

## TYPE II MIXED CRYOGLOBULINEMIA IN PATIENTS WITH HEPATITIS C VIRUS: TREATMENT WITH PEGYLATED-INTERFERON AND RIBAVIRIN

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### ABSTRACT

**Introduction:** Mixed Cryoglobulinemia is the most frequent extrahepatic disease in patients affected by Chronic Hepatitis C Virus infection. The association of pegylated-Interferon alpha-2a and Ribavirin could represent a rational and effective therapy for this extrahepatic disease. Was evaluated the safety and efficacy of pegylated-Interferon alpha-2a with Ribavirin for treatment of Hepatitis C Virus-related Mixed Cryoglobulinemia with detectable Hepatitis C Virus RNA, in patients with and without hepatic disease.

**Materials and methods:** 24 patients (14 with chronic hepatitis, 2 with Child-Pugh Class A cirrhosis, and 8 without hepatic disease), affected by Hepatitis C Virus -related type II Mixed Cryoglobulinemia underwent treatment with standard dose of peg-Interferon alpha-2a 180 mcg once weekly with Ribavirin.

**Results:** At the end of therapy, we observed a strict association between the eradication of hepatitis C virus and a complete clinical response (disappearance of cutaneous manifestations of cryoglobulinemic vasculitis) with a complete virological and clinical response in 12/16 (75%) and 8/8 (100%) patients with and without hepatic disease, respectively. In the first group 8/16 (50%) patients achieved a complete clinical response and sustained virological response, 4/16 (25%) were non-responders and 4/16 (25%) relapsers, while in the second group 7/8 (87.5%) patients achieved a complete clinical response and sustained virological response, and 1/8 (12.5%) was relapser. Therefore, we observed a higher rate of complete clinical response and sustained virological response (87.5% vs 50%) in patients without hepatic disease compared with patients with hepatic disease ( $p < 0.01$ ).

**Conclusion:** Peg-Interferon alpha-2a with Ribavirin seems to be safe and useful for the treatment of Hepatitis C Virus-related type II Mixed Cryoglobulinemia not only in patients with but also without hepatic disease. Moreover, in our study seems that the anti-viral therapy is more effective in patients affected by Hepatitis C Virus-related type II Mixed Cryoglobulinemia without hepatic involvement than in those with hepatic disease.

**Key words:** Hepatitis C Virus, Mixed Cryoglobulinemia, pegylated-Interferon alpha-2a, Ribavirin.

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### Introduction

Hepatitis C virus (HCV) is a RNA virus belonging to the Flaviviridae family<sup>(1)</sup>. It causes chronic infection in 200 million people worldwide<sup>(2)</sup>. As a hepatotropic virus, it can lead to Chronic Hepatitis (CH), Hepatic Cirrhosis (HC) and Hepatocellular Carcinoma (HCC)<sup>(3-8)</sup>, but, in some cases, it is not associated with hepatic disease. HCV+ patients without hepatic disease have not clinical, biochemical and histological signs of

liver involvement. The most frequent extrahepatic disease in HCV+ patients is mixed cryoglobulinemia (MC), a potentially life-threatening, systemic vasculitis affecting small and, less frequently, medium caliber arteries and veins, characterized by deposition of immune complexes on endothelial surface, eliciting vascular inflammation through poorly understood mechanisms<sup>(9,10)</sup>. Cryoglobulinemia is defined by the presence of circulating immunoglobulins that precipitate with cold temperature and solubilize when rewarmed.

Cryoglobulinemia is classified into three subgroups, according to Immunoglobulins (Ig) composition: Type I cryoglobulinemia is composed of only one isotype or subclass of immunoglobulin. Both type II and III mixed cryoglobulins are immune complexes composed of polyclonal IgGs, the autoantigens and mono or polyclonal IgMs, respectively; the IgMs are the corresponding autoantibodies with rheumatoid factor activity<sup>(10,11)</sup>.

Currently, there are insufficient epidemiological studies on the prevalence of cryoglobulinemia. The prevalence of mixed cryoglobulinemia is related to the endemic presence of HCV infection. Therefore, the prevalence varies from country to country. The incidence of HCV infection in mixed cryoglobulinemia in the Mediterranean Basin is 90%. The incidence is reportedly 1:100,000 persons with a female to male ratio of 3:1<sup>(10,12-16)</sup>. The mean age reported is 42-52 years. The disease is more common in Southern Europe than in Northern Europe or North America<sup>(12-15)</sup>. This distribution is associated with the prevalence of HCV infection. Cryoglobulins have been reported in a significant proportion of patients with chronic infections; 15-20% in patients with human immunodeficiency virus (HIV) infection and 15-25% in patients with connective tissue diseases. Upon examination, >50% of HCV-infected individuals are found to have circulating cryoglobulins, with approximately 5% presenting with cryoglobulinemic syndrome<sup>(16,17)</sup>.

Mortality and morbidity in individuals with cryoglobulinemia often depend on concomitant disease (eg, lymphoproliferative disorder, viral hepatitis); for example, the prognosis in patients with chronic hepatitis C infection depends on their response to treatment; manifested by their decrease in viral load. The overall prognosis is worse in persons with concomitant renal disease, lymphoproliferative disease, or plasma cell disorders. Mean survival is approximately 50% at 10 years after diagnosis. Morbidity due specifically to cryoglobulinemia may be significant, with infection and cardiovascular disease being major considerations<sup>(10)</sup>.

Hepatic failure may result from chronic viral hepatitis<sup>(18)</sup>.

MC tends to correlate with duration of HCV infection and older age. However, cryoglobulinemia and/or the presence of detectable cryoglobulins in the serum of HCV patients has been associated with increased risk of advanced fibrosis and cirrhosis in patients with chronic HCV infection,

irrespective of age or disease duration. The presence of serum cryoglobulins has been shown to correlate with the severity of hepatic steatosis on liver biopsy<sup>(18,19)</sup>.

Specific clinical manifestations associated with types II and III cryoglobulinemia include joint involvement (usually, arthralgias in the proximal interphalangeal [PIP] joints, metacarpophalangeal [MCP] joints, knees, and ankles), fatigue, myalgias, renal immune-complex disease, cutaneous vasculitis, peripheral neuropathy. Meltzer triad (ie, purpura, arthralgia, and weakness) was first described in 1966 by Meltzer and Franklin in cases of essential mixed cryoglobulinemia. This triad is generally seen with types II and III cryoglobulinemia and is seen in up to 25-30% of patients<sup>(20)</sup>.

#### ***Cutaneous manifestations***

These manifestations are nearly always present in cryoglobulinemia. Observed lesions have a predilection for dependent areas (particularly the lower extremities) and include erythematous macules and purpuric papules (90-95%), as well as ulcerations (10-25%)<sup>(21)</sup>.

#### ***Musculoskeletal manifestations***

Symptoms such as arthralgias and myalgias are rare in type I cryoglobulinemia and are common in types II and III disease. Frank arthritis and myositis are rare. Arthralgias commonly affect the proximal interphalangeal and metacarpophalangeal joints of the hands, knees, and ankles. Musculoskeletal symptoms are described in more than 70% of persons with cryoglobulinemia<sup>(22)</sup>.

#### ***Renal manifestations***

Renal disease may occur secondary to thrombosis (type I cryoglobulinemia) or immune complex deposition (types II and III). The incidence of renal disease varies from 5-60%. Histologically, membranoproliferative glomerulonephritis is almost always the lesion in mixed cryoglobulinemia. Clinically, isolated proteinuria and hematuria are more common than nephrotic syndrome, nephritic syndrome, or acute renal failure. Renal involvement is one of the most serious complications of cryoglobulinemia and typically manifests early in the course of the disease (within 3-5 y of diagnosis). Failure to treat may result in renal failure<sup>(23)</sup>.

### ***Pulmonary manifestations***

A reduction in forced expiratory flow rates and the presence of interstitial infiltrates revealed by chest radiographs are common in mixed cryoglobulinemia. Approximately 40-50% of patients are symptomatic with dyspnea, cough, or pleuritic pain. Severe pulmonary disease is rare<sup>(24)</sup>.

### ***Neuropathy***

Neuropathy is common in types II and III disease (as determined with electromyographic and nerve conduction studies), affecting 70-80% of patients. Symptomatic disease was once reported as less common (5-40%); however, more recently, subjective symptoms have been reported up to 91% of patients. Sensory fibers are more commonly affected than motor fibers, with pure motor neuropathy in approximately 5% of patients<sup>(25,26)</sup>.

The treatment of MC in HCV+ patients includes several drugs such as steroids, cyclosporines<sup>(27)</sup>, colchicines<sup>(27)</sup>, plasmapheresis<sup>(28)</sup> and others<sup>(29,30,31)</sup>, but given the documented association with HCV virus, the treatment of choice seems to be the antiviral therapy used for HCV-related CH (CCH)<sup>(32)</sup>. In the last decade, before the direct-acting antiviral agents (DAAs), antiviral treatment (AT) with pegylated interferon (Peg-IFN) plus ribavirin (RBV) was considered the first therapeutic option for CCH and HCV-MC<sup>(33-44)</sup>. Actually, limited and non-unique data are available regarding peg-IFN alpha-2a plus RBV for the treatment of MC in HCV+ patients<sup>(45,46)</sup>. The aim of this study was to evaluate safety and efficacy of peg-IFN alpha-2a in combination with RBV for the treatment of HCV-related MC with detectable HCV-RNA in patients with and without hepatic disease.

## **Materials and methods**

### ***Study design***

The study was a longitudinal prospective open-label uncontrolled study performed in cooperation with two Hepatology Units. Peg-IFN in combination with RBV was administered according to current guidelines for standard treatment of CCH. All patients provided their informed consent prior to their inclusion in the study.

### ***Patients***

Inclusion criteria for the study were: age 18 years or older, anti-HCV antibodies positivity, detectable HCV RNA, HCV Genotype 1b, clinical

and laboratory signs of MC vasculitis in the absence of any other condition known to cause vasculitis. Exclusion criteria for the study were: HBV infection, HIV infection, HCV genotype other than 1b, decompensated HC, significant atherosclerotic heart disease (defined as instrumental and clinical features of coronary heart disease and chronic heart failure); alcohol or drug abuse, history of hematological disorders or neoplastic diseases, pregnancy, psychiatric disorders or autoimmune diseases. All patients underwent HCV-RNA and HCV genotype determination. HCV-RNA levels were detected by polymerase chain reaction (PCR) of HCV-RNA 5' UTR using COBAS® AmpliPrep/COBAS® TaqMan® HCV Test (Roche Diagnostics Systems, Branchburg, N.J.; analytic sensitivity <20 IU/mL. HCV genotypes were determined by INNO-LiPA (Innogenetics) assay, using Simmond's classification. Anti-HCV antibodies were assayed by the second generation (four-antigen) immuno-enzymatic screening test ORTHO-HCV (Ortho Diagnostic Systems, Raritan, NJ, USA). Hepatitis B virus (HBV) and human immunodeficiency virus (HIV) markers were detected by enzyme-linked immunosorbent assay (ELISA) using commercially available kits. Values for the liver function tests Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) as well as hematological parameters (complete blood count, sodium, potassium, glycaemia, azotemia, creatininaemia) were determined by usual laboratory methods. Anti-nuclear (ANA), antimitochondrial (AMA), anti-smooth muscle (SMA), anti-liver/kidney microsome type 1 (LKM1) autoantibodies were measured using immunofluorescence assay (IFA) and semi-quantitative ELISA immobilizing enzyme test. Rheumatoid factor (RF), C3 and C4 fractions of complement were measured by rate nephelometry. Thyroid function was evaluated by levels of Thyroid Stimulating Hormone (TSH), free or total triiodothyronine (free T3) and free thyroxine (free T4), which were determined by immunoradiometric assay (IRMA); antibodies against thyroid peroxidase and thyroglobulin were measured by IFA. Cryoglobulin determination was performed according to standard methods<sup>(12,20)</sup>; the cryoprecipitate, diluted in 0.5 M NaCl, were fractionated by high-resolution gel electrophoresis to type cryoglobulins. Individual monoclonal bands were identified by immunofixation after electrophoresis using a cellulose acetate strip impregnated with antibodies specific for heavy and light chains. Mixed cryoglobulins were classified as type

II on the basis of the presence of monoclonal IgM immunoglobulins with RF activity complexed with polyclonal IgG. All patients underwent ultrasound-assisted percutaneous biopsy, obtained with Menghini modified needles (Automatic Aspiration Needle for Liver Biopsy, ACR 16G, 11 cm, manufactured by Sterylab Srl, Milan-Italy) within 6 months before the start of the study. Histological evaluation of the degree of necroinflammatory activity (grading) and fibrosis (staging) of hepatic tissue were assessed according to METAVIR scoring system: activity (A) was graded according the intensity of the necroinflammatory lesions: A0, no activity; A1, mild activity; A2, moderate activity; A3 and A4, severe activity. The stage of fibrosis (F) was graded as follows: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with some septa; F3, portal fibrosis with numerous septa; F4, cirrhosis. A skin biopsy of a purpuric lesion was performed in all patients. Each sample was placed in buffered formalin and stained with haematoxylin and eosin. Histological evaluation revealed leukocytoclastic vasculitis. On the basis of clinical, biochemical, virological, and histological data, from a total of 235 consecutive HCV+ patients evaluated at the Department of Clinical and Experimental Medicine and at the Department of Tropical Diseases, "Policlinico-Vittorio Emanuele" Hospital, University of Catania between 2008 and 2011, we selected 24 patients affected by HCV-related type II MC with detectable HCV RNA.

The diagnosis of MC was made according to serological, pathological and clinical criteria proposed in 1989 from the Italian Group for the Study of Cryoglobulinemia (GISC)<sup>(40)</sup>. Of these 24 patients with HCV-related type II MC, 16 patients [6 male and 10 female, mean age 57 years old (range 48-61), mean body weight 69 kg (range 61-80)] had chronic liver disease (14 with CH, 2 with Child-Pugh Class A HC) and 8 patients [3 male and 5 female, mean age 37 years old (range 34-40), mean body weight 68 kg (range 61-75)] had not liver involvement (normal level of liver enzymes, liver activity and fibrosis score < 1). All patients were Caucasian, heterosexuals and had no history of alcohol or drug abuse. All patients were enrolled and eligible to receive antiviral therapy with standard dose of peg-IFN alpha-2A 180 micrograms once weekly and weight based ribavirin (WBR) 1000-1200 mg/day for 48 weeks.

### ***Efficacy assessment***

The study protocol conformed to the ethical

guidelines of the 1975 Declaration of Helsinki (6<sup>th</sup> Revision, 2008). According to current guidelines<sup>(33)</sup> at time 0, all patients started therapy with subcutaneous peg-IFN alpha-2a, 180 micrograms once weekly, plus oral WBR in two separate doses (the total dose was 1200 mg daily for patients weighing >75 kg, and 1000 mg daily for those weighing <75 kg). Two weeks after the start of therapy and subsequently on a monthly basis, clinical and biochemical parameters were evaluated. The clinical evaluation included cutaneous manifestations such as purpura, Raynaud's phenomenon, and distal ulcers, while biochemical evaluation included complete blood count, sodium, potassium, glycaemia, fractionated bilirubin, azotemia, creatininaemia, serum AST and ALT levels, TSH, free T3, free T4, antibodies against thyroid peroxidase and thyroglobulin, RF, C3 and C4 fractions of complement, and cryoglobulin levels. In compliance with international guidelines and recommendations of the American Association for the Study of Liver Disease (AASLD) and the Italian Association for the Study of the Liver (AISF), HCV-RNA levels were measured in all patients at time 0 and at weeks 12, 24, 48 and 72<sup>(33,34)</sup>. Patients with a HCV-RNA reduction of at least 2 log<sub>10</sub> at week 12 in comparison with the pre-treatment HCV-RNA values were classified as early virological responders (EVR) and continued therapy. Patients who did not reach this target were classified as non-responders and stopped therapy. Relapse was defined as undetectable serum HCV-RNA levels <20 IU/mL at the end of treatment but positivity at the end of follow-up. A sustained virologic response (SVR) was defined as undetectable serum HCV RNA levels 24 weeks after the end of therapy (week 72). Clinical response was defined by analyzing the evolution of cutaneous manifestations of vasculitis. A complete clinical response was defined as disappearance of skin lesions. A partial clinical response was defined as a decrease of >50% of skin lesions compared with baseline. Patients who had neither a complete clinical response nor a partial clinical response were classified as non-responders. Relapse was defined as the partial or complete reappearance of cutaneous manifestations of vasculitis after the end of treatment.

Immunological response was defined by analyzing the serum levels of RF and cryoglobulins. A complete immunological response was defined as normalization of serum RF levels and disappearance of circulating cryoglobulins. A partial immunologic response was defined as a decrease in the serum

level of RF and cryoglobulins > 50% compared with baseline. Patients who had neither a complete nor a partial immunological response were classified as immunological non-responders. Relapse was defined as the partial or complete normalization of serum RF and cryoglobulins during therapy followed by return to higher values during follow-up. The efficacy of treatment was evaluated by analyzing clinical, virological, and immunological responses at time 0 and at weeks 12, 24, 48 and 72.

### Statistical analysis

Results are presented as means  $\pm$  standard deviation (SD), range and frequencies. Fisher's Exact test and Mann Whitney U Test for independent samples were applied. Two-tailed P values <0.01 were considered statistically significant. Significant predictors of a complete clinical response were evaluated by multiple logistic regression (MLR) analyses.

### Results

The baseline (week 0) characteristics are detailed in Table 1. The mean  $\pm$  SD age was higher in HCV-related type II MC patients with hepatic disease (56.9 $\pm$ 3.3 years) than without hepatic disease (37.7 $\pm$ 2.6 years). In both groups of patients there was a higher prevalence of female. The mean body weight in both groups was similar (about 69 Kg). All patients were Caucasian. The mean  $\pm$  SD duration of HCV infection was greater in patients with hepatic disease (17.5 $\pm$ 2.4 years) than without hepatic disease (8.9 $\pm$ 3 years). The HCV genotype was 1b in all 24 patients and the mean  $\pm$  SD serum HCV RNA levels were higher in patients with hepatic disease (1.050.000 $\pm$ 114.017 IU/ml) than without hepatic disease (450.000 $\pm$ 37.796 IU/ml). Moreover, serum transaminase levels were abnormally increased in patients with hepatic disease [AST (UI/l) = 93.7 $\pm$ 11.1; ALT (UI/l) = 128.4 $\pm$ 12.7] while normal in patients without hepatic disease [AST (UI/l) = 27.9 $\pm$ 4.1; ALT (UI/l) = 32.4 $\pm$ 5]. According to the Metavir criteria, the mean  $\pm$  SD liver activity (A) and fibrosis (F) score in patients with hepatic disease were A=1.75 $\pm$ 0.7 and F=2.1 $\pm$ 0.5, while A=0.4 $\pm$ 0.5 and F=0.5 $\pm$ 0.5 in the patients without hepatic disease. In the first group 2/16 patients were affected by Child-Pugh Class A HC. The type II cryoglobulins were found in all 24 patients and the mean cryoglobulin levels in both groups were similar (about 2 gm/l). Low C4 complement levels (<

Parameter	A HCV-MC patients with hepatic disease (n = 16)	B HCV-MC patients without hepatic disease (n = 8)	p-value (Odds Ratio 95% CI)
Gender - n° (%)			
Male	6 (37.5)	3 (37.5)	Ns
Female	10 (62.5)	5 (62.5)	Ns
Age - years	56.9 $\pm$ 3.3	37.7 $\pm$ 2.6	< 0.001
Weight - Kg	69.1 $\pm$ 5.4	68.2 $\pm$ 4.8	Ns
Race Caucasian - n° (%)	16 (100)	8 (100)	Ns
Duration of HCV infection - years	17.5 $\pm$ 2.4	8.9 $\pm$ 3	< 0.001
HCV genotype 1b - n° (%)	16 (100)	8 (100)	Ns
Serum HCV RNA level - IU/ml	1.050.000 $\pm$ 114.017	450.000 $\pm$ 37.796	< 0.001
AST serum level - IU/l	93.7 $\pm$ 11.1	27.9 $\pm$ 4.1	< 0.001
ALT serum level - IU/l	128.4 $\pm$ 12.7	32.4 $\pm$ 5	< 0.001
Liver activity score (0-4 scale)†	1.75 $\pm$ 0.7	0.4 $\pm$ 0.5	< 0.001
Liver fibrosis score (0-4 scale)†	2.1 $\pm$ 0.5	0.5 $\pm$ 0.5	< 0.001
Cryoglobulin level - gm/l	2.1 $\pm$ 1.1	2 $\pm$ 1.2	Ns
Type II cryoglobulins - n° (%)	16 (100)	8 (100)	Ns
Low C4 complement level - n° (%)	12 (75)	6 (75)	Ns
RF positive - n° (%)	12 (75)	6 (75)	Ns
Purpura - n° (%)	16 (100)	8 (100)	Ns
Asthenia - n° (%)	16 (100)	8 (100)	Ns
Arthralgia - n° (%)	12 (75)	6 (75)	Ns
Raynaud's phenomenon - n° (%)	8 (50)	4 (50)	Ns
Distal ulcers - n° (%)	2 (12.5)	1 (12.5)	Ns
Peripheral neuropathy - n° (%)	0 (0)	0 (0)	Ns
Renal involvement- n° (%)	0 (0)	0 (0)	Ns
Sicca syndrome - n° (%)	0 (0)	0 (0)	Ns
B cell lymphoma - n° (%)	0 (0)	0 (0)	Ns

**Table 1:** Baseline (week 0) characteristics of patients (A. HCV-MC patients with hepatic disease and B. without hepatic disease). Significant predictors of a complete clinical response evaluated by multiple logistic regression (MLR) analyses (n = 24).

*Abbreviations. HCV-MC = hepatitis C virus-associated mixed cryoglobulinemia; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; RF: Rheumatoid Factor; GI: gastrointestinal.*

*RF levels > 15 IU/ml were considered positive*

*C4 complement levels < 0.15 gm/l were considered low*

*Except where indicated otherwise, values are the mean  $\pm$ SD.*

*† Liver activity and fibrosis were graded according to the Metavir scoring system. All liver specimens were assessed by a local pathologist for histology status and reviewed by one expert pathologist, blinded about specimens group origin.*

*P was calculated by multiple logistic regression analysis. ANOVA was also performed within each group.*

0.15 gm/l) and elevated RF levels (> 15 IU/ml) were observed in the most patients: 12/16 with hepatic disease and 6/8 without hepatic disease. Clinical manifestations of MC included purpura and asthenia in 16/16 (100%) and 8/8 (100%), arthralgia in 12/16 (75%) and 6/8 (75%), Raynaud's phenomenon in 8/16 (50%) and 4/8 (50%), and distal ulcers in 2/16 (12.5%) and 1/8 (12.5%) patients with and without hepatic disease, respectively. Other clinical manifestations of MC such as peripheral neuropathy, renal involvement, sicca syndrome, and B cell lymphoma were not observed in our patients. The main treatment-related data are summarized in Table 2.

the end of follow-up (EFU), 8 patients out of 16 in the first group (50%) achieved a SVR, 4/16 (25%) non-responders and 4/16 (25%) relapsers, while 7 patients out of 8 in the second group (87.5%) achieved a SVR and 1/8 (12.5%) relapsers. In conclusion, at the end of follow-up, we observed a higher rate of SVR (87.5% vs. 50%) in patients without hepatic disease compared with patients with hepatic disease ( $p < 0.01$ ).

### Clinical response

At the EOT, a complete clinical response was found in 12/16 (75%) and 8/8 (100%) patients with

Parameter	A HCV-MC vasculitis patients with hepatic with hepatic disease (n = 16)					B HCV-MC vasculitis patients without hepatic disease (n = 8)				
	Baseline	12 weeks	24 weeks	48 weeks	End of follow-up (72 weeks)	Baseline	12 weeks	24 weeks	48 weeks	End of follow-up (72 weeks)
HCV RNA positive, no. (%)	16 (100)	4 (25)	4 (25)	4 (25)	8 (50)	8 (100)	0 (0)	0 (0)	0 (0)	1 (12.5)
AST serum level - IU/l	93.7±11.1	51.1± 14	38.6± 4.6	34.6± 3.6	65 ± 37.1	27.9±4.1	26.6±3	25.6±4.8	24.4±4.8	28.1±5.1
ALT serum level - IU/l	128.4±12.7	57.6± 11.7	38.6± 5.2	36.6± 2.7	72.1 ± 46.7	32.4±5	29.9±3	29.25±3	28±2.8	29.2±3.4
Cryoglobulin positive, no. (%)	16 (100)	10 (62.5)	8 (50)	6 (37.5)	8 (50)	8 (100)	6 (75)	4 (50)	2 (25)	4 (50)
RF positive, no. (%)	12 (75)	10 (62.5)	8 (50)	6 (37.5)	8 (50)	6 (75)	6 (75)	4 (50)	2 (25)	4 (50)
Low C4 complement level, no. (%)	12 (75)	10 (62.5)	8 (50)	6 (37.5)	8 (50)	6 (75)	6 (75)	4 (50)	2 (25)	4 (50)
Purpura, no. (%)	16 (100)	4 (25)	4 (25)	4 (25)	8 (50)	8 (100)	0 (0)	4 (50)	0 (0)	1 (12.5)
Raynaud's phenomenon, no. (%)	8 (50)	0 (0)	0 (0)	0 (0)	0 (0)	4(50)	0 (0)	0 (0)	0 (0)	0 (0)
Distal ulcers, no. (%)	2 (12.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (12.5)	0 (0)	0 (0)	0 (0)	0 (0)

**Table 2:** Laboratory parameters and clinical features of HCV-MC vasculitis patients. **A.** HCV-MC vasculitis patients with hepatic disease (n=16). **B.** HCV-MC vasculitis patients without hepatic disease (n=8)

*Abbreviations.* HCV-MC =hepatitis C virus-associated mixed cryoglobulinemia; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; RF: Rheumatoid Factor. Rheumatoid Factor levels > 15 IU/ml were considered positive C4 complement levels < 0.15 gm/l were considered low Cryoglobulin level > 0,05gm/l was considered positive Except where indicated otherwise, values are the mean ±SD.

### Virological response

At 12 weeks, 4/16 (25%) patients with hepatic disease (2 with CH and 2 with Child-Pugh Class A HC), were non-responders to therapy and stopped it. Twenty remaining patients (12 with CH, 8 without hepatic disease) early virological responders (EVR) continued therapy until to 48 weeks. At the end of treatment (EOT), HCV-RNA became undetectable in 12/16 (75%) and 8/8 (100%) patients with and without hepatic disease, respectively, and they were classified as ETR (end of treatment responders). At

and without hepatic disease, respectively, and they were classified as complete responders. At the EFU, 8 patients out of 16 in the first group (50%) were complete responders, 4/16 (25%) non-responders and 4/16 (25%) relapsers, while 7 patients out of 8 in the second group (87.5%) were complete responders and 1/8 (12.5%) relapsers.

In conclusion, at the end of follow-up, we observed a strict association between the eradication of HCV and a complete clinical response with a higher rate of complete clinical response (87.5% vs.

50%) in patients without hepatic disease compared with patients with hepatic disease ( $p < 0.01$ ).

### **Immunological response**

At the EOT, normalization of serum RF levels and disappearance of circulating cryoglobulins were observed in 10/16 (62.5%) and 6/8 (75%) patients with and without hepatic disease, respectively, and they were classified as complete responders. At the EFU, 8 patients out of 16 in the first group (50%) were complete responders, 6/16 (37.5%) non-responders and 2/16 (12.5%) relapsers, while 4 patients out of 8 in the second group (50%) were complete responders, 2/8 (25%) non-responders and 2/8 (25%) relapsers. In conclusion, at the end of follow-up, we observed the same rate of complete immunological response (50%) in patients with and without hepatic disease.

### **Adverse events**

Four patients out of 16 in the first group (25%) were non-responders to therapy and stopped it at 12 weeks. The side effects in the remaining 20 patients [12/16 (75%) with hepatic disease and 8/8 (100%) without hepatic disease] were fever, asthenia, loss of concentration, insomnia, anxiety, mild depression, mild anemia, mild leukopenia and thrombocytopenia. The fever was successfully treated with administration of paracetamol. Psychological Support Programs or, sometimes, administration of antidepressant drugs successfully controlled asthenia, loss of concentration, insomnia, anxiety, mild depression. No severe psychiatric side effects, such as major depression or psychosis have been reported during the antiviral treatment administration as well as in the follow-up. Mild anemia did not require adjuvant treatments<sup>(34-38)</sup>. No dosage reductions or interruptions of therapy were needed. No cases of death occurred during the study.

### **Multiple logistic regression analyses**

Significant predictors of a complete clinical response are presented in Table 2. In our regression model, factors significantly associated with a complete clinical response were: age  $< 40$  years old ( $p < 0.001$ ), duration of HCV infection  $< 12$  years ( $p < 0.001$ ), serum HCV RNA level  $< 500000$  IU/l ( $p < 0.001$ ), normal transaminases levels ( $p < 0.001$ ), liver activity and fibrosis scores  $< 1$  ( $p < 0.001$ ).

## **Discussion and conclusion**

Mixed Cryoglobulinemia is a systemic vasculitis affecting small and, less frequently, medium caliber arteries and veins<sup>(10)</sup>, characterized by deposition of immune complexes on endothelial surface, eliciting vascular inflammation through poorly understood mechanisms. HCV is the most frequent cause of MC and it is primarily associated with type II MC and less frequently, with type III MC<sup>(47, 48)</sup>. All HCV genotypes have been found in MC. The MC prevalence in HCV+ patients ranges widely from 10-70%. Female gender is slight prevalent<sup>(15)</sup>. MC is more frequent in Southern Europe than in Northern Europe and North America. In our study, we observed type II MC and genotype 1b HCV in all patients, the MC prevalence was 24/235 (10.2%) patients, F/M ratio was 15/9 and all patients were Caucasian (Italians). Several epidemiological and clinic-pathological observations suggest that MC is the result of a multifactorial and multistep pathogenetic process<sup>(10, 12-18)</sup>. While the immune-complex-mediated vasculitis is the final step of this complex process, B-lymphocyte expansion<sup>(49)</sup> may represent the remote disorder responsible for production of pathologic quantities of immunoglobulins with rheumatoid factor activity that form cryoglobulins. The deposition of cryoglobulins on endothelial surface of vessels elicits a vascular inflammation inducing vascular damage. The mechanisms responsible for the B-lymphocyte expansion remain still unknown<sup>(10,17,50-52)</sup>. A direct role of HCV in the B-cell expansion has been hypothesized on the basis of the high frequency of HCV-RNA positive lymphocytes in cryoglobulinemic patients demonstrated by RT-PCR (reverse transcription-polymerase chain reaction), in situ hybridization and immunohistochemical techniques<sup>(17,53-55)</sup>.

Since HCV is a RNA virus without reverse transcriptase activity, viral genome cannot integrate in the host genome and thus cannot regulate the cell replication and/or survival. Probably, HCV may exert its oncogenic potential, indirectly, through viral epitopes, autoantigen production, and/or molecular mimicry mechanism<sup>(56)</sup>. Besides HCV infection, other possible triggering agents, and/or environmental, and/or genetic co-factors remain still unknown. HCV is a hepatotropic virus that can lead to CH, HC and HCC<sup>(56,57,58,59,60)</sup>, but, in some cases, it cannot be associated with hepatic disease. HCV+ patients without hepatic disease have not clinical, biochemical and histological signs of liver involve-

ment. In our study, we presented 24 patients affected by genotype 1b HCV-related type II MC: 16 with hepatic disease (14 with CH, 2 with Child-Pugh Class A HC) and 8 without hepatic disease. Note that in the cohort studied there are only 2 patients with liver cirrhosis, however compensated (Child-Pugh Class A), because cirrhosis is an independent factor of poor response to combination therapy peg-IFN plus RBV and the comparison with the arm consists of subjects without liver disease would be methodologically incorrect.

Palpable purpura, arthralgia and asthenia are the typical clinical triad of MC<sup>(47)</sup> although it is rare. A lot of MC patients are asymptomatic. In our patients, we observed purpura and asthenia in 100% of cases, arthralgia, Raynaud's phenomenon and distal ulcers in 75%, 50% and 12.5% of cases, respectively. Circulating cryoglobulins are frequently detected in HCV+ patients (60-80%) whereas cryoglobulinemic vasculitis develops in only 5-10% of the cases (61). Serum RF, which is elevated in 16-70% of HCV+ patients<sup>(62)</sup>, is usually increased in HCV-related MC patients (>70%), and levels of complement, particularly C4, may be profoundly decreased.

In our study, we detected circulating cryoglobulins in 100% of patients, elevated serum RF levels and low C4 levels in 75% of patients. Diagnosis of MC was made according to serological, pathological and clinical criteria proposed in 1989 from the Italian Group for the Study of Cryoglobulinemia (GISC) (Table 1). In our study, all patients affected by genotype 1b HCV-related type II MC, with and without hepatic disease, underwent treatment with standard dose of peg-IFN alpha-2a 180 mcg once weekly and RBV 1000-1200 mg/day for 12 months. At 12 weeks, 4/16 (25%) patients with hepatic disease (2 with CH and 2 with Child-Pugh Class A HC) were non-responders to therapy and stopped it. Twenty remaining patients (12 with CH and 8 without hepatic disease) EVR continued therapy until to 48 weeks with complete clinical and virologic response in 12/16 (75%) and 8/8 (100%) patients with and without hepatic disease, respectively. At the end of follow-up (72 weeks), we observed a complete clinical and virologic response in 8/16 (50%) patients with hepatic disease and 7/8 (87.5%) in patients without hepatic disease.

Our results showed a higher rate of complete clinical and virologic responses in patients without hepatic disease (87.5%) compared with patients with hepatic disease (50%). Clinical and virologic

responses were closely correlated. HCV-RNA relapse is associated with recurrence of MC symptoms<sup>(63, 64)</sup>. Factors associated with a better response to peg-IFN alpha-2a and RBV therapy in patients without hepatic disease compared with those with hepatic disease were low levels of viremia before treatment (< 500000 IU/l), young age (< 40 years old), short duration of HCV infection (<12 years), normal transaminases levels and a low grade of liver activity and fibrosis (<1). Clearance of cryoglobulins and normalization of serum RF levels were noted in 50% of patients with and without hepatic disease. The persistence of cryoglobulins and RF after viral eradication supports the hypothesis of a continued HCV replication below the limit of detection or long-term persistence of viral antigen in the absence of replicating virus. Taking into account that IFNs are endowed with anti-viral and anti-proliferative properties (inhibition of lymphocytes B clone activity), it is likely that a longer treatment period, perhaps 18 or 24 months, could be necessary to get a complete immunological outcome<sup>(65)</sup>.

In conclusion, our data suggested that the therapy with standard dose of peg-IFN alpha-2A in combination with oral WBV seems to be safe and useful in patients affected by genotype 1b HCV-related type II MC. Moreover, it seems that the antiviral therapy is more effective in patients without hepatic involvement than in those with hepatic disease, although more data are necessary to confirm these results. However, a higher response rates could be obtained with different treatment schedules, such as higher drug dosages or longer treatment periods. New drugs, such as anti-CD20 monoclonal antibody or new immunosuppressive agents, should be considered for the future, hoping these new approaches will offer a better understanding of this disease and significant advantages for its therapy.

Peg-IFN plus RBV has been considered the standard antiviral therapy (AT) for about a decade<sup>(66)</sup>. Recently, the direct acting antiviral drugs (DAAs), inhibitor of the nonstructural 3/4A HCV protease, boceprevir (BOC), and telaprevir (TVR), in combination with Peg-IFN and RBV, have consistently increased the likelihood of response in patients infected with HCV genotype 1 (Gt 1a or 1b)<sup>(67,68)</sup>. Few data are still available for patients with HCV-related MCS treated with DAAs<sup>(69,73)</sup>.

The actual A.I.S.F. guidelines suggest therapy with sofosbuvir in association with simeprevir +/- ribavirin for 12 weeks, for the treatment of patients



suffering from chronic hepatitis C (genotype 1a, 1b) naïve or experienced plus mixed cryoglobulinemia. This is the therapy of choice in these patients, with greater efficacy and fewer side effects than other therapeutic profiles. The addition of RBV is recommended in patients with previous failures and, if possible, in patients with advanced disease<sup>(74,75)</sup>.

The development of this direct-acting antiviral agents of the second generation has revolutionized HCV treatment. DAAs, as well as new B-cell-depleting or B-cell-modulating monoclonal antibodies, will expand the panorama of treatment options for HCV-related extrahepatic manifestations including MC. In this context, a proactive, integrated approach to HCV therapy should maximize the benefits of HCV therapy, even when liver disease is mild<sup>(74)</sup>.

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