulins had significantly lower levels of 25(OH)vitamin D (5.61 ng/ml) than those without (13.65 ng/ml, p=0.029). At multivariate analysis, severe hypovitaminosis [i.e. 25(OH)vitamin D <13 ng/ml] was the only independent predictor of cryoglobulinemia (odds ratio=3.108). Conclusion: Severe deficiency of vitamin D was independently associated with mixed cryoglobulinemia in patients with HCV infection.*

According to recent estimates, more than 130 million people are infected with hepatitis C virus (HCV) worldwide (1, 2). The infection is associated with both hepatic and extrahepatic manifestations (3-6). Patients with chronic infection can progress to liver cirrhosis and hence to its complications and hepatocellular carcinoma (7-19). Several factors have been linked to the severity of chronic hepatitis C, including virus-related factors (genotype, duration of infection), co-morbidities (viral coinfection, insulin-resistance, liver steatosis, immunosuppression, iron overload) and lifestyle factors (alcohol intake) (20-24).

Mixed cryoglobulinemia (MC) is the most frequent extrahepatic manifestation and is associated with an increase of mortality in patients with HCV infection (5, 25-27). The key feature of MC is the presence in the serum of immunoglobulins that precipitate at 4˚C (28). These immunoglobulins are directed against self-antigens (notably other immunoglobulins) and these immune complexes can deposit on the surface of small- to medium-sized vessels giving rise to vasculitis which can be clinically symptomatic. Cryoglobulins are present in more than 50% of patients infected with HCV, but clinical manifestations are present in only 5%-10% of them (29). The exact pathogenic mechanism of MC has not precisely been defined, but the interaction between the HCV E2 envelope protein and CD81 (found on hepatocytes but also on the surface of lymphocytes) is considered a key trigger event that leads to chronic stimulation of lymphocytes. Finally, B-lymphocyte proliferation results in the production of antibodies and cryoglobulins (4). As not all patients with HCV infection develop MC, several viral or host features have been identified as risk factors for MC development. They include HCV genotype 2, female sex, older age, duration of infection, and Human-Leukocyte Antigen (HLA) DR phenotype (HLA-DR11 is associated with a high risk of MC, whereas HLA-DR7 with low risk) (30-32).

Treatment of MC includes eradication of HCV infection, use of immunosuppressive therapy, and of a low-antigen-content-diet (33). The clearance of HCV infection represents the etiological therapy of MC. However, data on the efficacy of direct anti-viral agents, which are very effective and well-tolerated in the treatment of HCV infection and in preventing hepatic progression of the disease, are lacking in this setting (34-47).

Correspondence to: Ivan Gentile, Department of Clinical Medicine,and Surgery, Section of Infectious Diseases, Federico II University,of Naples, via S. Pansini 5, I-80131 Naples, Italy. Tel: +39 0817463178, e-mail: ivan.gentile@unina.it

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It has been shown that the effects of vitamin D on human health are not limited to its well-known key role in calcium homeostasis and bone rearrangement. In fact, vitamin D is involved in the activation of more than 200 target genes and can modulate immune cells such as monocytes, macrophages, and T- and B-lymphocytes (48). For this reason, vitamin D is claimed to be an immunomodulatory agent in cases of autoimmune disorders. In the setting of extra-hepatic manifestation of HCV infection, a recent French article revealed that patients with low levels of vitamin D have higher rate of cryoglobulins and of symptomatic MC than those with high levels (49).

The aim of the present study was to evaluate whether patients with HCV with and without cryoglobulins differ in terms of vitamin D levels, measured as serum 25(OH) vitamin D, regardless of the stage of liver disease.

Patients and Methods

Patients. Patients with HCV infection admitted as in- or out-patients between March 2013 and April 2014 at the Department of Clinical Medicine and Surgery – Section of Infectious Diseases of the Federico II University of Naples, Italy, were enrolled.

The inclusion criterion was chronic HCV infection, defined as positivity of HCV-RNA twice in a 6-month interval.

Exclusion criteria were: a) inability to provide a valid informed consent; b) coinfection with HBV or HIV; c) previous or current immunosuppressive therapy; d) vitamin D2-, vitamin D3- or derivative-containing supplementations (current or during the previous 2 years).

A full medical history investigation and a physical examination were recorded for all patients. A complete laboratory panel work-up was also performed. It included a complete liver panel; urine analysis; blood count and immunological panel including C3, C4, rheumatic factor, serum cryoglobulins and 25(OH)vitamin D. Cirrhosis was defined histologically or by the means of noninvasive tools: patients who had an Aspartate Transaminase (AST) to platelet ratio index (APRI) of more than 2, evidence of esophageal or gastric varices at upper endoscopy, or typical patterns of cirrhosis or portal hypertension at ultrasound examination were also defined as cirrhotic. Cryoglobulinemic syndrome was defined by the presence of serum cryoglobulins with purpura and histological evidence of leukocytoclastic vasculitis, or by the presence of serum cryoglobulins plus one criterion among purpura, skin ulcers, membranoproliferative glomerulonephritis or peripheral neuropathy and the presence of rheumatoid factor as indicated elsewhere (4).

Laboratory method. 25(OH)Vitamin D levels were determined by LIAISON® 25 OH Vitamin D TOTAL Assay on an Liaison XL instrument (DiaSorin Inc.,1951 Northwestern Avenue, Stillwater, MN 55082-0285-USA). In accordance with a previous study, the serum 25(OH) vitamin D level was classified as follows: deficit: <13 ng/ml; insufficient: between 13 and 30 ng/ml; sufficient >30 ng/ml (49).

Detection of cryoglobulins was carried out as follows. Whole blood samples were collected, then the serum was separated from cells within 1 hour of collection by centrifugation and then dispensed into a plain tube and kept at 4˚C. Collection, shipment, storage and centrifugation were performed at 37˚C. Cryoglobulins, if present, appeared after 72 hours as white precipitates.

The amount of cryoglobulins was measured as the percentage of cryoprecipitates in a Wintrobe-tube after centrifugation at 4˚C.

Statistical analysis. We used the Kolmogorov-Smirnov test to check the distribution of quantitative variables. In cases of Gaussian distribution, we report data as the mean±standard deviation (SD) and used the Student's *t*-test for unpaired variables for comparisons. In cases of non-Gaussian distribution, data are reported as median and first and third quartile (interquartile range, IQR) and the Mann-Whitney *U*-test was used for comparison. The χ^2 test (or Fisher's exact test if appropriate) was used for qualitative variables (or quantitative variables that were categorized). For all tests, an alpha risk of less than 5% on a two-sided test was considered significant. The variables associated with cryoglobulin presence (or with $p<0.2$ at univariate analysis) were included in a binary logistic regression analysis model using the backward conditional stepwise method. The cut-off values used for the stepwise method were $p=0.05$ for entry into the model and $p=0.10$ for removal from the model. A cutoff of 13 ng/ml was used to dichotomize 25(OH) vitamin D levels Statistical Package for the Social Sciences version 18.0 (SPSS, Chicago, ILL, USA) was used for all calculations.

Results

One hundred and six patients were enrolled. All patients were Caucasian. Demographic, virological, clinical and laboratory data are shown in Table I.

Thirty patients (28.3% of the sample) had serum cryoglobulins. In Table II, we compare demographic, clinical, laboratory and virological data of the patients with and without cryoglobulins. Fifty-one patients had received prior treatment (standard or pegylated interferon with/without ribavirin). No patient had evidence of proteinuria or hematuria. Regarding collection of samples, 26.58% were gathered in the first trimester (January-March), 43.39% in the second (April-June); 10.38% in the third (July-September) and 22.64% in the fourth (October-December) of the year. Figure 1 shows the serum 25(OH) vitamin D variation across the trimesters of collection.

In Figure 2, we report serum 25(OH) vitamin D levels in patients with and without serum cryoglobulins.

We compared levels of 25(OH)vitamin D in patients with and without cirrhosis. The median level of 25(OH)vitamin D was 9.01 ng/ml (IQR=5.77-18.35 ng/ml) in patients with cirrhosis and 20.70 ng/ml (IQR=13.00-24.15 ng/ml) in those without cirrhosis ($p<0.01$).

At logistic regression analysis (see Table III), only severe vitamin D deficiency (defined as 25(OH)vitamin D <13 ng/ml) was identified as an independent risk factor for presence of serum cryoglobulins (Odds Ratio, OR: 3.108; confidence interval, CI: 1.163-8.302).

Discussion

This study shows that severe vitamin D deficiency (defined as serum levels lower than 13 ng/ml) is an independent risk

Table I. *Demographic, virological, clinical and laboratory data of patients (n=106).*

Characteristic	Value
Age (years)	69 (62.37-74.02)
Gender	
Male	n=66, 62.3%
Female	n=40, 37.7%
Serum iron (μg/dl)	104.50 (67.75-146.00)
Glucose (mg/dl)	98 (81-119)
Albumin (g/dl)	3.9 (3.4-4.4)
Total bilirubin (mg/dl)	0.93 (0.64-1.62)
Creatinine (mg/dl)	0.93 (0.80-1.20)
AST (U/l)	63.50 (39.75-100.00)
ALT (U/l)	54.00 (35.75-88.50)
P-CHE (U/l)	4,066 (2,246-6,351)
White blood cells (cells/μl)	5,295 (4,235-6,745)
Red blood cells (cells/μl)	4,450,000 (3,871,500-4,807,500)
Hemoglobin (g/dl)	13.25 (11.30-14.70)
Platelet (number/μl)	115,500 (77,750-164,750)
INR	1.16 (1.07-1.27)
Alpha-fetoprotein (ng/ml)	4.5 (2.2-11.2)
Ferritin (ng/ml)	196 (89-385)
C3 (g/l)	0.91 (0.75-1.08)
C4 (g/l)	0.15 (0.08-0.23)
Rheumatoid factor (IU/ml)	10.10 (10.10-12.40)
HCV RNA (IU/ml)	826,000 (109,147-1,944,720)
HCV Genotype	
1	n=85, 80.2%
2	n=21, 19.8%
Vitamin D (ng/ml)	10.95 (6.66-20.72)
Evidence of cryoglobulins	n=30, 28.3%
Defined cryoglobulinemic syndrome	n=5, 16.7%*
Purpura	n=3, 10%*
Neuropathy	n=2, 6.7%*
Presence of cirrhosis	n=77, 72.6%
Presence of hepatocellular carcinoma	n=16, 15.1%

AST: Aspartate Aminotransferase; ALT: alanine aminotransferase; P-CHE: pseudo-cholinesterase; INR: international normalized ratio; HCV-RNA: hepatitis C virus ribonucleic acid; HCV: hepatitis C virus. *Percentage calculated on the 30 patients with cryoglobulins.

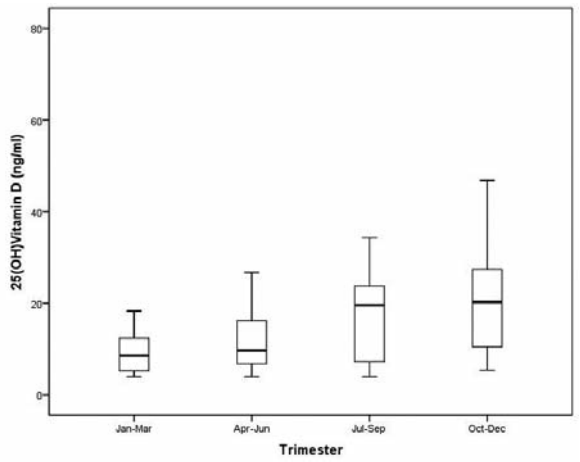

Figure 1. *Serum 25(OH)Vitamin D variation across the year. Data are reported as boxplots. The top and bottom of the box represent the third and first quartiles, and the line inside the box is the median. The whiskers represent the lowest and the highest datum within 1.5-fold interquartile range.*

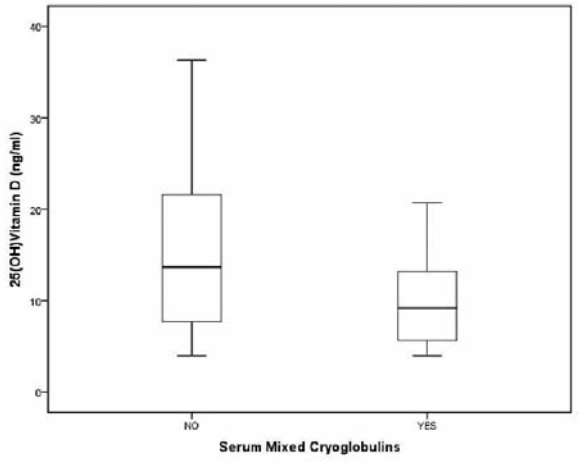

Figure 2. *Serum 25(OH) vitamin D levels in patients with and without serum mixed cryoglobulins. Data are reported as boxplots. The top and bottom of the box represent the third and first quartiles, and the line inside the box is the median. The whiskers represent the lowest and the highest datum within 1.5-fold interquartile range.*

factor for the presence of mixed cryoglobulins in a cohort of patients with chronic HCV infection in various stages of the disease.

However, it is noteworthy that a high prevalence of vitamin D deficiency was found in the entire cohort of the present study. In fact, the median serum 25(OH)vitamin D level was 10.95 ng/ml and 91.5% of the patients had a serum vitamin D level less than the lower limit of the normal range (*i.e.* 30 ng/ml). This finding is in agreement with previous studies that showed a prevalence of vitamin D deficiency in around 89% of patients with HCV infection (49). We underline that no consensus threshold of what should be considered a 'normal' level of vitamin D exists and, therefore, in our study we used previous contributions that considered thresholds of deficiency (<13 ng/ml), insufficiency (between 13 and 30 ng/ml) and sufficiency (>30 ng/ml), respectively (49).

It is well-known that vitamin D levels vary throughout the year according to the level of sun exposure. This study confirms this finding as the lowest levels were found in the first six months of the year, then they gradually increased. A peak of 25(OH)vitamin D is reached in September, after the Summer months during which solar radiation is usually greater.

Another interesting finding that emerges from our research is that 25(OH)vitamin D is significantly lower in patients with

Table II. *Demographic, laboratory, clinical and virological features of patients stratified by presence/absence of serum cryoglobulins (n=106).*

Characteristic	Absence (n=76)	Presence(n=30)	*p*-Value
Age (years)	68.08 (61.43-73.87)	69.87 (66.02-74.18)	0.328
Gender			
Male	68.42%	46.66%	0.037
Female	31.57%	53.33%	
Serum Iron (μg/dl)	108.00 (67.25-147.25)	103.00 (68.75-146.25)	0.702
Glucose (mg/dl)	93 (80-110)	106 (95-139)	0.014
Albumin (g/dl)	4.1 (3.4-4.4)	3.7 (3.5- 4.1)	0.174
Total bilirubin (mg/dl)	0.90 (0.60 -1.67)	1.10 (0.80-1.62)	0.422
Creatinine (mg/dl)	0.90 (0.80-1.10)	1.08 (0.77-1.30)	0.351
AST (U/l)	58.50 (37.00-92.75)	84.00 (50.75-112.00)	0.041
ALT (U/l)	52.50 (32.00-83.75)	59.50 (39.00-101.00)	0.373
P-CHE (U/l)	4,342 (2,261-6,802)	3,621 (2,061-5,590)	0.424
White blood cells (cells/ μl)	5,735 (4,392-6,835)	4,500 (3,510-6,825)	0.64
Red blood cells (cells/μl)	4,560,000 (3,960,000-4.952.500)	4,100,000 (3,560,000-4,515,000)	0.027
Hemoglobin (mg/dl)	13.70 (12.10-14.90)	12.75 (10.77-13.97)	0.100
Platelets (number/μl)	124,500 (85,250-173,500)	100,000 (58,000-137,750)	0.025
INR	1.14 (1.07-1.28)	1.17 (1.09-1.27)	0.862
Alpha-fetoprotein (ng/ml)	4.1 (1.9-10.0)	4.7 (3.1-13.6)	0.123
Ferritin (ng/ml)	197 (88-403)	181 (109-321)	0.774
C3 (g/l)	0.86 (0.72-1.00)	0.90 (1.03-1.36)	<0.0001
C4 (g/l)	0.15 (0.07-0.18)	0.21 (0.11-0.49)	0.003
Rheumatoid factor (IU/ml)	10.10 (10.10-11.05)	12.4 (10.1-30.4)	<0.0001
HCV-RNA (IU/ml)	910,416 (99,871-2,125,535)	117,714 (281,724-1,251,950)	0.254
HCV Genotype			
1	81.6%	76.9%	0.715
2	18.4%	23.1%	
25(OH)Vitamin D (ng/ml)	13.65 (7.69-21.80)	5.61 (9.17-13.52)	0.029
Presence of cirrhosis	n=51, 67.1%	n=26, 86.7%	0.42
Presence of HCC	n=11, 14.5%	n=5, 16.7%	0.776

AST: Aspartate Aminotransferase; ALT: alanine aminotransferase; P-CHE: pseudo-cholinesterase; INR: international normalized ratio; HCV-RNA: hepatitis C virus ribonucleic acid; HCV: hepatitis C virus; HCC: hepatocellular carcinoma.

Table III. *Logistic regression analysis for the presence of serum cryoglobulins.*

Factor	Regression coeffient	Standard error	OR (95% CI)	*p*-Value
Vitamin D deficiency*, yes *vs*. no	1.134	0.501	3.108 (1.163-8.302)	0.024
Gender, female *vs* male.	0.802	0.460	2.230 (0.905-5.490)	0.081
Glycemia (mg/dL)	0.008	0.004	1.008 (0.999-1.016)	0.070

*Serum 25(OH) Vitamin D concentration <13 ng/ml vs ≥13 ng/ml.

cirrhosis that in those without it. This finding is in agreement with a previous study (50). As the presence of cirrhosis was correlated both with vitamin D deficiency and higher rate of cryoglobulins, we performed a multivariate analysis through a logistic regression model. Based on this analysis, severe deficiency of vitamin D [25 (OH)vitamin D <13 ng/ml] was the only independent predictor of mixed cryoglobulinemia. What is a possible mechanism that links vitamin D deficiency and cryoglobulins? Actually, many different roles have been attributed to vitamin D apart from its classical role as a key regulator of calcium homeostasis. Among them, it is noteworthy that vitamin D is considered a modulator of the inflammatory pathways driven by the immune reaction to an exogenous trigger (48, 51). It can be hypothesized that in a

patient with a genetic and an environmental predisposition (such as vitamin D deficiency), HCV infection may cause over-response of the immune system, that leads to the production of self-reactive immunoglobulins, as cryoglobulins. However, an alternative hypothesis is that the presence of cryoglobulins may be the cause and not the effect of vitamin D deficiency.

We acknowledge that our study has some limitations which include the lack of a healthy control group, and its mono-centric and cross-sectional design. Moreover, we also found serum C3 and C4 paradoxically to be higher in patients with serum cryoglobulins than in those without. We cannot provide an explanation for this finding.

In conclusion, our study shows that 25(OH)vitamin D deficiency (<13 ng/ml) is associated with the presence of serum cryoglobulins in patients with chronic HCV infection, regardless of the stage of disease.

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