

High Serum Levels of CXCL11 in Mixed Cryoglobulinemia Are Associated with Increased Circulating Levels of Interferon- γ

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ABSTRACT. Objective. No study has evaluated circulating chemokine C-X-C motif ligand (CXCL)11 in patients with “mixed cryoglobulinemia and chronic hepatitis C infection” (MC+HCV). We measured CXCL11, and correlated this measurement to the clinical phenotype.

Methods. Serum CXCL11, interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α) were assayed in 97 MC+HCV patients and in 97 sex- and age-matched controls.

Results. MC+HCV patients showed significantly higher mean CXCL11 serum levels than controls (254 ± 295 , 68 ± 16 pg/ml, respectively; $p = 0.0002$; ANOVA). CXCL11 was significantly increased in 36 cryoglobulinemic patients with compared to those without active vasculitis (303 ± 208 vs 179 ± 62 pg/ml, respectively; $p < 0.001$; ANOVA). IFN- γ levels were significantly higher in MC+HCV than in controls [6.1 (range 0.8–114.5), 1.4 (range 0.7–2.4) pg/ml, respectively; $p < 0.05$; Mann-Whitney U test]. Serum TNF- α mean levels were significantly higher in MC+HCV than in controls [13.4 (range 1.8–369), 1.1 (range 0.7–3.2) pg/ml, respectively; $p < 0.0001$; Mann-Whitney U test]. A multiple regression analysis considering CXCL11 as a dependent variable, and age, alanine aminotransferase, IFN- γ , and TNF- α as independent variables, showed in MC+HCV patients a significant association only with IFN- γ ($p < 0.0001$).

Conclusion. Our study demonstrates markedly high serum levels of CXCL11 in patients with MC+HCV compared to healthy controls overall in the presence of active vasculitis. A strong relationship between circulating IFN- γ and CXCL11 was shown, strongly supporting the role of a T helper 1 immune response in the pathogenesis of MC+HCV. (First Release July 1 2011; J Rheumatol 2011;38:1947–52; doi:10.3899/jrheum.110133)

Key Indexing Terms:

CXCL11/ITAC
CXCL10

INTERFERON- γ
HEPATITIS C

TUMOR NECROSIS FACTOR- α
CRYOGLOBULINEMIA

Interferon-inducible T cell α chemoattractant (I-TAC), or chemokine C-X-C motif ligand (CXCL)11, is a non-ELR (lacking the Glu-Leu-Arg tripeptide motif) CXC chemokine¹. CXCL11 is 36% and 37% identical in primary structure to the

other non-ELR CXC chemokines interferon- γ (IFN- γ) inducible protein 10 (IP-10)/CXCL10, and monokine induced by IFN- γ (MIG)/CXCL9, respectively¹. In humans, CXCL11, CXCL10, and CXCL9 all map to the same locus on chromosome 4^{1,2,3}.

CXCL11 utilizes CXC chemokine receptor 3 (CXCR3)^{1,4}, a G protein-coupled receptor expressed primarily on activated T cells, yet also found on endothelial cells⁵. CXCL10 and CXCL9 also bind CXCR3, but with lower affinity^{1,4} and less potency¹ than CXCL11.

Among activated T cells, CXCR3 is more highly expressed on the T helper (Th1) subset⁶. CXCL11 expression is strongly increased in response to IFN- β or IFN- γ and is presumed to be involved in pathologies characterized by the presence of activated T cells. CXCL11 recruits activated Th1 lymphocytes to sites of inflammation.

Recent evidence has shown that CXC α chemokines (Th1), especially CXCL10, play an important role in the active phases of mixed cryoglobulinemia (MC). Indeed, circulating CXCL10 is high overall in cryoglobulinemic patients with active vasculitis, suggesting a prevalence of the Th1 immune

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response in this phase^{7,8,9}. Further, the level of CXCL10 is higher in MC patients with autoimmune thyroiditis versus those without^{10,11}.

Many studies have linked a Th1 immune response with MC and chronic hepatitis C infection (HCV+). Th1 response leads to increased IFN- γ and tumor necrosis factor- α (TNF- α) production that in turn stimulates CXCL10 secretion by the target cells, thus perpetuating the immune cascade. This process may lead to the appearance of MC in genetically predisposed subjects¹².

In HCV+, only a few studies have evaluated serum levels of CXCL11, with discordant results. Increased circulating levels of CXCL11 were observed in some studies^{13,14,15}, while other studies were not able to show any significant difference from controls¹⁶. *In vitro*, CXCL11 mRNA and protein expression were inducible in Huh-7 cells following either IFN- α or IFN- γ stimulation and synergistically with TNF- α . Further, transfection of Huh-7 cells with either poly(I:C) or HCV RNA representing the HCV subgenomic replicon induced CXCL11 mRNA expression¹⁷. These results suggest CXCL11, one of the most potent chemoattractants for activated T cells, is produced by hepatocytes in the HCV-infected liver and plays an important role in T cell recruitment and ultimately in the pathogenesis of HCV+.

To our knowledge, no study has evaluated serum levels of CXCL11 in patients with “mixed cryoglobulinemia and HCV chronic infection” (MC+HCV)¹⁸. The aim of our study was to evaluate serum levels of CXCL11 and of the Th1 cytokines IFN- γ and TNF- α , in a series of MC+HCV patients and to correlate these measures to clinical features of the disease.

MATERIALS AND METHODS

Patients. Ninety-seven MC+HCV patients (68 women and 29 men; mean age 60 ± 13 SD yrs; mean disease duration 15 ± 10 SD yrs), consecutively referred to our Rheumatology Unit, were recruited for the study between 2002 and 2009. The diagnosis of MC+HCV was based on the presence of serum mixed (IgG-IgM) cryoglobulins and the classical clinical triad — purpura, weakness, arthralgias — and on the exclusion of other well known systemic disorders, such as immunorheumatic and neoplastic diseases^{7,8,9}.

Our study included only patients with MC+HCV, without liver cirrhosis or hepatocellular carcinoma (by histology, laboratory evidence of liver failure, and/or ultrasound-proven portal hypertension), in whom a thyroid screening (history, physical examination, thyrotropin, free triiodothyronine, free thyroxine, anti-thyroglobulin and anti-thyroid peroxidase antibody measurements, and ultrasonography) excluded the presence of associated thyroid autoimmune disorders, a well known cause of high serum CXCR3 chemokines^{10,11}.

The main demographic and clinico-serological features of MC+HCV patients are reported in Table 1. Among them, 29 had been previously treated with IFN- α for an average of 7.3 months (range 1–17); the time elapsed from the last course of IFN- α treatment ranged from 6 to 68 months (mean 30 ± 16). No statistically significant differences were observed in the main demographic and clinico-serological features of MC+HCV patients treated or untreated with IFN- α .

At the time of study, 66 MC+HCV patients were taking low doses of corticosteroids (< 6 mg/die methylprednisolone), 12 had previously received corticosteroids, and 19 had never been treated with corticosteroids. No MC+HCV patient had plasma exchange treatment in the year before the

Table 1. Demographic and clinico-serological features of 97 patients with mixed cryoglobulinemia (MC) and chronic hepatitis C infection.

Characteristic	
Age, yrs	60 \pm 13
Men/women	29/68
Disease duration with MC, yrs	15 \pm 10
Purpura, %	88
Active vasculitis, %	37
Weakness, %	98
Arthralgias, %	94
Arthritis, %	13
Raynaud's phenomenon, %	45
Sjögren's syndrome, %	48
Peripheral neuropathy, %	76
Renal involvement ^a , %	13
Aminotransferase elevation and/or histologic activity ^b , %	81
Cryocrit, %	4.8 \pm 8.9
CH50 (normal 160–220 units)	111 \pm 42
C3 (normal 0.6–1.3 g/l)	0.77 \pm 0.42
C4 (normal 0.2–0.55 g/l)	0.14 \pm 0.08
Autoantibodies ^c , %	31

^a Serum creatinine > 132.6 μ mol/l and/or proteinuria > 0.5 g/24 h.

^b Increase of liver enzyme (alanine aminotransferase) and/or histological alterations. ^c Presence of antinuclear and/or anti-mitochondrial and/or anti-smooth muscle and/or anti-extractable nuclear antigen autoantibodies.

study. In both patients and controls, a careful medical history was collected, in particular with regard to family history of thyroid disease, smoking habit, and drugs. The presence of Raynaud's phenomenon, Sjögren's syndrome, skin ulcers, peripheral neuropathy, and renal and liver involvement in MC+HCV patients was evaluated as described^{7,8,9}. Routine blood chemistry was carried out by standard methods.

Controls. Each of the 97 MC+HCV patients eligible for the study was matched, by sex and age, one-to-one with a control group of healthy subjects of the general population from the same geographic area (North-West Tuscany). This control group was extracted from a larger sample of 1640 subjects in a population-based survey of thyroid disorders; only HCV-negative subjects, without clinical and laboratory evidence of thyroid and liver disorders and autoimmune diseases and not treated with immunomodulators, were included.

Extraction of the control group from the original population was performed by finding the closest age match (± 2 yrs) to each case within either gender⁷. When more than one age match was available per case, the choice was made at random.

The study protocol was approved by the local ethics committee. All subjects gave their informed consent to enter the study.

Immunological studies. Cryocrit was measured as the percentage of packed cryoglobulins after cold centrifugation of the serum; cryoglobulin composition was determined by including the presence in cryoprecipitates of monoclonal or polyclonal IgM-rheumatoid factor (i.e., MC type II or MC type III); hemolytic complement C3–C4 fractions were measured as described⁸; antinuclear, anti-smooth muscle, and anti-mitochondrial autoantibodies were detected by current techniques^{7,8,9}. Sera with a titer > 1:40 were considered positive. Anti-extractable nuclear antigen antibodies, including anti-Sc170, antibodies to Sm antigen, anti-RNP, anti-SSA/SSB, anti-proliferating cell nuclear antigen, anti-histidyl-tRNA synthetase (-SL and -Jo1) specificities, were detected by counter-immunoelectrophoresis^{7,8,9}.

Virological studies. Antibodies against HCV (anti-HCV) and HCV RNA were determined on serum clotted and centrifuged at 37°C and stored at -70°C. Anti-HCV and HCV RNA (polymerase chain reaction technique) in serum were investigated as described^{7,8,9}.

Analytical measurements. Alanine aminotransferase (ALT), γ -glutamyltrans-

ferase (γ -GT), aspartic-aminotransferase (AST), alkaline phosphatase, bilirubin, and platelet count were assayed by conventional methods^{7,8,9}.

Cytokines and chemokine assays. Serum CXCL11 levels were assayed by a quantitative sandwich immunoassay using a commercial kit (R&D Systems, Minneapolis, MN, USA), with a sensitivity ranging from 0.41 to 21.5 pg/ml and a mean minimum detectable dose of 11.9 pg/ml. The intra- and interassay coefficients of variation were 4.1% and 7.4%.

Serum IFN- γ and TNF- α concentrations were measured using commercial kits (R&D Systems). The mean minimum detectable dose was 8 pg/ml for IFN- γ and 0.12 pg/ml for TNF- α ; the intra- and inter-assay coefficients of variation were 2.9% and 6.3% for IFN- γ , 5.8% and 10.2% for TNF- α . Samples were assayed in duplicate. Quality control pools of low, normal, or high concentration for all parameters were included in each assay.

Data analysis. Values are given as mean \pm SD for normally distributed variables, or as median \pm interquartile range (IQR) for non-normally distributed variables. Group values were compared by univariate analysis of variance (ANOVA), for normally distributed variables, or by Kruskal-Wallis (≥ 3 groups) or Mann-Whitney U (2 groups) tests. Proportions were compared by chi-square test. Post-hoc comparisons on normally distributed variables were carried out using the Bonferroni-Dunn test. Univariate analysis was performed by simple regression. A multiple regression analysis considering CXCL11 as dependent variable, and age, ALT, IFN- γ , and TNF- α as independent variables was performed in MC+HCV patients. Statistical power (ex-post analysis) (stat-power) was calculated.

RESULTS

Patients with MC+HCV showed significantly higher mean CXCL11 serum levels than controls (254 ± 295 , 68 ± 16 pg/ml, respectively; $p = 0.0002$, ANOVA; stat-power = 1; Figure 1).

To better define the role of increased serum CXCL11 in MC+HCV, mean levels of this chemokine were separately evaluated by ANOVA among MC+HCV patient subgroups defined according to main demographic and clinical features (age > 55 yrs; gender; disease duration > 10 yrs; presence or absence of purpura, active vasculitis, weakness, arthralgias, arthritis, Raynaud's phenomenon, Sjögren's syndrome, peripheral neuropathy, renal involvement, aminotransferase

elevation and/or histologic activity in the liver). Significantly higher levels of CXCL11 were observed in 36 patients with active vasculitis (defined as necrotizing vasculitis with or without vasculitic skin ulcers)^{8,9} at the time of the study, in comparison to those without active vasculitis (303 ± 208 vs 179 ± 62 pg/ml, respectively; $p < 0.001$; ANOVA; Figure 2; stat-power = 1); no other significant result was found.

By defining high CXCL11 level as a value of at least 2 SD above the mean value of the control group (> 100 pg/ml), 86% of patients with MC+HCV and 8% of control subjects had high CXCL11 ($p < 0.0001$ vs controls; chi-square).

No significant correlations were observed between CXCL11 and serological findings of MC+HCV (levels of cryocrit and complement, presence/absence of autoantibodies) or previous/ongoing treatments.

IFN- γ was detectable in the serum of 8% of controls and 28% of MC+HCV patients. IFN- γ levels, in patients with detectable IFN- γ , were significantly higher in MC+HCV than in controls [6.1 (range 0.8–114.5), 1.4 (range 0.7–2.4) pg/ml, respectively; median (interquartile range); $p < 0.05$; Mann-Whitney U test; stat-power = 0.9].

Serum TNF- α was detectable in 87% of controls and in all MC+HCV patients; mean levels were significantly higher in MC+HCV than in controls [13.4 (range 1.8–369), 1.1 (range 0.7–3.2) pg/ml, respectively; $p < 0.0001$; Mann-Whitney U test; stat-power = 1]. No correlation was found between serum TNF- α and CXCL11, or ALT, or the presence of active vasculitis, or the other demographic, serological and clinical features of MC.

No correlation was found among IFN- γ levels and TNF- α or ALT, or the presence of active vasculitis, or other demographic, serological, and clinical features of MC. No relationship was found among IFN- γ or TNF- α levels and previous/ongoing treatments.

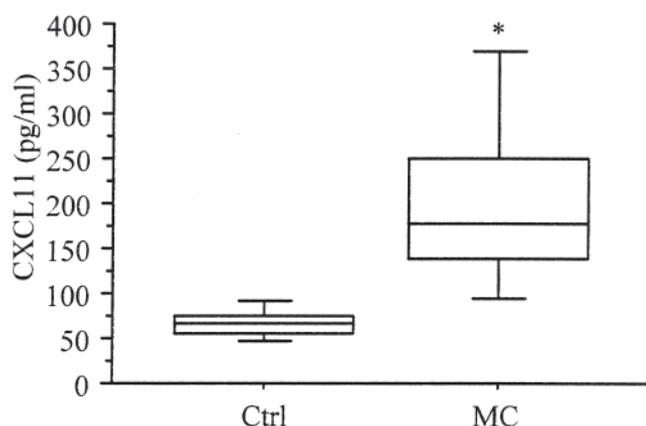


Figure 1. CXCL11 secretion in patients with mixed cryoglobulinemia and chronic hepatitis C infection (MC+HCV) compared to healthy controls. MC+HCV patients (MC) showed significantly higher mean CXCL11 than controls (ANOVA; $p < 0.0001$). Box indicates lower and upper quartiles, central line is the median value; horizontal bars show 2.5% and 97.5% values. * $p < 0.05$ or less versus control by Bonferroni-Dunn test.

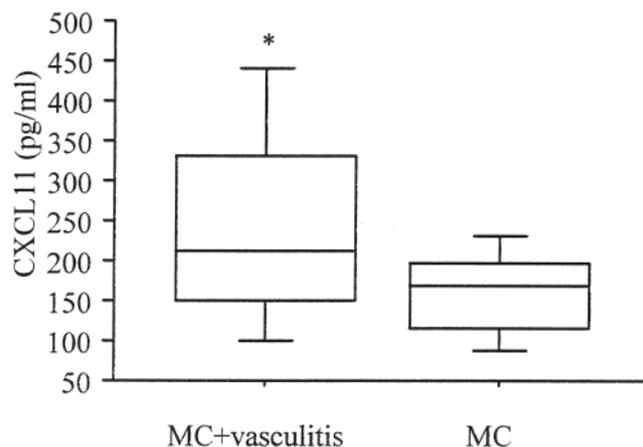


Figure 2. CXCL11 secretion in patients with mixed cryoglobulinemia and chronic hepatitis C infection (MC+HCV) in presence or absence of active vasculitis. Serum CXCL11 (ANOVA; $p < 0.001$) was significantly increased in MC+HCV patients with active vasculitis (MC+vasculitis) compared to those without (MC; ANOVA; $p < 0.001$). * $p < 0.05$ or less versus MC+HCV patients without active vasculitis by Bonferroni-Dunn test.

A simple regression analysis showed a significant correlation between circulating CXCL11 and IFN- γ levels ($r = 0.46$; $p < 0.0001$); no other association was observed by simple regression.

CXCL11, evaluated by classes of circulating levels of IFN- γ (IFN- $\gamma < 1$; $1 < \text{IFN-}\gamma < 3$; $3 < \text{IFN-}\gamma < 5$; $< 5 \text{ IFN-}\gamma$; pg/ml), showed a progressive increase, with significantly higher values, for circulating levels of IFN- γ higher than 3 and 5 pg/ml versus IFN- γ values lower than 1 pg/ml (ANOVA; $p < 0.01$, for both; Figure 3; stat-power = 0.9).

A multiple regression analysis considering CXCL11 as a dependent variable, and age, ALT, IFN- γ , and TNF- α as independent variables was performed in MC+HCV patients, showing a significant association only with IFN- γ [coefficient (β), 0.74; coefficient (regression coefficient), 13.1; 95% CI 9.5, 16.7; $p < 0.0001$].

DISCUSSION

Our study is the first to demonstrate significantly high serum levels of CXCL11 in patients with MC+HCV compared to healthy controls. Interestingly, among MC+HCV the CXCL11 levels were significantly higher in patients with signs of active vasculitis compared to those without. To our knowledge, this is the first study showing that circulating levels of CXCL11 are associated with serum IFN- γ levels, strongly supporting the role of a Th1 immune response in the pathogenesis of MC+HCV.

The involvement of CXCL11 in hepatic HCV infection has been shown in many studies. It has been previously demonstrated that CXCL11 mRNA was expressed in hepatocytes of patients with HCV+. Further, transfection of Huh-7 cells with either poly(I:C) or HCV RNA representing the HCV subgenomic replicon induced CXCL11 mRNA expression¹⁷. These results suggest CXCL11, one of the most potent chemoattractants for activated T cells, is produced by hepatocytes in the HCV-infected liver and plays an important role in T cell recruitment and ultimately in the pathogenesis of HCV+.

Bièche, *et al* studied the expression of 240 genes in first-stage liver fibrosis in patients with HCV+¹⁹, demonstrating that the 3 most discriminatory genes were CXCL10, CXCL11, and IFI27.

Other findings suggest that the CXCR3-associated chemokines CXCL10 and CXCL11 may play an important role in the development of necroinflammation and fibrosis in the liver parenchyma in HCV+²⁰.

The importance of the CXCR3 chemokines, in particular CXCL11, was highlighted by replicating HCV (JFH-1) to selectively upregulate its expression in response to IFN- γ and TNF- α ¹⁶. In summary, the CXCR3 (CXCL10, CXCL11) chemokines are the most significantly expressed chemokines in HCV+ and most likely play a role in positioning T cells in the liver. Further, HCV+ can selectively increase CXCL11 expression in response to IFN- γ and TNF- α stimulation, which may play a role in the pathogenesis of HCV-related liver disease.

In HCV+, few studies have evaluated serum levels of CXCL11, with discordant results. Increased circulating levels of CXCL11 were observed in some studies^{13,14,15}, while other studies were not able to show any significant difference with controls¹⁶. Chemokine levels were measured in samples collected before, during, and after antiviral therapy from a group of 29 patients infected with HCV+. Levels of CXCL10 and CXCL9 decreased following successful antiviral therapy; CXCL11 did not decline significantly during or in the first 6 months after therapy¹³.

In our MC+HCV patients serum levels of CXCL11 were significantly higher than in controls. The possible contribution of HCV to high CXCL11 serum levels in MC+HCV patients cannot be excluded. Indeed, CXCL11 levels in MC+HCV patients without active vasculitis are higher than those found in controls. Other studies will be needed to evaluate if CXCL11 levels in MC+HCV patients are different from those of HCV patients without MC, and if CXCL11 may have a causative role in MC+HCV.

The significantly higher serum CXCL11 in MC+HCV patients with active vasculitis compared to those without suggests that a further, significant increase of this chemokine, expression of a Th1 immune response, is particularly relevant in the pathogenesis of cryoglobulinemic vasculitis. These data agree with findings in other non-MC+HCV vasculitic syndromes^{18,21,22}.

Further, the increase of CXCL11 is in agreement with recent evidence, which has shown that CXCL10 plays an important role in the active phases of MC. Indeed, circulating CXCL10 is high overall in cryoglobulinemic patients with active vasculitis, suggesting a prevalence of the Th1 immune response in this phase^{7,8,9}.

Other studies have shown the involvement of CXCL10 in different types of vasculitic diseases^{23,24,25}. However, to our

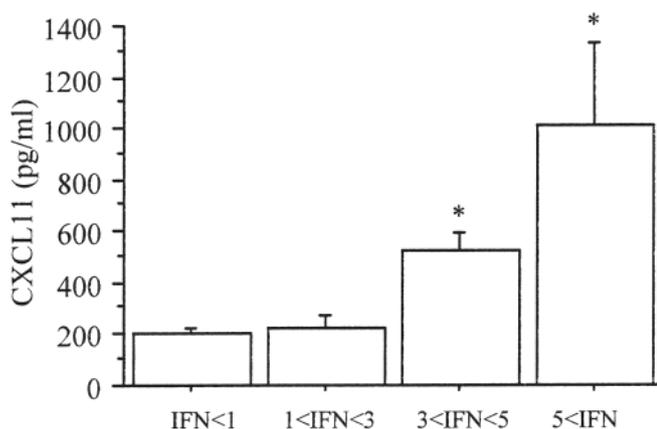


Figure 3. CXCL11 secretion, evaluated by classes of IFN- γ , in patients with mixed cryoglobulinemia and chronic hepatitis C infection. CXCL11, evaluated by classes of circulating levels of IFN- γ (IFN < 1; $1 < \text{IFN} < 3$; $3 < \text{IFN} < 5$; $< 5 \text{ IFN}$; pg/ml), showed a progressive increase with significantly higher values for circulating levels of IFN- γ higher than 3 and 5 pg/ml versus IFN- γ values lower than 1 pg/ml (ANOVA; $p < 0.01$, for both). Bars are mean \pm SEM. * $p < 0.05$ or less versus IFN- γ values lower than 1 pg/ml (IFN < 1) by Bonferroni-Dunn test.

knowledge, ours is the first study showing an involvement of CXCL11 in a vasculitic disease such as cryoglobulinemia, opening the way to the evaluation of CXCL11 as an inflammatory marker of vasculitic disorders.

Change in serum chemokine levels in the course of other autoimmune disorders has been described. Recent experimental evidence has demonstrated that CXC chemokines and particularly CXCL10 play an important physiopathological role in the initial phases of autoimmune thyroid disorders^{26,27,28}, with an inverse correlation between circulating CXCL10 levels and disease duration in Graves' disease.

In our study we found no relation between serum CXCL11 levels and the duration of MC+HCV, probably because the disease is characterized by a relapsing clinical course whose most common expression is in vasculitic symptoms; these complications may appear at any time during followup, possibly triggered by multiple pathogenetic co-factors²⁹. Clinico-pathological alterations of MC+HCV may recognize at least 2 synergical pathogenetic mechanisms triggered by HCV. First, B-cell proliferation leads to immune-complex production, mainly HCV-containing cryoglobulins³⁰, which are responsible for immune complex-mediated vasculitis^{29,31,32,33}; while high serum CXCL11 levels secondary to both HCV-related vascular and hepatic cell injury strongly amplify the inflammatory process through Th1-mediated immune response.

The increase of CXCL11 in the active phase of the disease is in agreement with findings from previous reports in which serum CXCL10 was found to be high in the active phase of Graves' disease³⁴.

Longitudinal studies evaluating serum CXCL11 levels in larger MC+HCV patient series will be necessary to evaluate if serum CXCL11 measurement could represent an easily detectable prognostic marker for clinical management of MC+HCV patients.

More recently, it has been shown that high plasma CXCL10 levels correlate with a poor outcome of antiviral therapy in patients with HCV+. Indeed, a low baseline CXCL10 level was significantly associated with low baseline viral load, rapid viral response, and sustained viral response in HCV+ patients treated with IFN- α ^{13,35,36,37}. Since IFN- α is a well known effective therapy for MC+HCV³³, pretreatment evaluation of serum CXCL11 levels might suggest the virological response to IFN also in MC+HCV patients; however, more studies are needed to further evaluate this point.

IFN- γ serum levels were significantly increased in MC+HCV patients (with detectable IFN- γ). These findings agree with data on upregulation of IFN- α , IFN- β , and IFN- γ in HCV+¹⁹. Circulating TNF- α was higher in MC+HCV than in controls. These data agree with those obtained in a limited number of patients with MC+HCV³⁸. The increase of TNF- α in MC+HCV patients may be due to liver disease; however, since no correlation was found between TNF- α levels and ALT, a definitive conclusion is not possible. However, other

studies have shown increased production of TNF- α by lymphocytes of MC+HCV patients^{39,40}.

Interestingly, serum CXCL11 levels were strongly associated with high levels of circulating IFN- γ . Different studies have shown that *in vitro* IFN- γ alone stimulates, TNF- α alone has no effect, and the combination of IFN- γ plus TNF- α has a synergistic effect (vs IFN- γ alone) on CXCL11 secretion in different types of cells, such as hepatocytes, thyrocytes, fibroblasts, preadipocytes, and others^{16,17,41,42}. Our results, obtained in MC+HCV patients, are fully in agreement with these studies.

This is the first demonstration of a strong relationship between circulating IFN- γ and CXCL11 in a human pathology such as MC+HCV. Our finding strongly supports the role of a Th1 immune response in the pathogenesis of MC+HCV⁴³.

Our study demonstrates markedly high serum levels of CXCL11 in patients with MC+HCV compared to healthy controls overall in the presence of active vasculitis. A strong relation between circulating IFN- γ and CXCL11 in MC+HCV was shown, strongly supporting the role of a Th1 immune response in the pathogenesis of MC+HCV. Future studies in larger patient series will be needed to evaluate the relevance of serum CXCL11 determination as a clinico-prognostic marker of MC+HCV, as well as its usefulness in the therapeutic approach to these patients.

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