Chemokine (CXC motif) ligand 9 serum levels in mixed cryoglobulinaemia are associated with circulating levels of IFN-gamma and TNF-alpha

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Abstract

Objectives

No study evaluated circulating chemokine (CXC motif) ligand (CXCL)9 in "patients with mixed cryoglobulinaemia and hepatitis C virus chronic infection" (MC+HCV). We aimed to measure CXCL9, IFN-γ and TNF-α in a series of MC+HCV to correlate these parameters to different clinical phenotypes.

Methods

Serum CXCL9, IFN-γ and TNF-α were assayed in 54 MC+HCV, in 54 patients with HCV chronic infection (HCV+) and in 54 sex- and age-matched controls.

Results

MC+HCV showed significantly higher mean CXCL9 than HCV+ patients (p=0.01; ANOVA) or controls (p=0.0001; ANOVA), in particular in 21 cryoglobulinaemic patients with active vasculitis compared to those without (p<0.001; ANOVA). Serum IFN-γ (in patients with detectable IFN-γ) and TNF-α were significantly higher in MC+HCV than in controls (p<0.05, Mann-Whitney U test; p<0.0001, Mann-Whitney U-test; respectively). CXCL9, evaluated by classes of IFN-γ (IFN-γ<2; 2<IFN-γ<5; IFN-γ>5 pg/mL), or TNF-α (TNF-α<2; 2<TNF-α<10; TNF-α>10 pg/mL), showed a progressive, but not significant, increase of circulating values. When the combination of high circulating levels of IFN-γ and TNF-α (IFN-γ>2 and TNF-α>10 pg/mL vs. IFN-γ<2 and/or TNF-α<10 pg/mL) was evaluated, significantly higher CXCL9 levels were observed (p<0.01; ANOVA).

Conclusion

We demonstrated markedly high serum levels of CXCL9 in MC+HCV (vs. HCV+ patients or healthy controls), significantly associated with the presence of active vasculitis. A strong relation among high levels of circulating IFN-γ, TNF-α and serum CXCL9 has been shown in MC+HCV. Larger patients' series will be needed to evaluate the relevance of serum CXCL9 determination as clinico-prognostic marker of MC+HCV.

Key words

CXCL9, hepatitis C, cryoglobulinaemia, IFN-γ, TNF-α, CXCL10, chemokine, cytokine

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Introduction

Monokine induced by IFN-γ (MIG), also known as chemokine (CXC motif) ligand (CXCL)9 (CXCL9), along with IFN-γ-induced protein 10 (IP-10)/CXCL10 and IFN-inducible T-cell α chemoattractant (ITAC)/CXCL11, is a member of a CXC chemokine subfamily that lacks an ELR (Glu-Leu-Arg) motif in front of the first cysteine. Whereas ELR+CXC chemokines attract neutrophils and promote angiogenesis *via* CXC receptor (CXCR)1 and CXCR2, ELR-CXC chemokines recruit activated lymphocytes and retard angiogenesis via CXCR3 (1, 2).

CXCR3 is a seven transmembrane G-protein coupled receptor expressed on activated T-helper (Th)1, but not Th2 lymphocytes (2, 3).

Expression of CXCL9 is induced by the prototypical Th1 cytokine, IFN-γ, and it is dramatically enhanced by addition of tumour necrosis factor (TNF)-α (4). A wide range of cells and tissues exhibits IFN-γ-dependent CXCL9 production, including monocytes/macrophages, neutrophils, keratinocytes, endothelial cells, epithelial cells (1, 5).

Not surprisingly, CXCL9 has been implicated in pathologies characterised by the accumulation of activated Th1 lymphocytes. These include acute allograft rejection, glomerulonephritis, autoimmunity, rheumatoid arthritis, atherosclerosis, psoriasis, and allergic contact dermatitis (5-12).

Recent evidence has shown that CXC α-chemokines (Th1) play an important role in the initial phases of autoimmune thyroid diseases. Patients with newly diagnosed autoimmune thyroiditis (AT) show an increased serum CXCL10, in particular in the presence of a more aggressive thyroiditis and hypothyroidism (13, 14).

Our group has recently suggested the important role played by CXC α-chemokines (Th1), especially CXCL10, in the active phases of mixed cryoglobulinaemia (MC). In fact, circulating CXCL10 is high in particular in cryoglobulinaemic patients with active vasculitis, suggesting a prevalence of the Th1 immune response in this phase (15-17).

Furthermore, CXCL10 is higher in cryoglobulinaemic patients with AT, with respect to those without (18, 19).

A link between a Th1 immune response and MC in HCV infection was evidenced by numerous studies: Th1 response leads to increased IFN-γ and 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 subjects (20).

In hepatitis C chronic infection (HCV-CI), few studies have evaluated serum levels of CXCL9, with discordant results.

Some authors observed increased circulating levels of CXCL9 compared with controls (21-25), however, this has not been confirmed by others (26).

It has been shown that the most noteworthy changes in gene expression of HCV patients with first stage of liver fibrosis mainly affected the transcriptional network regulated by IFNs, including IFN-γ-inducible genes (CXCL9, CXCL10, CXCL11) (27).

Furthermore, plasma levels of CXCL9, TNF receptor (sTNFR)1, and sTNFR2 were independently associated with liver histological changes, suggesting a role of TNF activation and Th1-type cell-mediated immune response in the pathogenesis of HCV infection (24).

These results suggest CXCL9, 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-CI.

To our knowledge, no study has evaluated serum levels of CXCL9 in patients with "mixed cryoglobulinaemia and HCV chronic infection" (MC+HCV).

The aim of this study was to evaluate serum levels of CXCL9, and of the Th1 cytokines IFN-γ and TNF-α, in a series of MC+HCV patients, and to correlate these parameters to the clinical features of the disease.

Methods

Patients

Fifty-four MC+HCV patients (41 F and 13 M; mean age 58±12 standard deviation [SD] years; mean disease duration 13±12 SD years), consecutively re-

ferred 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 immuno-rheumatic and neoplastic diseases (15-17).

Only patients with the following features were included in the study: diagnosis of 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 triiodo-thyronine, free thyroxine, anti-thyroglobulin and anti-thyroid peroxidase antibodies measurements, and ultrasonography) excluded the presence of associated thyroid autoimmune disorders, a well known cause of high serum CXCR3 chemokines (18, 19).

The main demographic and clinico-serological features of MC+HCV patients are reported in Table I.

Twenty-five MC+HCV patients had sicca syndrome. Among them, only 5 had a diagnosis of Sjögren's syndrome (SS) according to the American-European classification criteria (28).

Among 5 MC+HCV patients with SS, 2 had anti-Ro/SSA and 1 had antiLa/SSB; all the others were negative. A positive minor salivary gland biopsy was present in 3/5 MC+HCV patients in whom biopsy was performed.

Among our MC+HCV patients, 16 had been previously treated with IFN-α for an average of 7.3 months (range 2–21); the time elapsed from the last course of IFN-α treatment ranged from 6 to 74 months (mean 28±19). 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, 38 MC+HCV patients were taking low doses of corticosteroids, 5 had previously been on corticosteroids and 11 had never been treated with corticosteroids. No MC+HCV patient had had plasma exchange treatment in the last year before the study. In both patients and controls, a careful medical history was collected, in particular with regard to family history of thyroid disease, smoking habits, and drugs. The presence of Raynaud's phenomenon (RP), sicca syndrome or SS, skin ulcers, peripheral neuropathy, renal and liver involvement in MC+HCV patients was evaluated as previously described (15-17). Routine blood chemistry was carried out by standard methods.

Table I. Demographic and clinico-serological features of 54 MC+HCV patients.

Age (years)	58 ± 12
Men/women	13/41
Disease duration with MC (years)	13 ± 12
Purpura	86%
Active vasculitis	36%
Weakness	96%
Arthralgias	92%
Arthritis	16%
Raynaud's phenomenon	44%
Sicca syndrome	46%
Peripheral neuropathy	78%
Renal involvement*	16%
Aminotransferases elevation and/or histologic activity†	87%
Cryocrit (%)	4.1 ± 7.7
CH50 (normal: 160–220 units)	108 ± 37
C3 (normal: 60–130 mg/dL)	78 ± 34
C4 (normal: 20–55 mg/dL)	15 ± 9
Autoantibodies‡	36%

*Serum creatinine >1.5 mg/dL and/or proteinuria >0.5 gr/24h.
†Increase of the liver enzyme (alanine aminotransferase) and/or histological alterations.
‡Presence of anti-nuclear and/or anti-mitochondrial and/or anti-smooth muscle and/or anti-extractable nuclear antigen autoantibodies.

Patients with HCV chronic infection (HCV+)

HCV+ patients (n=54) at first presentation were matched by age and gender with MC+HCV patients (41 F and 13 M; mean age 56±13 SD years). The diagnosis of chronic hepatitis C was based on abnormal serum aminotransferase levels for longer than 6 months and positive anti-HCV serology and HCV-RNA.

Only HCV+ patients, not previously treated with IFN, without liver cirrhosis (by histology, laboratory evidence of liver failure and/or ultrasound-proven portal hypertension), without hepatocellular carcinoma, in whom a thyroid screening (history, physical examination, thyrotropin, free triiodo-thyronine, free thyroxine, anti-thyroglobulin and anti-thyroid peroxidase antibodies measurements, and ultrasonography) excluded the presence of associated thyroid autoimmune disorders (18, 19), and without other well-known systemic disorders, such as immuno-rheumatic, neoplastic, and infectious diseases were included in this group.

Controls

Each of the 54 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 evidences of thyroid and liver disorders and autoimmune diseases and not treated with immunomodulators, were included.

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 Commitee. 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); haemolytic complement C3-C4 fractions were measured as previously described (29); anti-nuclear, anti-smooth muscle, and anti-mitochondrial autoantibodies were detected by current techniques (15-17). Sera with a titre>1:40 were considered positive. Anti-extractable nuclear antigen antibodies, including anti-Scl70, -Sm, -RNP, -SSA/SSB, -PCNA, -SL and -Jo1 specificities, were detected by counter-immunoelectrophoresis (15-17).

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 antibodies and HCV RNA (by PCR technique) in the serum were investigated as previously described (15-17).

Analytical measurements

Alanine aminotransferase (ALT), γ-glutamyltransferase (γ-GT), aspartic-aminotransferase (AST), alkaline phosphatase, bilirubin, and platelet count were assayed by conventional methods (15-17).

Cytokines and chemokines assays

Serum CXCL9 levels were assayed by a quantitative sandwich immunoassay using a commercially available kit (R&D Systems, Inc., Minneapolis, MN, USA), with a sensitivity ranging from 1.3–11.3 pg/mL and a mean minimum detectable dose of 3.8 pg/mL. The intra- and inter-assay coefficients of variation were 4.2% and 6.8%.

Serum IFN-γ and TNF-α concentrations were measured in serum using commercially available 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±SD for normally distributed variables, or as median±interquartile range (IQR) for not 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 the χ^2 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 CXCL9 as a dependent variable, and age, ALT, IFN-γ and TNF-α as independent variables, was performed in MC+HCV patients.

Results

Mean CXCL9 serum levels were significantly higher in patients with MC+HCV than controls ($p<0.0001$; ANOVA) (Fig. 1).

To better define the role of increased serum CXCL9 in MC+HCV, mean levels of this chemokine were separately evaluated by ANOVA among MC+HCV patients' subgroups defined according to main demographic and clinical features (age>55 years; gender; disease duration>10 years; presence or absence of purpura, active vasculitis, weakness, arthralgias, arthritis, RP, sicca syndrome or SS, peripheral neuropathy, renal involvement, aminostransferases elevation and/or histologic activity in the liver). Significantly higher levels of CXCL9 were observed in 21 patients with active vasculitis at the time of the present study in comparison to those without ($p<0.001$; ANOVA) (Fig. 2); no other significant result was found.

By defining high CXCL9 level as a value of at least 2 SD above the mean value of the control group (>100 pg/mL), 91% of patients with MC+HCV, 6% of the controls had high CXCL9 ($p<0.0001$ *vs*. controls; χ^2 test).

No significant correlations were observed between CXCL9 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 6% of controls, and 28% of MC+HCV. IFN-γ levels, in patients with detectable IFN-γ, were significantly higher in MC+HCV than in controls [6.3 (0.8–121, range) pg/mL, 1.2 (0.7–2.1, range) pg/mL, respectively; median and (interquartile range); $p<0.05$; Mann-Whitney U-test].

Serum TNF-α was detectable in 84% of controls and in all MC+HCV patients; mean levels were significantly higher in MC+HCV than in controls [13.8 (1.6–391, range) pg/mL, 1.0 (0.7–2.9, range) pg/mL, respectively; $p<0.0001$; Mann-Whitney U-test]. No correlation was found between serum TNF-α and CXCL9, or ALT, or the presence of active vasculitis, or the other demographic, serological and clinical features of MC.

A simple regression analysis showed no significant correlation between circulating CXCL9 and IFN-γ or TNF-α levels; no other association was observed by simple regression.

CXCL9, evaluated by classes of IFN-γ circulating levels (IFN-γ<2; 2<IFN-γ<5; IFN-γ>5 pg/mL), showed a progressive, but not significant, increase of circulating values. Also when CXCL9 was evaluated by classes of TNF-α circulating levels (TNF-α<2; 2<TNF-α<10; TNF-α>10 pg/mL), a progressive, but not significant, increase was observed. However, when it was evaluated by the combination of high circulating levels of IFN-γ and TNF-α (IFN-γ>2 and TNF-α>10 pg/mL, *vs*. IFN-γ<2 and TNF-α<10 pg/mL), significantly higher levels of serum CXCL9 were observed in association ($p<0.01$; ANOVA) (Fig. 3).

A multiple regression analysis considering CXCL9 as dependent variable, and age, ALT, IFN-γ and TNF-α as independent variables, was performed in MC+HCV patients, and it showed no significant association.

Mean CXCL9 serum levels were significantly higher in HCV+ patients than in controls ($p<0.001$; ANOVA) (Fig. 1), but lower than MC+HCV patients ($p<0.01$; ANOVA) (Fig. 1).

By defining high CXCL9 level as a value of at least 2 SD above the mean value of the control group (>100 pg/mL), 78% of HCV+ patients had high CXCL9 ($p<0.0001$ *vs*. controls and MC+HCV patients; χ^2 test).

Discussion

Our study first demonstrates significantly higher serum levels of CXCL9 in patients with MC+HCV compared to healthy controls. Interestingly, among MC+HCV the CXCL9 levels were significantly higher in patients with signs of active vasculitis compared to those without. Moreover, to the best of our knowledge, we have first shown that CXCL9 circulating levels are associated with the combination of high serum IFN-γ and TNF-α levels, strongly supporting the role of a Th1

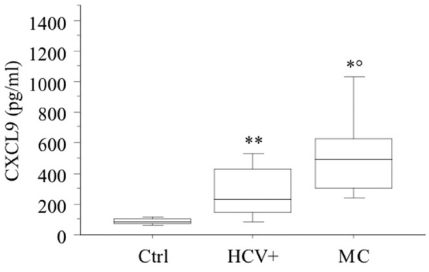

Fig. 1. MC+HCV patients (MC) showed significantly (*) higher mean CXCL9 than HCV+ patients (p<0.01; ANOVA) or controls (°) (Ctrl) (p<0.0001; ANOVA). HCV+ patients have higher CXCL9 serum levels than controls (**) (Ctrl) (p<0.001; ANOVA). The box indicates the lower and upper quartiles and the central line is the median value; the horizontal lines at the end of the vertical lines are the 2.5% and 97.5% values.

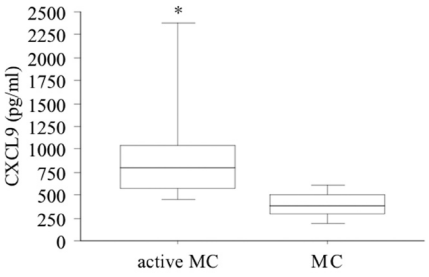

Fig. 2. Serum CXCL9 (p<0.001; ANOVA) was significantly (*) increased in MC+HCV patients with "active vasculitis" (active MC) compared to those without (p<0.001; ANOVA). Data are displayed as box-and-whisker plots.

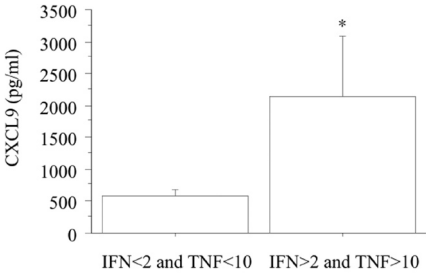

Fig. 3. The combination of high circulating levels of IFN-γ and TNF-α (IFN-γ>2 and TNF-α>10 pg/mL, *vs*. IFN-γ<2 and TNF-α<10 pg/mL), was significantly (*) associated with higher levels of serum CXCL9 (p<0.01; ANOVA).

immune response in the pathogenesis of MC+HCV.

Interestingly, CXCL9 serum levels were significantly higher in MC+HCV patients than in HCV+ patients, suggesting that the coexistence of both the conditions, mixed cryoglobulinaemia and HCV infection, is associated with higher CXCL9 circulating levels in MC+HCV patients.

Our results agree with those of other studies in patients with HCV infection without cryoglobulinaemia. In fact, the levels of circulating CXCL9 in HCV+ patients were similar to those found in other studies (21-25). However, another study was not able to show any significant difference with controls (26).

Bieche *et al*. studied the expression of 240 genes in first stage liver fibrosis in patients with HCV chronic infection (27). The most notable changes in gene expression mainly affected the transcriptional network regulated by IFNs, including both IFN-α/β-inducible genes (STAT1, STAT2, etc.) and IFN-γ-inducible genes (CXCL9, CXCL10, CXCL11). Moreover, they showed mRNA level up-regulation for these genes. Furthermore, plasma levels of CXCL9, sTNFR1, and sTNFR2 were independently associated with liver histological changes, suggesting a role of TNF activation and Th1-type cell mediated immune response in the pathogenesis of HCV infection (24). These results suggest CXCL9, 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-CI.

CXCL9 serum levels were significantly higher in our MC+HCV patients than in controls; a possible contribution of HCV-CI to high CXCL9 serum levels in MC+HCV patients cannot be excluded, since CXCL9 levels in MC+HCV patients without active vasculitis are higher than those found in controls.

In agreement with findings obtained in other not MC+HCV vasculitic syndromes (30, 31), the significantly higher serum CXCL9 detected in our 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 cryoglobulinaemic vasculitis. Moreover, the increase of CXCL9 is in agreement with recent evidences, which have shown that CXCL10 plays an important role in the active phases of MC. Indeed, circulating CXCL10 is high in particular in cryoglobulinaemic patients with active vasculitis, suggesting a prevalence of the Th1 immune response in this phase (15-17).

No significant association was observed between CXCL9 serum levels and sicca syndrome in MC+HCV patients. Our finding is in line with the literature (32, 33). In fact, the involvement of CXCL9 in sicca syndrome has been demonstrated in salivary epithelial cells and tears, but, to the best of our knowledge, no study evaluated CXCL9 serum levels in patients with SS.

However, changes of serum chemokine levels in the course of other autoimmune disorders have been demonstrated (34-37).

Recent experimental evidence has demonstrated that CXC chemokines, and particularly CXCL10, play an important physiopathological role in the initial phases of autoimmune thyroid disorders (13, 14, 38), with an inverse correlation between circulating CXCL10 levels and disease duration in GD.

No relationship between serum CXCL9 levels and the duration of MC+HCV was found in our study, probably because the disease is characterised by a relapsing clinical course whose most common expression are vasculitic symptoms; these complications may appear at any time during the follow-up, possibly triggered by multiple pathogenetic co-factors (39). Clinico-pathological alterations of MC+HCV may recognise at least two synergical pathogenetic mechanisms triggered by HCV.

Firstly, B-cell proliferation leads to immune-complexes production, mainly HCV-containing cryoglobulins (40), which are responsible for immune-complex-mediated vasculitis (29, 39, 41, 42), while high serum CXCL9 levels secondary to both HCV-related vascular and hepatic cell injury strongly amplify the inflammatory process through Th1-mediated immune response.

Detection of increased CXCL9 levels in the active phase of the disease is in agreement with findings arisen from previous reports in which serum CXCL10 has been found high in the active phase of multiple sclerosis. In particular, previously reported inverse correlation between CXCL10 levels and time from last clinical relapse together with the

finding that CXCL10 is upregulated at disease onset and during relapse in multiple sclerosis (43, 44) strongly supports this hypothesis.

Moreover, our group has recently shown that increased serum CXCL10 levels in patients with GD are associated mainly with the active phase of GD, not related to hyperthyroidism itself, but mainly to autoimmune response (45).

To evaluate if serum CXCL9 measurement could represent an easily detectable prognostic marker for clinical management of MC+HCV patients, longitudinal studies evaluating serum CXCL9 levels in large MC+HCV patients' series are mandatory.

In fact, chemokine levels were measured in samples collected before, during, and after antiviral therapy from a group of 29 patients infected with HCV infection. Levels of CXCL10 and CXCL9 decreased following successful antiviral therapy (21).

IFN-γ serum levels were significantly increased in MC+HCV patients (with detectable IFN-γ), which agrees with the findings of up-regulation of IFN-α,-β and -γ in HCV-CI (27).

Circulating TNF-α was higher in MC+HCV than in HCV-CI in accordance with data obtained in a limited number of patients with MC+HCV patients (46). Although a definitive conclusion is not possible, since no correlation was found between TNF-α levels and ALT, the increase of TNF-α in MC+HCV patients may be due to a more aggressive liver disease. However, other studies have shown an increased production of TNF-α by lymphocytes of MC+HCV patients (47, 48).

Interestingly, serum CXCL9 levels were strongly associated with high levels of circulating IFN-γ and TNF-α. This finding is in agreement with previuos data showing that the combination of IFN-γ and TNF-α is able to induce CXCR3-chemokines secretion in hepatocytes *in vitro* (26).

Furthermore, it has been recently shown that the combination of IFN-γ and TNF-α was able to produce a potent synergistic effect on the secretion of CXCL9, both in human thyrocytes and fibroblasts, *in vitro* (37).

However, to the best of our knowledge, this is the first demonstration of a strong relation between circulating IFN-γ and TNF-α and CXCL9, in a human pathology, such as MC+HCV.

This finding strongly supports the role of a Th1 immune response in the pathogenesis of MC+HCV.

In conclusion, our study demonstrates markedly higher serum levels of CXCL9 in patients with MC+HCV compared to HCV+ patients or healthy controls; in MC+HCV patients increased CXCL9 levels were significantly associated with the presence of active vasculitis. Moreover, a strong relation between high levels of circulating IFN-γ and TNF-α and serum CXCL9, in MC+HCV has been shown. Larger patients' series will be needed to evaluate the relevance of serum CXCL9 determination as clinico-prognostic marker of MC+HCV, as well as its usefulness in the therapeutic approach to these patients.

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