The presence of autoimmune thyroiditis in mixed cryoglobulinemia patients is associated with high levels of circulating interleukin-6, but not of tumour necrosis factor-alpha

A. Antonelli[1], C. Ferri[2], S.M. Ferrari[1], A. Di Domenicantonio[1], P. Ferrari[1], C. Pupilli[3], A. Nicolini[1], A.L. Zignego[4], S. Marchi[1], P. Fallahi[1]

[1]Department of Internal Medicine, University of Pisa School of Medicine, Pisa, Italy; [2]Department of Internal Medicine, Rheumatology Unit, University of Modena and Reggio Emilia School of Medicine, Modena, Italy; [3]Endocrinology Unit, Azienda Ospedaliera Careggi and University of Florence, Florence, Italy; [4]Department of Internal Medicine, University of Florence, Florence, Italy.

Alessandro Antonelli, MD
Clodoveo Ferri, MD
Silvia M. Ferrari, MSc
Andrea Di Domenicantonio, BSc
Paola Ferrari, MD,
Cinzia Pupilli, MD
Andrea Nicolini, MD
Anna Linda Zignego, MD
Santino Marchi, MD
Poupak Fallahi, MD

Please address correspondence to:
Dr Alessandro Antonelli,
Department of Internal Medicine,
University of Pisa – School of Medicine,
Via Roma 67,
56100 Pisa, Italy.
E-mail: alessandro.antonelli@med.unipi.it

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ABSTRACT

Objectives. *To our knowledge, no study has evaluated serum levels of interleukin-6 (IL-6), together with tumour necrosis factor-alpha (TNF-α), in a large series of patients with "mixed cryoglobulinemia and HCV chronic infection" (MC+HCV) in relation to the presence of autoimmune thyroiditis (AT). The aims of the study were to evaluate serum levels of IL-6 in MC+HCV patients and to correlate this parameter with the presence of AT and with circulating levels of TNF-α.*

Methods. *Serum IL-6 and TNF-α were assayed in 41 MC+HCV patients, in 41 MC+HCV patients with autoimmune thyroiditis (MC+AT), in 41 sex- and age-matched controls, and 20 AT patients.*

Results. *MC+HCV patients showed significantly ($p<0.01$; Mann-Whitney U-test) higher IL-6 (median 8.1ng/l, range 0.7–651) serum levels than controls (median 0.6ng/l, range 0.5–41), or AT (median 2.8ng/l, range 0.5–67). MC+AT showed significantly ($p<0.01$; Mann-Whitney U-test) higher mean IL-6 (median 15.8ng/l, range 0.5–781) than controls, AT and MC+HCV. Serum TNF-α levels were significantly higher in MC+HCV (median 9.9ng/l, range 1.5–283) or MC+AT (median 11.2ng/l, range 1.6–412) than in controls (median 1.0ng/l, range 0.6–6.4), or AT (median 1.7ng/l, range 0.6–11.8) ($p<0.01$, for each comparison).*

Conclusion. *Our study demonstrates significantly higher serum levels of IL-6 and TNF-α in patients with MC+HCV and MC+AT compared to healthy controls. Furthermore, the study first shows a significant increase in circulating IL-6 observed in MC+AT patients with respect to MC+HCV. Future studies in larger patients' series will be needed to evaluate the relevance of serum IL-6 and TNF-α determination as clinico-prognostic markers of MC+HCV patients and its usefulness in the therapeutic approach to these patients.*

Introduction

An involvement of the immune system in the pathogenesis of "mixed cryoglobulinemia (MC) and hepatitis C virus (HCV) chronic infection" (MC+HCV) has been shown (1).

Cytokines might be of particular relevance in this context as they play a central role in the immune response to viral agents (2-4).

Furthermore, we have recently shown markedly high serum levels of chemokine (C-X-C motif) ligand 10 (CXCL10) and tumour necrosis factor-alpha (TNF-α) in patients with MC+HCV compared to patients with HCV (HCV+) and healthy controls; moreover, in MC+HCV patients increased CXCL10 levels were significantly associated with the presence of active vasculitis (5).

Interleukin-6 (IL-6) is a multifunctional protein produced by lymphoid and non-lymphoid cells, including fibroblasts, hepatocytes and vascular endothelial cells (6, 7). The effect of IL-6 on B cells is stimulation of differentiation and antibody secretion (8), while it affects T cells to stimulate IL-2 production and receptor expression. It stimulates production of acute phase proteins by hepatocytes (9) and has colony-stimulating activity on hematopoietic stem cells (10, 11).

The various activities of IL-6 suggest that this factor has a major role in the mediation of the inflammatory and immune responses. Elevated IL-6 levels have been reported to be associated with a variety of diseases, including autoimmune diseases such as arthritis (7), mesangial proliferative glomerulonephritis (7) and inflammatory bowel disease (12).

Until now, to our knowledge, few studies have evaluated the importance of IL-6 in MC patients. IL-6 serum concentrations have been shown to be increased in HCV-infected patients with or without MC (13-17).

We have previously demonstrated a high frequency of autoimmune thyroid disorders in cryoglobulinemic patients (1). Furthermore, it has been shown that CXCL10 in patients with "mixed cryoglobulinemia and autoimmune thyroiditis (AT)", (MC+AT) is significantly higher than MC+HCV (18).

However, the immunological base of this association remains to be further investigated. Increased serum IL-6 concentration has been shown in patients with subacute thyroiditis, in amiodarone-induced thyroiditis and in patients with Graves' ophthalmopathy (19-21). Furthermore, in patients with Hashimoto's thyroiditis (HT), serum IL-6 levels are positively associated with thyroxine replacement dose and negatively associated with 3,5,3'-triiodothyronine (T3) (22). More recently, it has been shown that IL-6 serum levels are increased in patients with HT (23).

To our knowledge, no study has evaluated serum levels of IL-6 and TNF-α in MC+HCV patients in presence or absence of AT. The aims of the study were to evaluate serum levels of IL-6 and TNF-α in a series of MC+HCV patients in the presence or absence of AT and to relate their levels to the clinical phenotype of these patients, features of the disease and presence or absence of AT.

Methods

Patients

The diagnosis of MC+HCV was based on the presence of serum mixed (type II or III) cryoglobulins and the classical clinical triad – purpura, weakness, arthralgias – and on the exclusion of other well-known systemic disorders, such as immuno-rheumatic, neoplastic and infectious diseases other than HCV infection (1). HCV infection was systematically evaluated in all patients, and HCV negative subjects were excluded. Only patients with MC+HCV were included, without hepatocellular carcinoma and/or liver cirrhosis (identified by histology, laboratory evidence of liver failure and/or ultrasound-proven portal hypertension) (24). The presence of sicca syndrome, skin ulcers, peripheral neuropathy and renal and liver involvement in MC+HCV patients was evaluated, as previously described (25). Routine blood chemistry was carried out by standard methods (25). No MC+HCV patient has had plasma exchange treatment in the last year before the study. Vasculitis activity, neuropathy, arthritis and liver histological activity were evaluated, as previously reported (5, 16).

Seven out of eleven patients with renal involvement were evaluated with renal biopsy.

A thyroid screening included history, physical examination, thyroid stimulating hormone (TSH), free triiodo-thyronine (FT3), free thyroxine (FT4), anti-thyroglobulin (AbTg) and anti-thyroid peroxidase (AbTPO) antibodies measurements and neck ultrasonography.

All study subjects gave their informed consent to the study, which was approved by the local Ethics Committee.

Patients with mixed cryoglobulinemia without autoimmune thyroiditis (MCo)

Forty-one MC+HCV patients (males/females, 8/33; age, 60±13 years) consecutively referred to the Rheumatology Unit were recruited into the study between 2001 and 2006. Only patients with MC+HCV, in whom a thyroid screening excluded the presence of associated thyroid autoimmune disorders (26), were included in this group (MCo).

The main demographic and clinico-serological features of MCo patients are reported in Table I. Among them, 16 had been previously treated with interferon alpha (IFN-α) for an average of 8.1 months (range 1 to 17), at a mean dosage of 9.9MU/week (range 3–10); the time elapsed from the last course with IFN-α treatment ranged from 6 to 81 months (mean 44). No statistically significant difference was observed in the main demographic and clinico-serological features of MCo patients treated or untreated with IFN-α.

At the time of the study, 21 MCo patients were taking low doses of corticosteroids, 11 had previously been on corticosteroids and 9 had never been treated with corticosteroids.

Patients with mixed cryoglobulinemia and autoimmune thyroiditis (MC+AT)

Forty-one MC+HCV patients (males/females, 5/36; age, 59±14 years) consecutively referred to the Rheumatology Unit were recruited into the study between 2001 and 2006. Only patients with MC, in whom a thyroid screening revealed the presence of associated thyroid autoimmune disorders, were included in this group (MC+AT).

The main demographic and clinico-serological features of MC+AT patients are reported in Table I. Among them, 19 had been previously treated with INF-α for an average of 6.7 months (range 1–10), at a mean dosage of 7.7MU/week (range 3–9); the time elapsed from the last course of IFN-α treatment ranged from 6 to 83 months (mean 41). No statistically significant difference was observed in the main demographic and clinico-serological features of MC+AT patients treated or untreated with IFN-α.

At the time of study, 19 MC+AT patients were taking low doses of corticosteroids, 11 had previously been on corticosteroids and 11 had never been treated with corticosteroids.

Controls

The control group consisted of 41 subjects (males/females, 8/33; age, 61±15 years), extracted from a random sample of the general population within the same geographic area (27) without HCV infection or other liver disorders, coupled by gender and age (that is a well known confounding factor) with MCo patients, in whom a complete thyroid work-up was available and excluded the presence of thyroid autoimmune disorders, or any kind of immunomodulant therapy.

Controls – autoimmune thyroiditis

The control group consisted of 20 subjects (males/females, 4/16; age, 58±14 years), extracted from a random sample of the general population within the same geographic area (27) without HCV infection or other liver disorders, or any kind of immunomodulant

Table I. Clinical characteristics of 41 patients with hepatitis C virus-related mixed cryoglobulinemia (MC) without autoimmune thyroiditis (MCo) and 41 with autoimmune thyroiditis (MC+AT). No significant differences were observed about the undermentioned characteristics in the 2 groups.

	MCo n=41	MC+AT n=41
Age (years)	60±13	59±14
Males/Females	8/33	5/36
Disease duration with MC (years)	12±12	10±12
Purpura	84%	87%
Weakness	92%	90%
Arthralgias	92%	88%
Arthritis	15%	17%
Sicca syndrome	45%	50%
Peripheral neuropathy	77%	74%
Renal involvement*	13%	16%
Aminotransferases elevation and/or histologic activity§	81%	78%
Cryocrit (%)	4.1±9.6	4.3±8.9
C3 (normal 60–130 mg/dl)	83±41	87±39
C4 (normal 20–55 mg/dl)	15±13	13±14
Autoantibodies†	27%	29%

*Serum creatinine >1.5mg/dl and/or proteinuria >0.5gr/24h; §Increase of the liver enzyme alanine transaminase and/or histological alterations. †Presence of antinuclear antibodies (1:160 or less, without extractable nuclear antigens positivity).

therapy, matched by gender and age (a well known confounding factor) with MCo patients, in whom a complete thyroid work-up was available and that showed the presence of thyroid autoimmune disorders (presence of circulating AbTg, AbTPO, or thyroid hypoechogenecity).

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); C3-C4 fractions were measured as previously described; antinuclear, anti-smooth muscle and antimitochondrial autoantibodies were detected by current techniques. 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 (5, 18, 25).

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 -PCR-technique) in the serum were investigated as previously described (5, 18, 25).

Ultrasonography of the neck and fine-needle aspiration

Thyroid ultrasonography was performed both in patients and controls. Neck ultrasonography was performed by the same (blinded) operator using an Esaote AU5 (Florence, Italy) with a sectorial 7.5MHz transducer. Thyroid volume was calculated using the ellipsoid formula, as described (18, 26). The presence of hypoechoic and dyshomogeneous echogenicity was arbitrarily rated at three levels (0=normal echogenicity; 1=slight hypoechoic and dyshomogeneous pattern; 2=severely hypoechoic and dyshomogeneous pattern) in order to evaluate structural abnormalities of thyroid tissue associated with AT (18, 26). The presence of thyroid nodules was recorded and nodules with a diameter >10mm were submitted to ultrasonography-guided fine-needle aspiration, which was performed by the same operator, using the free-hand method as already described (18, 26).

Thyroid blood flow

Thyroid blood flow (TBF) by colour-flow Doppler was studied in all patients (18, 26). The TBF pattern was defined as followed: a) normal (or type 0) when TBF was limited to peripheral thyroid arteries; b) type I when TBF was mildly increased; c) type II when TBF was clearly increased; d) type III when TBF was markedly increased.

Laboratory evaluation

Laboratory evaluation included measurement of serum levels of TSH (reference range 0.3–3.6 μU/ml), FT3, FT4, AbTg and AbTPO. Circulating FT3 and FT4 were measured by commercial RIA kits (AMERLEX-MAB FT3/FT4 Kit; Amersham, Little Chalfont, Buckinghamshire, UK). Serum TSH (Dia-Sorin, Stillwater, MN, USA), AbTPO and AbTg (ICN Pharmaceuticals, Costa Mesa, CA, USA) were evaluated by Immunoradiometric Assay (IRMA) methods. For AbTg, AbTPO, positivity was set at >100UI/ml and >100UI/ml, respectively (5, 18, 25). Values are given as mean±SD for normally distributed variables.

Alanine aminotransferase (ALT) was assayed by conventional methods (5).

Cytokines, chemokines and analytical assays

Serum IL-6 levels were assayed by a quantitative sandwich immunoassay using a commercially available kit (R&D Systems, Minneapolis, MN, USA), with a sensitivity of 0.5pg/ml. The intra- and inter-assay coefficients of variation were 2.5% and 3.0%.

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

Data analysis

To verify the distribution of the evaluated variables, the Shapiro-Wilk test was used. Values are given as mean±SD for normally distributed variables, or as median and range for not normally distributed variables (IL-6, TNF-α). Group values were compared by univariate

ANOVA, for normally distributed variables; or by Kruskal-Wallis (≥3 groups) or Mann-Whitney U- (2 groups) tests, proportions were compared by the 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 multivariate logistic regression analysis was performed in MC+HCV patients, considering age, gender, ALT, presence or absence of active vasculitis, and of AT as independent variables and presence or absence of high levels of IL-6 or TNF-α as the dependent variable.

Results

Patients with MCo showed significantly (Mann-Whitney U-test) higher mean IL-6 serum levels (median 8.1ng/l, range 0.7–651) than controls ($p<0.01$) (median 0.6ng/l, range 0.5–41), or AT ($p<0.01$) (median 2.8ng/l, range 0.5–67). Patients with MC+AT showed significantly ($p<0.01$; Mann-Whitney U-test) higher mean IL-6 (median 15.8ng/l, range 0.5–781) than controls, or AT or MCo ($p<0.05$; Mann-Whitney U-test) (Fig. 1a). AT patients had higher IL-6 circulating levels than controls (Mann-Whitney U-test, $p<0.05$).

By defining high IL-6 level as a value higher than the 95th percentile of the control group (>2.4pg/ml), 45% of AT patients, 61% of patients with MCo, 5% of the control subjects and 80% of MC+AT patients had high IL-6 ($p<0.01$, MCo *vs*. controls; $p<0.01$, MC+AT *vs*. controls; $p<0.05$, MCo *vs*. MC+AT, or MC+AT *vs*. AT; $p<0.01$, AT *vs*. controls; Chi-square).

In order to better define the role of increased serum IL-6 in MC+HCV, mean levels of this chemokine were separately evaluated (by Mann-Whitney U-test) 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, Raynaud's phenomenon, sicca syndrome, peripheral neuropathy, renal involvement, aminostransferases elevation and/or histological activity in the liver), but no significant difference was observed. No significant correlations were observed between IL-6 and serological findings of MC+HCV (levels of cryocrit and complement, presence/absence of autoantibodies) or previous/ongoing treatments. No significant correlations were observed among IL-6 and TSH, FT3, FT4, AbTg and AbTPO titres; however, IL-6 was significantly higher in MC+AT patients in the presence of thyroid hypoechogenicity and/or positivity of AbTg or AbTPO.

Serum TNF-α was detectable in 34/41 (83%) of controls, 19/20 AT (95%), and in all MCo and MC+AT patients; mean levels were significantly (Mann-Whitney U-test) higher in MCo (median 9.9ng/l, range 1.5–283) or MC+AT (median 11.2ng/l, range 1.6–412) than in controls (median 1.0ng/l, range 0.6–6.4), or AT (median 1.7ng/l, range 0.6–11.8) ($p<0.01$, for each comparison). No significant difference in TNF-α serum levels was observed between MCo and MC+AT (Fig. 1b), or between controls and AT. No significant correlations were observed among TNF-α and TSH, FT3, FT4, AbTg, AbTPO, thyroid hypoecogenicity, presence or absence of AbTg or AbTPO. No correlation was found between serum TNF-α and IL-6, or ALT, or liver histology activity index, or stage of liver fibrosis, or the presence of active vasculitis, or the other demographic, serological and clinical features of MC.

There was no significant difference in serum ALT between MCo and MC+AT patients. The multivariate logistic regression analysis performed in MC+HCV patients, considering age, gender, ALT, presence or absence of active vasculitis, and of AT as independent variables and presence or absence of high levels of IL-6 or TNF-α as the dependent variable, showed a significant association with AT ($p=0.021$; coef=1.189, 95% lower=1.104, 95% upper=9.778). The activity index (grade) and the stage in MCo and MC+AT patients were not significantly different.

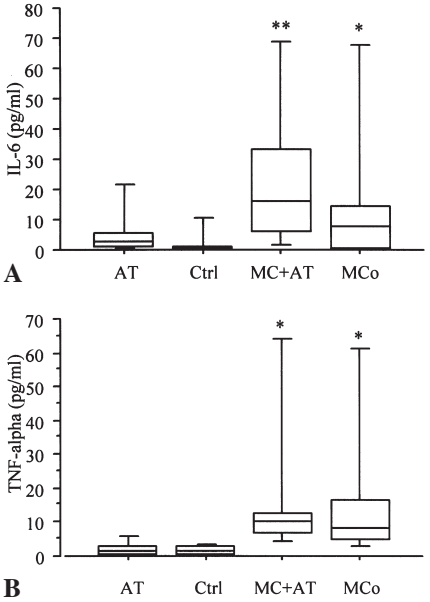

Fig. 1. A. Patients with MCo showed significantly (*) (Mann-Whitney U-test) higher IL-6 serum levels (box-plot: median, interquartile range and outlier) than controls ($p<0.01$), or AT ($p<0.001$). Patients with MC+AT (**) showed significantly ($p<0.01$; Mann-Whitney U-test) higher IL-6 than controls, or AT or MCo ($p<0.05$; Mann-Whitney U-test). AT patients had higher IL-6 circulating levels than controls (Mann-Whitney U-test, $p<0.05$).
B. Serum TNF-α levels were significantly (*) (Mann-Whitney U-test) higher in MCo, or MC+AT than in controls, or AT ($p<0.01$, for each comparison). No significant difference in TNF-α serum levels was observed between MCo and MC+AT, or between controls and AT.

Discussion

Our study demonstrates significantly high serum levels of IL-6 in MCo and MC+AT patients compared to healthy controls and confirms significantly high serum levels of TNF-α in patients with MCo and MC+AT patients compared to healthy controls (5). Furthermore, the study first shows a significant increase in circulating IL-6 in MC+AT patients with respect to MCo.

IL-6 is a typical pleiotropic cytokine that plays roles in the immune system and inflammation. It has been hypothesized that a deregulated, high-level production of IL-6 could induce an undesired inflammatory state in many organs. In fact, a number of reports implicate IL-6 in the pathogenesis of many human disorders such as rheumatoid arthritis (28), Sjögren's syndrome (29), systemic lupus erythematosus (30) and others. Moreover, IL-6 appears to be essential for the progression of experimentally induced-immunological disorders in animals, making IL-6 an attractive therapeutic target (31).

Our results showing high levels of circulating IL-6 in MC+HCV are in agree-

ment with two other studies present in literature (13, 14). IL-6 serum concentrations were increased in HCV-infected patients with or without MC (13). Furthermore, peripheral blood mononuclear cells of HCV patients associated with non-Hodgkin lymphoma B (B-NHL), MC (n=14), uncomplicated hepatitis C (n=12) and healthy volunteers (n=12) were incubated with the recombinant HCV proteins E2, core and secreted non-structural protein 3 (NS3) to study induction of cytokine production. HCV core was the only studied protein that induced production of IL-6 in CD14(+) mononuclear cells. In a second study (14) serum levels of IL-6 were evaluated in HCV+ patients with (n=30) and without (n=30) MC. IL-6 serum levels were higher in the MC+ group than the MC- group such as in our study. Interestingly, the observed IL-6 levels were similar to those in our MC patients.

Other studies have shown an increase of IL-6 in inflammatory and autoimmune thyroid disorders, such as subacute thyroiditis, amiodarone-induced thyroiditis and Graves' ophthalmopathy (19-21). Furthermore, in patients with HT serum, IL-6 levels were negatively associated with T3 (22). Other studies (31, 32) have not found an involvement of IL-6 in autoimmune thyroid disorders. In fact, no significant differences in IL-6 levels were found in most of the patient groups with thyroid dysfunctions (32, 33).

However, a recent study assessed the serum levels of different cytokines and IL-6 (involved in the differentiation of Th17 cells) of 18 patients with HT as well as 10 healthy controls. Increased serum concentrations of IL-6 were observed in HT patients (23). Our results confirm high levels of circulating IL-6 levels in AT patients with respect to controls, and suggest a determining role of AT in inducing higher levels of IL-6 in MC+AT with respect to MCo.

These findings are in agreement with the results of the present study, that first show that IL-6 serum levels are higher in MC+AT patients than in MCo patients, suggesting an involvement of IL-6 in the pathogenesis of AT in MC and possibly in HCV chronic infection.

The increase of IL-6 in MC+HCV is in agreement with findings arisen from previous reports in which serum CXCL10 has been found high in the active phase of multiple sclerosis and Graves' disease (GD). In particular, it has been shown that CXCL10 is upregulated at disease onset and during relapse in multiple sclerosis (34-36). Furthermore, we have recently shown that increased serum CXCL10 levels in patients with GD are associated mainly with the active phase of GD (37, 38). Since IL-6 in MC patients is higher in the presence of another autoimmune disorder such as AT, it will be necessary to evaluate if IL-6 higher levels are associated with other autoimmune manifestations of MC. Longitudinal studies evaluating serum IL-6 in large MC patients' series will be necessary in order to evaluate if serum IL-6 measurement could represent an easily detectable prognostic marker of autoimmunity for clinical management of MC patients.

Moreover, our study confirms that serum TNF-α resulted high in MC in accordance to other studies in hepatitic C patients (39-41). It is unlikely that the increase of TNF-α in MC patients is due to a more aggressive liver disease; in fact, in our study no correlation was found between TNF-α levels and ALT, or degree of liver inflammation (5). Other studies have shown an increased production of TNF-α by lymphocytes of MC+HCV patients (42, 43), suggesting that the increase of TNF-α may be due to lymphoid cells.

The finding of no significant change of TNF-α in the presence of AT is in agreement with the results of two recent studies in patients with AT (22, 44).

In conclusion, our study demonstrates significantly higher serum levels of IL-6 and TNF-α in patients with MCo and MC+AT compared to healthy controls. Furthermore, the study first shows a significant increase in circulating IL-6 in MC+AT patients with respect to MCo. Future studies in larger patients' series will be needed to evaluate the relevance of serum IL-6 and TNF-α determination as clinico-prognostic markers of MC+HCV patients, as well as its usefulness in the therapeutic approach to these patients.

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