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Interleukin-17A and B-cell activating factor in chronic hepatitis C patients with or without asymptomatic mixed cryoglobulinemia: effects of antiviral treatment and correlations with vitamin D

Polydoros Konstantinides, Alexandra Alexopoulou, Emilia Hadziyannis, Theoni Kanellopoulou, Spyridon P. Dourakis

Medical School, National and Kapodistrian University of Athens, Hippokration General Hospital, Athens, Greece

Abstract

Background Several studies have provided conflicting results regarding the immune responses in chronic hepatitis C (CHC) patients with mixed cryoglobulinemia (MC). The importance of B-cell activating factor (BAFF) in MC has been described, but the role of interleukin (IL)-17A is less clear.

Methods Serum concentrations of IL-17A, BAFF and 25-OH vitamin D were measured in CHC patients at baseline, end of treatment, and 6 months post-treatment with pegylated interferon-α and ribavirin, versus 12 healthy controls.

Results Thirty-four patients (20 male, mean age 40.7±9.2 years, 12 of genotype 1 or 4, 22 of genotype 2 or 3) were included, of whom 64.7% achieved a sustained virological response (SVR). MC was detected in 52.9% of the patients. Higher levels of both cytokines were found in patients with MC compared to those without. Patients who achieved SVR had higher pretreatment IL-17A and lower BAFF levels compared to those without SVR. IL-17A was downregulated during and following treatment in responders, whereas upregulation was observed in non-responders. CHC patients demonstrated low vitamin D levels compared to HC. Moreover, the changes in IL-17A over the treatment period were significantly associated with vitamin D changes (β=-0.04, SE=0.02, P=0.046). No difference in IL-17A, BAFF and vitamin D values was seen between patients with cirrhosis (n=14) and those without.

Conclusions CHC patients with asymptomatic MC have increased levels of IL-17A and BAFF. IL-17A levels decline significantly while BAFF increases during treatment in responders. An interplay between IL-17A and vitamin D concentrations was revealed during the antiviral treatment.

Keywords Interleukin 17A, B-cell activating factor, vitamin D, chronic hepatitis C, mixed cryoglobulinemia

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Introduction

Globally, the prevalence of chronic hepatitis C (CHC) patients is about 2.5%, corresponding to >177 million infections worldwide [1]. Mixed cryoglobulinemia (MC) has been recognized as the most common extrahepatic manifestation of hepatitis C virus (HCV) infection. It is an autoimmune disorder characterized by the presence of circulating cold-precipitating immune complexes [2]. These complexes are detected with a prevalence of 40-50% in CHC patients, but most of them are asymptomatic [3].

The intrinsic mechanisms inducing cryoglobulin production are still unclear. A common hypothesis is that chronic stimulation by HCV antigens, mainly the HCV core antigen, facilitates the expansion of rheumatoid factor-producing B lymphocytes [4]. It is possible that both cytokines and the host's genetic background may also contribute to lymphoproliferation in CHC [5,6]. B-cell activating factor (BAFF), also called B-lymphocyte stimulator (BLyS), has been identified as a novel ligand of the tumor-necrosis factor

2nd Department of Internal Medicine and Research Laboratory, Medical School, National and Kapodistrian University of Athens, Hippokration General Hospital, Athens, Greece

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Correspondence to: Polydoros Konstantinides, 15 Eynardou St, 15237 Athens, Greece, e-mail: polidorosk@hotmail.com

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superfamily. It is produced by dendritic cells, monocytes, macrophages and activated T lymphocytes, and stimulates survival of B lymphocyte and autoantibody production [7]. Antiviral treatment with interferon has been shown to significantly increase BAFF levels [8].

Previous studies have also demonstrated that T-cell immunoregulatory cytokines contribute to liver damage [9] and that HCV may directly modulate both B- and T-cell functions [10]. Among immune deregulations, those related to subtype 17 of the T-helper lymphocyte (Th17)/interleukin-17 (IL-17) axis have been recognized as key immune-pathological and prognostic elements in patients with chronic viral hepatitis. The proinflammatory cytokine IL-17A may activate neutrophils, leading to tissue inflammation and fibrosis, and promote the progression of autoimmune ddiseases [11,12]. Recently-developed direct acting antiviral agents (DAAs) have achieved excellent therapeutic results in CHC patients. However, DAA therapy may not be adequate for the treatment of MC and combination with immunosuppressive therapy may be necessary [13,14]. Notably, limited data are available on the efficacy of antiviral treatment in HCV-related cryoglobulinemia [15]. On the other hand, due to financial reasons, pegylated interferon-α (pegIFNa) and ribavirin (RBV) therapy is still the standard treatment for CHC patients in some countries [16].

Vitamin D is a critical regulator of immunity, playing a role in both innate and cell-mediated immune responses [17]. Numerous studies have demonstrated a decline in vitamin D concentrations in CHC patients [18], but the causes of this deficiency have not yet been defined. Moreover, vitamin D deficiency is considered as a risk factor of fibrosis and treatment failure [19].

The objectives of this study were to investigate the kinetics of IL-17A and BAFF in HCV patients with or without MC, at baseline (BSL) and during the antiviral treatment, and to evaluate their interactions with viral clearance, vitamin D levels, and fibrosis.

Patients and methods

Consecutive CHC patients were prospectively evaluated from March 2011 to March 2014 in the Hepatology Department of Hippokration General Hospital, Athens, Greece. The study protocol was approved by the Medical School of Athens and the local Ethics Committee of Hippokration General Hospital.

CHC was confirmed by HCV RNA polymerase chain reaction (PCR) and HCV genotyping was performed in all subjects. Inclusion criteria were: a) written informed consent provided by the patients; b) age ≥18 years (without any upper age limit); and c) chronic HCV infection. Exclusion criteria were: a) coinfection with hepatitis B virus or human immunodeficiency virus; b) symptomatic cryoglobulinemia with vasculitis; and c) decompensated cirrhosis.

Forty-six individuals who met the inclusion and exclusion criteria were initially evaluated, of whom 34 were ultimately judged eligible for enrollment. Twelve healthy individuals matched for age and sex were used as the control group (HC). All patients received pegIFNa/RBV treatment for 48 weeks (genotype 1 and 4) or 24 weeks (genotype 2 and 3).

The following parameters were recorded from each patient participating in the study:

i) HCV viral load, quantified by real-time HCV PCR assay with a lower limit of quantification of 13 IU/mL (Versant kPCR, Siemens, Tarrytown, NY, USA) and HCV genotype (line probe assay, VERSANT HCV Genotype 2.0, Siemens, Tarrytown, NY, USA).
ii) Cryoglobulins: Blood samples of approximately 20 mL were collected into pre-warmed tubes and clotted at 37°C for >1 h. The serum was separated from clots by centrifugation at 3000 rpm for 20 min and was incubated for 7 days at 4°C. The presence of cryoglobulin was first assessed by visual inspection of a precipitate and was confirmed by its dissolution at 37°C. The cryoglobulin concentration was measured after centrifugation as the cryocrit.
iii) Liver stiffness was assessed by transient elastography (TE) and patients with a TE>12.5 kPa were identified as cirrhotic.
iv) Complete blood count and biochemistry panel, including 25-OH vitamin D, using immunoassay kit.
v) Abdominal ultrasound: Groups were categorized by: 1) the presence or not of MC at BSL; 2) response to treatment (with or without sustained virological response, SVR); 3) the presence or not of cirrhosis; and 4) level of viremia at BSL with low or high HCV-RNA level (after interpretation of our data we used a cut-off point of 400,000 IU/mL, similarly to other studies, showing an improved discrimination between high and low probability of achieving an SVR) [20].

Blood samples were obtained at three time points: before starting treatment (BSL), at the end of treatment (EOT), and 6 months after the end of treatment (6Mo-EOT). Blood was collected in tubes with ethylenediamine tetraacetic acid and centrifuged at 800 *g* at room temperature. Plasma was collected, aliquoted and stored at -80°C until assayed. Concentrations of IL-17A and BAFF were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (eBioscience, human BAFF, instant ELISA, BMS2007INST and eBioscience, human IL-17A platinum ELISA, BMS2017TEN).

Statistical analysis

All continuous variables are presented as mean ± standard deviation. Quantitative variables are presented with absolute and relative frequencies. Spearman's correlation coefficients were used to explore the association of two continuous variables. Chi-square test was used for the comparison of proportions. Differences between different groups in changes of study variables during the follow-up period were evaluated using repeated measurements analysis of variance (ANOVA). Bonferroni correction was used in case of multiple testing. IL-17A and BAFF had skewed distribution and were log-transformed for the analysis. To longitudinally assess if changes in BAFF, IL-17A and vitamin D

during follow up were correlated, mixed linear regression models were fitted, accounting for multiple measurements per individual obtained at different time points. Regression coefficients (β) with standard errors (SE) were computed from the results of the mixed models. All P-values reported are two-tailed. Statistical significance was set at 0.05 and analyses were conducted using SPSS statistical software (version 22.0).

Results

Twenty male and 14 female patients, mean age 40.7±9.2 years were included. Patients' characteristics are presented in Table 1. Twelve cases had genotype 1 or 4 and 22 genotype 2 or 3. Cryoglobulins were detected in 52.9% of the cases. Twenty two patients (64.7%) achieved SVR and the rate was higher in HCV genotypes 2 and 3 (77.3%) compared to genotypes 1 and 4 (41.7%) (P=0.003). Additionally, the percentage of patients who achieved SVR was similar in those with and without MC (62.5% vs. 66.7%, P=0.8). Cirrhosis (TE>12.5 kPa) was found in 14 patients.

Table 1 Sample characteristics

Characteristic	N (%)
Sex	
Male	20 (58.8)
Female	14 (41.2)
Age, mean±SD	40.7±9.2
Nationality	
Greek	24 (70.6)
Other	10 (29.4)
BMI, mean±SD	25.8±4.4
AST/ALT, mean±SD	55±28.6/85±54
HCV-RNA IU/mL (baseline)	
<400,000	11 (32.4)
>400,000	23 (67.6)
Genotype	
1+4	12 (35.3)
2+3	22 (64.7)
MC	
No	16 (47.1)
Yes	18 (52.9)

MC, mixed cryoglobulinemia; SD, standard deviation; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV-RNA, hepatitis C virus ribonucleic acid

The mean BSL IL-17A values in patients with CHC were higher than in HC (P=0.020). The mean BAFF values tended to be higher in CHC patients compared to HC (P=0.076). Low vitamin D concentrations were measured in CHC patients (mean: 22.21±8.87 ng/mL). Vitamin D deficiency (<20 ng/mL) or insufficiency (20-29 ng/mL) at BSL was observed in 55% and 17% of patients, respectively.

The changes in IL-17A, BAFF and vitamin D over time are illustrated in Table 2. IL-17A remained unchanged, while BAFF increased significantly at the EOT and vitamin D decreased significantly at 6Mo-EOT. However, no significant change in vitamin D was found between BSL and 6Mo-EOT values.

Table 3 shows the changes in IL-17A, BAFF and vitamin D levels over the study period in relation to SVR. At BSL, patients with SVR had significantly higher IL-17A (P=0.040) and lower BAFF levels (P=0.045) compared to those with no SVR. IL-17A declined significantly from BSL to EOT in the SVR group and this decline persisted thereafter. In contrast, IL-17A increased significantly from BSL to EOT in subjects without SVR (P=0.022). The IL-17A kinetics during the follow-up period differed significantly (P=0.001) between those with and without SVR (Fig. 1). BAFF was significantly elevated in responders compared to non-responders; this elevation persisted after treatment discontinuation only in responders but it did not reach statistical significance. The kinetics of BAFF levels differed significantly (P=0.01) over the study period between responders and non-responders (Fig. 2). Vitamin D declined significantly only from EOT to 6Mo-EOT in patients with SVR.

Values and changes in IL-17A, BAFF and vitamin D levels in relation to the presence of MC are presented in Table 4. Patients with MC had significantly higher IL-17A and BAFF levels compared to those without. No significant differences in the three parameters were found between the MC and non-MC groups over the total period of treatment and follow up.

Results from mixed linear models showed that changes in BAFF were not significantly associated with the corresponding

Table 2 Values of IL-17A (pg/mL), BAFF (ng/mL) and vitamin D (ng/mL) levels in all HCV patients and changes during the therapy

Parameter	Mean±SD					Significance			
	BSL	EOT	6Mo-EOT	Change BSL to EOT	Change EOT to 6Mo-EOT	P[1]	P[2]	P[3]	P[4]
IL-17A[+]	16.6 (31.95)	9.66 (22.02)	8.67 (22.6)	−6.94 (18.35)	−0.99 (6.09)	1.000	0.700	1.000	0.392
BAFF[+]	0.11 (0.31)	0.3 (0.62)	0.34 (1.07)	0.19 (0.54)	0.04 (0.57)	0.043	0.991	0.366	0.004
Vit. D	22.21 (8.87)	22.93 (7.21)	18.01 (6.91)	0.72 (8.39)	−4.92 (5.87)	1.000	0.011	0.167	0.002

[1]P-value for time effect (BSL to end of therapy measurement); [2]P-value for time effect (end of therapy to 6 months measurement); [3]P-value for time effect (BSL to 6 months measurement); [4]P-value for overall time effect; +based on logarithmic transformations

BSL, baseline; EOT, end of therapy; 6ML-EOT, 6 months after the end of therapy; IL-17A, interleukin 17A; BAFF, B-cell activating factor; HCV, hepatitis C virus

Table 3 Values of IL-17A (pg/mL), BAFF (ng/mL) and vitamin D (ng/mL) levels in patients with and without SVR and changes during therapy

Parameter	Mean±SD					Significance			
	BSL	EOT	6MA-EOT	Change BSL to EOT	Change EOT to 6MA-EOT	P[2]	P[3]	P[4]	P[5]
IL-17[+]									
SVR									
No	4.58 (12.37)	7.42 (6.92)	7.17 (9.06)	2.84 (12.05)	−0.25 (7.36)	0.020	1.000	0.076	0.001
Yes	23.15 (37.37)	10.88 (27.06)	9.48 (27.53)	−12.27 (19.2)	−1.4 (5.42)	0.022	0.591	0.016	
P[1]	0.040	0.799	0.489						
BAFF[+]									
SVR									
No	0.24 (0.5)	0.28 (0.5)	0.19 (0.43)	0.04 (0.21)	−0.09 (0.12)	1.000	0.453	1.000	0.010
Yes	0.03 (0.07)	0.32 (0.68)	0.42 (1.3)	0.28 (0.64)	0.1 (0.69)	0.017	1.000	0.089	
P[1]	0.045	0.978	0.652						
Vitamin D									
SVR									
No	19.55 (6.57)	20.84 (4.02)	18.36 (4.02)	1.28 (6.14)	−2.48 (4.66)	1.000	0.419	1.000	0.029
Yes	23.66 (9.74)	24.06 (8.33)	17.83 (8.16)	0.41 (9.52)	−6.24 (6.14)	1.000	<0.001	0.001	
P[1]	0.202	0.218	0.834						

[1]P-value for group effect; [2]P-value for time effect (BSL to end of therapy measurement); [3]P-value for time effect (end of therapy to 6 months measurement); [4]P-value for time effect (BSL to 6 months measurement); [5]Repeated measurements ANOVA. Effects reported include significant differences between the two groups in the degree of change in each particular variable during the study period; [+]analysis was based on logarithmic transformations

BSL, baseline; EOT, end of therapy; 6Mo-EOT, 6 months after the end of therapy; IL-17A, interleukin 17A; BAFF, B-cell activating factor; SVR, sustained virological response

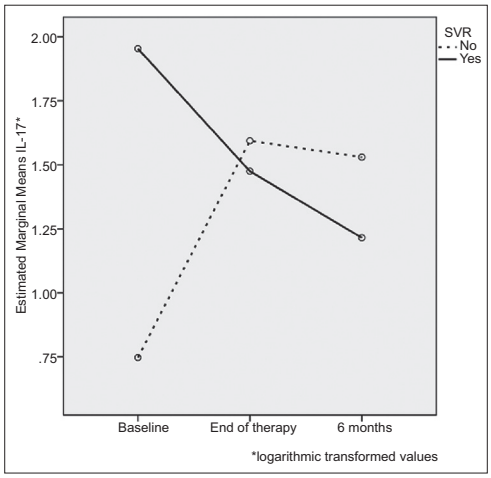

Figure 1 Changes in interleukin 17A levels during follow up in relation to sustained virological response (SVR)

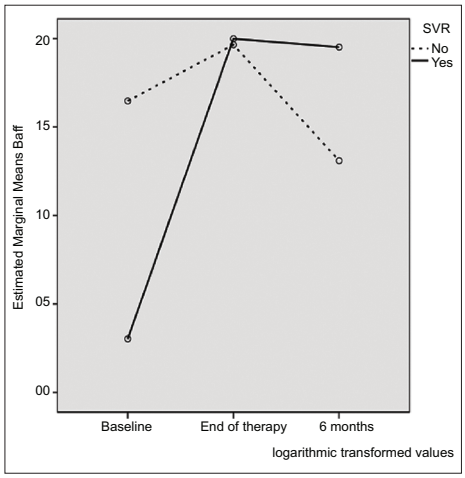

Figure 2 Changes in B-cell activating factor levels during follow up in relation to sustained virological response (SVR)

changes in IL-17A (β=-0.23, SE=0.44, P=0.601). Additionally, no significant association of vitamin D changes with corresponding BAFF changes was demonstrated during follow up (β=0.82, SE=2.59, P=0.751). In contrast, it was found that IL-17A changes were significantly associated with vitamin D changes (β=-0.04, SE=0.02, P=0.046).

Correlation coefficients between IL-17A, BAFF and vitamin D showed that vitamin D levels at BSL were negatively correlated with BAFF at EOT (P=0.017) and at 6MoA-EOT (P=0.03). No correlation was found between vitamin D and BAFF at BSL or between vitamin D and IL-17A at any time point. Finally, no significant differences in IL-17A, BAFF

Table 4 Values of IL-17A (pg/mL), BAFF (ng/mL) and vitamin D (ng/mL) levels in patients with and without MC and changes during therapy

Parameter	Mean±SD					Significance			
	BSL	EOT	6Mo-EOT	Change BSL to EOT	Change EOT to 6Mo EOT	P[2]	P[3]	P[4]	P[5]
IL17[+]									
MC									
No	6.73 (10.85)	4.2 (4.42)	4.17 (8.02)	−2.52 (9.1)	−0.03 (6.42)	1.000[+]	0.730[+]	1.000[+]	0.855[+]
Yes	25.38 (41.29)	14.51 (29.51)	12.67 (29.98)	−10.86 (23.36)	−1.85 (5.83)	1.000[+]	1.000[+]	1.000[+]	
P[1]	0.021[+]	0.180[+]	0.079[+]						
BAFF[+]									
MC									
No	0.02 (0.04)	0.11 (0.23)	0.06 (0.15)	0.09 (0.23)	−0.05 (0.18)	0.903	1.000	1.000	0.351
Yes	0.18 (0.42)	0.47 (0.79)	0.58 (1.44)	0.29 (0.7)	0.1 (0.76)	0.047	1.000	0.243	
P[1]	0.047	0.071	0.093						
vitD									
MC									
No	21.34 (10.44)	22.07 (8.69)	16.99 (7.91)	0.73 (7.64)	−5.08 (5.95)	1.000	0.005	0.072	0.909
Yes	22.98 (7.44)	23.68 (5.75)	18.92 (5.98)	0.71 (9.23)	−4.76 (5.98)	1.000	0.006	0.076	
P[1]	0.599	0.524	0.426						

[1]P-value for group effect; [2]P-value for time effect (BSL to end of therapy measurement); [3]P-value for time effect (end of therapy to 6 months measurement); [4]P-value for time effect (BSL to 6 months measurement); [5]Repeated measurements ANOVA. Effects reported include significant differences between the two groups in the degree of change in each particular variable during the study period; [+]analysis was based on logarithmic transformations
BSL, baseline; EOT, end of therapy; 6Mo-EOT, 6 months after the end of therapy; IL-17A, interleukin 17A; BAFF, B-cell activating factor; MC, mixed cryoglobulinemia

and vitamin D were found between groups with or without cirrhosis (P=0.363, P=0.567, P=0.956, respectively) and those with low or high HCV RNA levels (P=0.737, P=0.3, P=0.317, respectively).

Discussion

Several studies have suggested that cytokines play an important role in the pathogenesis, progression and treatment outcome of HCV-infected patients. The immune responses are highly complex and not well defined. Extrahepatic autoimmune involvement with abnormal immune responses mediated by both T and B lymphocytes is observed in CHC, mainly in those with cryoglobulinemia [21,22]. Nevertheless, the role of different IL-17A-secreting T cells on CHC patients with MC is less clear.

Th17 cells are a subpopulation of CD4+ T cells that have been implicated in the disease progression of many autoimmune and inflammatory disorders [23]. In the present study, we compared IL-17A and BAFF levels between CHC patients with and without asymptomatic MC, as well as between CHC patients and HC. Both cytokines were found to be significantly elevated in MC compared to the non-MC group. Other studies evaluating the immune phenotype and its relationship with B cells in these patients have reported elevated serum BAFF levels in patients with MC [24,25]. To our knowledge, there is no other study showing increased IL-17A levels in MC, suggesting that IL-17A might participate in HCV-related autoimmunity. There is one study in which increased expression of IL-17 was associated with HCV infection but not with asymptomatic MC [26].

We observed a significant increase in the IL-17A levels in HCV-infected patients compared to HC, confirming recent reports [27,28]. Interestingly, further analysis showed high levels of IL-17A in patients who responded to antiviral treatment versus non-responders [29]. Hence, upregulation of IL-17A might be considered as a predictor of the response to therapy with pegIFN/RBV. It is difficult to propose a cutoff point and larger studies are required to further assess these findings. When we further analyzed IL-17A kinetics during the treatment period, we noticed that antiviral therapy significantly downregulated IL-17A in the SVR group (Fig. 1). This finding persisted in responders beyond the cessation of antiviral treatment, suggesting that the suppressed cytokine release could be attributed to the viral clearance itself and not to the antiviral treatment. Likewise, Sousa *et al* suggested that pegIFN/RBV treatment resulted in a significant down-modulation of IL-17, mainly in responders [30].

On the other hand, IL-17A was upregulated in the non-SVR group. Our results suggest that a worsening proinflammatory

condition (IL-17A) during antiviral treatment may have a deleterious effect and may lead to treatment failure. The study of Fathy *et al* described a normalizing immune response during treatment concerning IL-17A, in both responders and non-responders, but in a very small number of patients and over a short period of time [31], while Sousa *et al* described a non-significant downregulation in their non-responder group. Menezes *et al* reported that patients with SVR displayed lower IL-17 levels compared with non-responders [32].

Innate and adaptive immunity are associated with the prognosis of HCV infection and the response to antiviral therapy. Suppressed cellular immunity against HCV may contribute to the development of chronic infection [5,33]. Similarly to what Lake-Bakaar *et al* described [34], the current study demonstrated that BAFF kinetics during antiviral treatment differed between responders and non-responders. On the other hand, unlike the earlier study, we included asymptomatic patients with MC and extended the investigation of BAFF kinetics to 6 months after the end of treatment. Interferon therapy in responders induced an enhanced immune response, as indicated by increasing BAFF levels, resulting in viral clearance. Previous investigators revealed that antiviral therapy induced increased serum BAFF values, irrespectively of treatment response [8].

It is well established that BAFF levels seem to be higher in patients with MC, especially when vasculitis is present. In the current study, which included asymptomatic patients with or without MC, BAFF levels were higher in the MC group, but they were comparable between patients and HC. Other studies also showed that HCV patients without vasculitis had levels similar to those of healthy individuals [35,36]. Higher BAFF levels compared to HC were evident in patients with cryoglobulinemia [34].

Vitamin D is an immune modulator that reduces inflammation while enhancing protective immune responses. Severe vitamin D deficiency (25-OH-vitamin D serum level <20 ng/mL) is a common feature in chronic hepatitis C, even in the absence of advanced liver fibrosis [37]. A high rate of HCV patients in the current study had vitamin D deficiency (<20 ng/mL) or insufficiency (20-29 ng/mL). Increased IL-17A values were evident in patients with MC compared to those without, but no difference in vitamin D levels at BSL was noticed between these groups. However, using mixed linear models we found that changes in IL-17A were significantly associated with changes in vitamin D levels (β=-0.04, SE=0.02, P=0.046), indicating an interaction between vitamin D and IL-17 expression [38].

This study had some limitations. First, the small size of the sample and HC group and the short post-treatment period of follow up may have prevented some results from reaching statistical significance. On the other hand, this study may serve as preliminary data for further investigation into this issue. Second, in the DAA era, the effect of antiviral treatment with pegIFN/RBV may appear outdated. However, many changes in the variables measured at BSL and at the EOT persisted for long after treatment, suggesting a normalization of the immune response achieved by viral clearance.

In conclusion, different kinetics of IL-17A, BAFF and vitamin D were assessed between responders and non-responders to antiviral treatment resulting in normalization of the immune response in the former group after treatment. An interplay between IL-17A and vitamin D concentrations was apparent during the antiviral treatment period, a finding that may explain the low levels of vitamin D in this group of patients. Finally, overexpression of both BAFF and IL-17A is associated with the presence of MC in CHC patients. MC poses therapeutic challenges even in the DAA era. In this respect, any additional information on this topic definitely enhances our ability to understand the complex mechanisms involved in cryoglobulinemia and its effect on HCV patients.

Summary Box

What is already known:

- Mixed cryoglobulinemia (MC) in chronic hepatitis C (CHC) is associated with abnormal immune responses mediated by T and B cells
- Interleukin (IL)-17 and B-cell activating factor (BAFF) levels are higher in the serum of CHC patients than in healthy controls
- Treatment with pegylated interferon-α (pegIFN) and ribavirin (RBV) downregulates IL-17A and upregulates BAFF
- An increase in IL-17 is correlated with a significant decrease in vitamin D

What the new findings are:

- IL-17A was shown to be significantly higher in CHC patients with MC
- High levels of IL-17A or low levels of BAFF appear to be associated with a sustained virological response to IFN/RBV
- Downregulation of IL-17A and upregulation of BAFF was observed after antiviral treatment in responders
- There is an interaction between vitamin D and IL-17A levels associated with antiviral treatment

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