Efficacy and safety of peginterferon alfa-2b plus ribavirin for HCV-positive mixed cryoglobulinemia: a multicentre open-label study

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Abstract

Objective

The aim of the present study is to provide information on clinical outcome of the patients affected by HCV-positive mixed cryoglobulinemia (MC) treated with PEG-IFN and Ribavirin for 6 or 12 months according to the HCV genotype.

Methods

Eighty-six patients (42 women and 44 men) were enrolled in 8 Italian centres. All the patients had MC in the active phase of the disease. The patients received Peginterferon alfa-2b 1.5 mcg/kg/once a week (QW) and daily oral Ribavirin (800/1,000/1,200) according to their body weight for 48 weeks for genotype 1 and 4 and for 24 weeks for genotypes 2 and 3.

Results

In the 44 patients who underwent 12 months of therapy, 17 cases (39%) could be considered as "non-responders" and 11 relapsed, therefore only 16 patients (36%) obtained a sustained virological response. In the 42 patients who underwent six months of therapy only 7 cases (17%) could be considered as "non-responders" and 8 relapsed, therefore 27 patients (64%) obtained a sustained virological response. Purpura score dropped in both group ($p<5.79 \times 10^{-17}$) and only 5 cases of the group A (11%) and 5 of the group B (12%) did not show any improvement. Arthralgias showed a similar behaviour. Many patients relapsed after the end of the treatment.

Conclusion

This study documents a lower response rate than that observed in the clinical trials with HCV chronic hepatitis, but the presence of comorbidities and older age should be taken into consideration. Most patients (88.5%) showed a complete and persistent recovery from clinical symptoms.

Key words

hepatitis C virus, mixed cryoglobulinemia, antiviral therapy, HCV genotype, cryoglobulinemic glomerulonephritis

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Introduction

At present, the current standard treatment for chronic hepatitis C is well established *i.e.* the combination of pegylated interferon (IFN) alfa and ribavirin. Even the efficacy endpoint of hepatitis C treatment is well defined as the "sustained virological response" (SVR), *i.e.* the absence of detectable HCV RNA in serum as assessed by an HCV RNA assay 24 weeks after the end of treatment (1). The decision to treat patients with chronic hepatitis C depends on multiple parameters, including a precise assessment of the severity of liver disease and of its foreseeable outcome, the presence of absolute or relative contra-indications to therapy, and the patient's willingness to be treated (2). The HCV genotype is systematically determined before treatment, since it determines the indication, the duration of treatment, the dose of ribavirin and the virological monitoring procedure (3). The monitoring of HCV-RNA load during the treatment allows clear stopping rules to avoid useless and expensive antiviral therapy (4). All these guidelines are the product of the careful and precise follow-up of hundred thousands of patients treated in dozen of multicentre randomised trials carried out in several hepatological centres scattered in USA, Europe and Japan.

Despite many HCV carriers show detectable levels of cryoglobulins, only a small proportion of patients with chronic hepatitis C are also carriers of a symptomatic mixed cryoglobulinemia (MC) (5). Given the great heterogeneity of the disease, where several pathological conditions and multi-organ involvement can be present in the same patient (6), clear indications for therapy, shared by most clinicians, are lacking. For these cases no clear rules have been established, neither for when to initiate the antiviral treatment, nor which is the endpoint of the therapy. In fact, some patients do not show any hepatic damage, while having renal or neurological involvement (7), and others show severe hepatic disease associated with autoimmune disorders (and therefore with major contraindications to IFN therapy) (8, 9). In addition, the number of subjects affected by MC is small and most cases come from Italy and France (10-13). Given this situation, to carry out randomised trials is not easy, therefore, the "Gruppo Italiano per lo Studio della Crioglobulinemia" (GISC) promoted the collection of data concerning the treatment of MC with pegilated-interferon (PEG-IFN) and ribavirin in the main Italian centres. The aim of the present study is to provide further information on clinical outcome of HCV-positive patients treated with PEG-IFN.

Patients

Eighty-six patients (42 women and 44 men, mean age 54±11, range 29–75 years) affected by MC were enrolled in the study between 2004 and 2008 in 8 Italian centres. The inclusion criteria for the study were as follows: 1) chronic active HCV infection (*i.e.* presence of anti-HCV antibodies associated with detectable levels of serum HCV-RNA), 2) signs of MC vasculitis in the absence of any other condition known to cause vasculitis, 3) treatment with PEG-IFN alfa-2 plus ribavirin for a minimum of 6 months, and 4) a minimum of 6 months of follow-up after discontinuation of the combination anti-HCV treatment. The only exclusion criteria were the presence of either hepatitis B surface antigen or anti-human immunodeficiency virus antibodies. A fraction of these patients (27 cases 31%) had received antiviral therapy in the past (usually IFN 3MU three time a week for 6 or 12 months), but none had been treated with PEG-IFN. Most patients received steroids or other immunosuppressive agents, but none had been treated with anti-CD20 antibodies. This study, therefore, is not randomised and all the patients were treated in open manner. Since most centres used the same PEG-IFN with the same treatment schedule, the collected data are comparable, giving thus the opportunity to determine the efficacy and the safety of the antiviral therapy in the largest MC population ever collected.

Diagnosis was based on standard criteria, and most patients showed the presence of an active disease *i.e.* vasculitic purpura, arthritis, asthenia and skin ulcers. In addition to these common symptoms, 8 patients (9%) showed

Raynaud's phenomenon, 9 cases (10%) had "sicca syndrome", 22 cases (25%) had peripheral sensitive neuropathy (7 of them also motorial) and 9 cases (10%) had nephropathy (1 had mesangioproliferative glomerulonephritis while the others membranoproliferative glomerulonephritis).

Five patients had non-Hodgkin's lymphoma (6%). Four of them had lymphoplasmacytoid lymphomas, confirmed by bone marrow biopsy, and one had a lymphoma of the kidney (not well histologically defined).

All patients were Caucasian, heterosexuals and only two (2.3%) had history of intravenous drug abuse; the source of infection was transfusion of blood products in 14 cases (16.3%), surgical procedures in 19 cases (22%), medical care occupation in 7 cases (8.1%), contact with an HCV-positive relatives in 3 cases (3.5%) and unknown in the others (45 cases: 52.3%). All patients gave their informed consent before entry into the study.

Methods

Values for the liver function tests as well as haematological parameters were determined by usual laboratory methods. Rheumatoid factor (RF), C3 and C4 fractions of complement were measured by rate nephelometry. In most centres, the cryoglobulin determination was performed according to standard methods as previously reported (14): the cryoprecipitates, 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, and as type III in the presence of polyclonal immunoglobulins.

Purpura scoring system

To assess the severity of vasculitis, the following clinical scoring system was used: A score of 0 indicated the absence of skin lesions; a score of 1 the presence of less than 10 purpura spots on the lower leg; a score of 2 the presence of more than 10 spots on the lower leg; a score of 3 the extension of the spots to the upper leg and/or the abdomen; and a score of 4 the presence of skin ulcers and/or gangrene.

Arthralgias scoring system

To assess the severity of the arthralgias a clinical score was used: 0 no arthralgias, 1 occasional arthralgias, 2 continuous arthralgias, 3 intense arthralgias with impairment of the movements. A liver biopsy was performed in the patients showing clinical and laboratory signs of chronic liver disease. Samples were placed in buffered formalin, stained with haematoxylin and eosin, and, for reticulum, with Gomori stain. In each biopsy the disease activity and fibrosis were assessed according to METAVIR (15) scoring system. The activity (A) was graded according the intensity of the necro-inflammatory lesions: A0: no activity; A1: mild activity; A2: moderate activity; A3: 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

Virological studies

Hepatitis B virus (HBV) and human immunodeficiency virus (HIV) markers were detected by enzyme-linked immunosorbent assay (ELISA) using commercially available kits. The presence of anti-HCV antibodies was assayed by the second generation (four-antigen) immuno-enzymatic screening test ORTHO-HCV (Ortho Diagnostic Systems, Raritan, NJ, USA). The presence of HCV-RNA in the serum was assessed by nested PCR amplification of the conserved 5' untranslated region (5'UTR) of HCV. The HCV genotypes were determined with the line probe assay (Inno-Lipa HCV; Innogenetics, Zwijnaarde, Belgium).

Therapy

The subjects, according the most recent guidelines, were treated on the basis of the HCV genotype:

– Peginterferon alfa-2b (PegIntron, Schering-Plough,) 1.5 mcg/kg/once a week (QW) for 48 weeks for genotype 1 and 4 and for 24 weeks for genotypes 2 and 3.
– Ribavirin (Rebetol, Schering-Plough) 800 mg for the patients with a body weight less than 65 kg, 1,000 mg/daily for those with body weight 65–85 kg, and 1,200 mg for those above this weight, for 48 weeks for genotype 1 and 4 and for 24 weeks for genotypes 2 and 3.

Criteria for therapy evaluation

As indicated in previous reports (16, 17), the response to treatment was split into four separate categories: 1) Virological response 2) Biochemical response 3) Immune response 4) Clinical response. All the patients were evaluated at the end of treatment (EOT) and at the end of follow-up (EFU). All patients were followed for at least 12 months after the end of therapy.

Virological response: effect of treatment on HCV-RNA. Sustained virological response (SVR): loss of HCV-RNA at the end of follow-up. Relapse: loss of HCV-RNA at the end of treatment but positivity at the end of follow-up (6 months). No response: persistent positivity during therapy and at the end of follow-up.

Biochemical response: effect of therapy on ALT; normal value was considered 40 IU/L. Complete response: normalisation of the serum ALT level during treatment followed by normal ALT values lasting for 6 months after discontinuation of therapy. No response: ALT out of normal value during treatment and follow-up. Relapse: normalisation of the serum ALT level during treatment followed by return to abnormal values during follow-up. In some patients this parameter was not considered, since the ALT level was normal at the beginning of the treatment.

Immune response: effect of therapy on serum RF concentration and cryocrit level; normal value for RF was considered as 0-40 IU/mL. Complete response: normalisation of serum RF concentration and disappearance of circulating cryoglobulins. Partial response: reduction (but not normalisation) of RF and cryoglobulins ≥50%.

No response: Reduction <50% of RF and cryocrit levels or stable levels. Relapse: partial or complete normalisation of serum RF and cryoglobulins during therapy followed by return to higher values during follow-up.

Clinical response: effect of therapy on the clinical manifestations of the disease (including purpura, arthralgia and weakness). Complete response: disappearance of all clinical signs of the disease. Partial response: improvement of the clinical symptoms (reduction of the purpura score ≥50%). No response: reduction of the purpura score <50% or stable disease. Relapse: partial or complete normalisation of clinical symptoms during therapy followed by return to higher score after the end of treatment.

In addition to the above-indicated criteria for therapy evaluation, additional criteria were added for the patients affected by cryoglobulinemic glomerulonephritis:

1) Complete response: normalisation of serum creatinine, normalisation of proteinuria.
2) Partial response: decrease of creatinine and of proteinuria more than 50%,
3) Minimal response: decrease of creatinine, decrease of proteinuria less than 50%
4) No response: no modification or improvement less than 10% of creatinine and of proteinuria

Follow-up

Biochemical and clinical parameters were determined each two weeks during first two months of therapy and, subsequently, each month. During the follow-up, the patients were referred to the centre each two months up to the 12th month. Auto-antibodies were determined every 3 months and thyroid function tests every 6 months. Determinations of HCV-RNA were performed before starting the therapy, at week 4th, 12th, at the end of treatment (EOT) and at the end of follow-up (EFU). All patients were followed for at least 12 months after the end of therapy.

Statistical analysis

Descriptive statistics were performed, including proportions, means and standard deviations (SD) of relevant variables.

Results

Biochemical and histological findings

The main clinical, laboratory, and histological findings of patients are presented in Table I. The age of the patients is expressed as years at the start of therapy. The liver biopsy was obtained from most patients (92%). The liver biopsy was not performed in 7 patients: in six of them the biopsy was not done since they showed normal liver function tests over 2 years of follow-up before therapy and normal liver at the ultrasonography, while one patient with abnormal liver function tests refused the biopsy. A chronic liver disease of variable severity was found in these subjects (Table I). The low prevalence of liver cirrhosis in our group of patients (4 cases only, 5%) is due to the enrolment of cases mainly from rheumatological instead from hepatological centres.

Virological findings

HCV-RNA was detected in all subjects before therapy. HCV genotyping showed a genotype distribution different from that observed in patients affected by chronic hepatitis. Genotype 1 was detected in 42 cases (49%), while a large number of infections from genotype 2 (33 cases, 39%) was found (Table II). In one patient (1%) the HCV genotype was not determinable.

Therapy outcome

Forty-four patients (51%) received combination therapy for twelve months (all the cases with genotype 1 and 4 plus the case with undetermined genotype and 2 cases with genotype 3) (Group A), while the others (42 cases, 49%) (all the cases with genotype 2 and 7 out 9 of genotype 3) received therapy for six months (Group B). The two groups were not different for age (55±10 *vs*. 56±12 years), male/female ratio (1.1 *vs*. 0.9), length of the disease (8±2 *vs*. 8±2 years), severity of the disease *i.e*. the same cryocrit level (5.6±5.7 *vs*. 6.3±7.6 % p: 0.3 NS), the same purpura score (2.2±0.7 *vs*. 2.0±0.7 p: 0.11 NS) the same C4 level (8.8±6.2 *vs*. 9.5±5.8 mg/dl p: 0.3 NS), while the rheumatoid factor was higher in group A than in group B (256±378 *vs*. 168±164 U/L p: 0.01). Even the severity of the liver disease was the same as indicated by the ALT level (114.5±70.2 *vs*. 135.4±134.5 U/L p: 0.2 NS) and by the liver histology (A: 1.88±0.84 *vs*. 1.87±0.81; F: 1.95±0.97 *vs*. 1.97±0.91).

Virological response

In the 44 patients who underwent twelve months of therapy (group A), at the end of the treatment, the HCV-RNA became undetectable in 27 cases (61%), therefore 17 cases (39%) could be considered as "non-responders". During the follow-up, 11 patients (41% of responders) relapsed, therefore only 16 patients (37%) obtained a sustained virological response.

In the 42 patients who underwent six months of therapy (group B), at the end of the treatment, the HCV-RNA became undetectable in 35 cases (83%), therefore only 7 cases (17%) could be considered as "non-responders". At the end of follow-up, eight patients (23% of responders) relapsed, therefore 27 patients (64%) obtained a sustained virological response.

Biochemical response

Five patients in group A (11%) and 7 in group B (17%) had normal ALT at baseline, hence these cases were not considered for this assessment. In group A, at the end of the treatment, 25 patients showed normal ALT levels (64%); at the end of the follow-up, 14 subjects only showed complete biochemical response (36%) while 11 were relapses (28%). In group B, at the end of the treatment, 32 cases showed normal ALT levels (91%). Since one patient only relapsed (3%), most cases (89%) showed a complete biochemical recovery.

Immune response

At the end of the treatment, the mean cryocrit level was significantly reduced in both groups (p<0.000009 and p<0.0000004, respectively), but cryglobulins were undetectable in only 14 cases in group A (32%) and in 20 cases in group B (48%). In six patients (three in group A and three in group B)

Table I. Clinical, biochemical, characteristics of the 86 HCV-MC patients at baseline.

Parameter	All MC patients (n=86)
Age ,years (median ± SD)	56 ± 11
Female, n (%)	42 (48.8)
HCV genotype 1, n (%)	40 (46.5)
Elevated level ALT, U/L, mean n (%)	121 ± 70,13 (15.1)
Liver necroinflammation score (0–3 scale)	1.9 ± 0.8
Liver fibrosis score (0–4 scale)	2.0 ± 0.9
Cirrhosis, n (%)	4 (4.6)
Purpura, n (%)	78 (90.6)
Arthralgias, n (%)	76 (88.3)
Peripheral neuropathy, n (%))	22 (25.5)
Sicca sindrome, n (%)	9 (10.4)
Raynaud's phenomenon, n (%)	10 (11.6)
Renal involvement , n (%)	10 (11.6)
B cell lymphoma, n (%)	5 (5.8)
Cryoglobulin level (%)	5.6 ± 6.0
Type II cryoglobulins, n (%)	10 (11.6)
Rheumatoid factor (U/L), n (%)	78 (90.6)
Low C4 complement level, n (%)	77 (89.5)
Previous antiviral therapy, n (%)	23 (26.7)
Previous corticosteroids, n (%)	14 (16.2)
Previous immunosuppressive agents, n (%)	4 (4.6)
Previous plasmapheresis, n (%)	3 (3.4)

ALT: alanine-aminotransferase; Liver necro-inflammation and fibrosis were graded according to the METAVIR scoring system.

Table II Clinical, biochemical data of the 86 MC patients at baseline, at the end of the treatment, and the end of the follow-up.

Parameter	Baseline				End of therapy				End of follow-up			
Purpura score	0	1	2	3	0	1	2	3	0	1	2	3
n. of patients	5	9	46	26	74	5	7	0	59	9	16	2
Arthralgias score	1.02 ± 0.41				0.09 ± 0.29				0.29 ± 0.46			
Peripheral neuropathy (%)	26%				13%				16%			
Renal involvement (%)	12%				5%				7%			
Sicca syndrome (%)	9%				6%				8%			
Raynaud's phenomenon (%)	9%				2%				6%			
ALT (U/L)												
Mean ± SD	125 ± 106				40 ± 28				54 ± 51			
Median ± SD	100 ± 106				32 ± 28				32 ± 51			
Median (IQR)	100 (104)				32 (19)				32 (46)			
Rheumatoid factor (U/L)	211 ± 320				175 ± 324				197 ± 348			
Cryglobulin level (%)	5.6 ± 6.0				2.4 ± 4.5				3.8 ± 4.2			
C4 serum level (mg/ml)	9.8 ± 5.9				11.5 ± 7.7				12.3 ± 8.8			

ALT: alanino-aminotransferase; C4: complement component four.

we observed a delayed response, since cryocrit was detectable at the end of the therapy, but undetectable at the end of the follow-up. However, all these "late responders" relapsed after some months. At the end of the follow-up, the statistically significant cryocrit reduction, although inferior, was still observed (p<0.00002 and p<0.000003 respectively), while cryoglobulins were undetectable in 10 cases in group A (23%) and in 18 cases of group B (43%).

Three cases in group A (7%) and 2 in group B (5%) showed normal RF levels at baseline. In both groups the RF levels decreased during combination therapy but of less extent in group A (p<0.01) than in group B (p<0.0002). At the end of the follow-up, the mean RF level returned to pre-treatment value in group A (256±378 *vs*. 244±400 p<0.15 NS), while in group B, after the end of the therapy, a slight increase was observed but the mean RF level is still significantly lower than basal level (168±164 *vs*. 123±180 IU p<0.04).

At the end of the follow-up, only 3 patients of group A (7 %) end 9 patients of group B (22%) normalised RF levels.

On the basis of the above reported criteria, only 3 patients of group A (7%) and 7 of group B (17%) can be considered complete responders, 12 cases of group A (27%) and 20 in group B (48%) could considered as partial responders, 4 cases of group A relapsed (9%) as well as 2 cases of group B (5%). Twenty-five cases in group A (57%) and 13 in group B (31%) did not show response, hence were considered as "non-responders".

Clinical response

Purpura score dropped in both groups (p<5.79 x 10^{-17}) since near all patients showed a quick disappearing of all skin lesions. In fact, only 5 cases of group A (11%) and 5 of group B (12%) did not show any improvement of the purpura score, and therefore were considered as "non responders". However, two of these non-responders (1 in group A and 1 in group B) healed from purpura at the end of the follow-up. A large fraction of the patients of group A (16 cases, 36%) and a small fraction of group B (2 cases, 5%) relapsed after the end of the treatment. Arthralgias showed a similar behaviour. On the basis of the above reported criteria, 23 cases of group A (59%) and 34 in group B (83%) obtained a complete clinical response.

Peripheral neuropathy

Twenty-two patients (26%) had clinical signs of peripheral neuropathy at baseline. Electromyography, performed in the majority of patients (17 cases: 72%) confirmed the diagnosis in all cases and showed the presence of sensitive neuropathy in all patients, while 7 of them showed also motor neuropathy. After the treatment, 9 patients (41%) showed a complete clinical recovery from neuropathy, 2 patients had a partial response (9%), 10 patients did not show any improvement (45%) and 1 patient worsened (5%). At the end of the follow-up three patients relapsed (27% of responders), therefore only 7 cases obtained complete response (32%) and one a partial response (5%).

Table III. Virological parameters of the 85 HCV-MC patients (in one case the genotype was not determinable) at baseline, at the end of the treatment, and at the end of the follow-up.

Parameter	Baseline	End of therapy (% of non responders)	End of follow-up (% of non-responders + relapsers)
HCV-RNA (% of positivity)	100%	28%	51%
Genotype 1a	6 (7%)	2 (33%)	3 (50%)
Genotype 1b	36 (42%)	15 (42%)	25 (69%)
Genotype 2	33 (39%)	7 (21%)	14 (42%)
Genotype 3	9 (11%)	0	1 (11%)
Genotype 4	1 (1%)	0	0

At this time (EFU), electromyography was repeated in only a small fraction of cases (5 cases: 23%).

Cryoglobulinemic glomerulonephritis

Ten patients (12%) had clinical and laboratory alterations of renal function: mainly proteinuria ranging from 2 to 10 g/24 hours (mean value 5.1±2.6). The kidney biopsy showed the presence of membrano-proliferative glomerulonephritis type I in 9 cases (90%) and a mesangio-proliferative glomerulonephritis in one case (10%). During the treatment, a reduction of daily proteinuria was observed in the large majority of patients (from 5.1±2.6 to 2.1±1.2 g/day $p<0.05$). However, at the end of the treatment, 6 patients (60%) showed a major (complete or partial) response, while 4 (40%) did not show any improvement of proteinuria. At the end of the follow-up, 2 patients relapsed (33%), therefore only 4 cases (40%) could be considered long-term responders. In these patients, a second renal biopsy was not performed to confirm the recovery from the kidney disease.

Side effects of the therapy

Five patients (9%; three in group A and two in group B) interrupted the treatment: one at third month for severe depression, one at fourth month for severe weight loss, moderate depression and skin lesions, one at forth month for the worsening of peripheral neuropathy, one at fifth month for increasing title of anti-nuclear antibodies (without clinical symptoms), and one at ninth month for thrombocytopenia and neutropenia. A dose reduction of Peginterferon therapy was required for two subjects (one in group A and one in group B) showing neutropenia and/or thrombocytopenia: overall dosage of Peginterferon was 81% and 94%, respectively. All the other patients completed therapy with 100% dose compliance. Ribavirin dose was reduced for 3 subjects (2 in group A and one in group B), that reached 90%, 87% and 92% of the forecasted total dosage. One of these cases required erythropoietin therapy. All other patients achieved 100% compliance.

Discussion

In previous study (17), we used a lower Peg-interferon alfa-2b dosage in comparison to what described on HCV updated guidelines and the treatment duration was 12 months for all viral genotypes. This therapeutic schedule was adopted in order to enhance drug tolerability and to minimise drop-out rate. Since the complete virological responders were disappointingly low (44%), in this large cohort of patients affected by MC the length of the therapy was 6 or 12 months, according the HCV genotype, and the Peginterferon alfa-2b and ribavirin dosage regimens were the same used in HCV-chronic hepatitis without cryoglobulins. This approach is particularly useful since, as previously indicated by Zignego and co-workers (18), the HCV genotype 2 shows a significantly higher prevalence in the patients with MC than in patients with chronic hepatitis who did not have MC, allowing a shorter treatment period in a large fraction of MC cases. It is not clear whether this genotype is endowed with a greater lymphotropism or induces a different host immune reactivity, but some observations that genotype 2 is particularly frequent in patients with anti-liver-kidney microsomal autoantibody-positive type 2 autoimmune hepatitis further supports the possibility of a peculiar pathogenetic role for this genotype (19).

Table IV. Comparison of group A (HCV-genotype 1 or 4) and group B (HCV-genotype 2 or 3) at baseline, and behaviour of the main parameters the end of the treatment, and at the end of the follow-up.

	Age years	A Activity score	F Fibrosis score	ALT U/L	Cryo %	RF U/L	Purpura score	C4 mg/dl
A *vs*. B (at baseline)	NS	NS	NS	0.2 : NS	0.3 : NS	$p<0.01$	0.1 : NS	0.3 : NS
Group A baseline	55 ± 10	1.9 ± 0.8	1.9 ± 0.9	114 ± 70	5.6 ± 5.7	256 ± 378	2.2 ± 0.7	8.8 ± 6.2
Group A therapy end	-	-	-	49 ± 34*	2.5 ± 4.5°	225 ± 385§	0.2 ± 0.6*	10.3 ± 8.2
Group A follow-up	-	-	-	70 ± 61**	2.7 ± 3.7°°	244 ± 400	0.7 ± 0.9	10.4 ± 9.2
Group B baseline	56 ± 12	1.9 ± 0.8	1.9 ± 0.9	135 ± 134	6.3 ± 7.6	168 ± 64	2.0 ± 0.7	9.5 ± 5.8
Group B therapy end	-	-	-	31 ± 16°	2.0 ± 3.7°	113 ± 143§	0.2 ± 0.6*	11.5 ± 7.4
Group B follow-up	-	-	-	31 ± 27°°	4.1 ± 6.5°°	123 ± 180	0.3 ± 0.7	12.3 ± 7.8

Groups A and B are homogeneous at baseline, only RF levels are significantly higher in group A. At the end of the treatment ALT activity, Cryoglobulin level, Rheumatoid factor levels and purpura score dropped significantly in both groups. *$p<0.0000006$; °$p<0.000009$; § $p<0.01$; **$p<0.00003$; °°$p<0.00002$; §§$p<0.0002$.

Our results show a overall rates of complete virologic responses of 36% of the patients with genotype 1 and 4 and of 64% of the patients with genotype 2 and 3. To compare this limited number of MC patients with the very large number of patients with HCV chronic hepatitis is rather difficult. However, the number of long-term responders of our study is largely lower than that observed in the three most relevant and quoted studies on this topic *i.e*. Manns *et al*. (2316 patients) (20), Fried *et al*. (1459 patients) (21) and Hadziyannis *et al*. (1736 patients) (22). In these papers the response rate for geno-type 1–4 ranges from 42 to 52% and for genotype 2–3 from 76 to 80%. The lower proportion of complete responses does not necessary indicate that mixed cryoglobulinemia subjects have a more advanced liver disease coupled with negative prognostic factors (immunological alterations could be blamed for HCV enhanced resistance to anti-viral treatment), but this can be secondary to a different patient enrolment. In fact, in the above-reported papers, a very large number of patients were not enrolled (786, 310 and 425, respectively) for several medical conditions such as renal disease, or thrombocytopenia or peripheral neuropathy or others; on the contrary, in our series, a significant fraction of our cases was affected by these disorders (12% had renal disease, 26% neuropathy, 5% Raynaud phenomenon, 8% thrombocytopenia), this means that at least 50% of our patients should not be admitted in those trials. In addition, cryoglobulinemic patients are generally older than chronic hepatitis C patients without MC (55±11 years in our series as compared with 42±10 in Fried *et al*. or with 47±8 in Manns *et al*., and Hadziyannis *et al*.). On the contrary, the comparison of our patients with the largest cohort of HCV chronic liver disease so far collected in Italy *i.e*. the PROBE Project (2149 Italian patients) shows minor clinical difference: the mean age of this unselected cohort is 49±13 years, and the response rate is not so different: 41% for genotypes 1–4 and 70% for genotypes 2–3 (23). In the PROBE Project the overall response rate (all genotype) is 56.5%, but this percentage decreases to 48% in the subjects over 65 years of age, indicating the importance of the patient age, and decreases to 34% in the patients with comorbidities (obesity, diabetes, renal diseases etc.). Since most patients with MC have, by definition, at least one comorbidity (purpura secondary to vasculitis) the lower virological response rate is thus easily explained.

Apart from the rather satisfactory virological response, most patients (88.5%) showed a complete and persistent recovery from clinical symptoms (purpura, arthralgias and weakness). This is exactly the same result obtained by Cacoub *et al*. (86.3%) with a prolonged treatment with combination therapy of variable length (13±4 months) (24). The clinical and virologic responses were not closely correlated, since a large fraction (46%) of virological non-responders experienced a complete recovery from purpura, that did not relapse at the end of the treatment. Only four non-responders (17%) did not show any improvement of clinical symptoms. On the contrary, among responders/relapsers only 4 patients did not have relief from purpura and the other clinical symptoms. As shown in this and in many previous reports, the patients with HCV-MC may remain in clinical remission despite the persistence of viremia (25, 26). Besides its antiviral activity, IFN also has antiproliferative and immunomodulatory properties (27), which may account for the clinical response in the non responders from a virological point of view.

The results of therapy on the immunological parameters are difficult to explain. The disappearance of circulating cryoglobulins (the goal of the treatment) was obtained in only a fraction of the patients who cleared HCV-RNA, this means that a large fraction of virological responders had measurable levels of cryoglobulins, even without clinical symptoms. The behaviour of RF is even less satisfactory since only a very small fraction of patients (12 cases, 14%) showed normal levels at the end of the follow-up. Taken together, these results indicate a scarce activity of antiviral therapy on immunological alterations of MC. This is in contrast with the excellent clinical results. The persistence of cryoglobulins and rheumatoid factor after viral eradication supports the hypothesis that antiviral treatment interferes partially with lymphocyte B clone activity. In fact, the persistence of subtle immunological alterations, such as abnormal sIL2r (28, 29) or sCD30 serum levels (30, 31) have been observed by some authors after the HCV-RNA clearance. Alternatively, "cryptic" HCV replication (as occurs in HBV infection) might be present in the immune system (spleen, bone marrow, lymph nodes) giving a viremia below the sensitivity of the available assay, but sufficient to induce the production of RF or cryoglobulins (32-34). Given this situation, it is likely that a longer treatment period, perhaps 18 or 24 months, could be necessary to obtain a complete immunological outcome, as suggested by Cacoub *et al*. (24) who treated with PEG-IFN plus ribavirin 40 MC patients for over 14 months and, at the end of the follow-up, obtained high immunological (but not virological) responses (57%).

Patients with kidney involvement had less probability to respond to antiviral therapy, in fact, only 4 cases (40%) could be considered as long-term responders. A significant decrease in proteinuria was observed in virologic responders and relapsers, whereas no significant change in the serum creatinine level was seen. Nephropathy is a major cause of morbidity and mortality in MC (35, 36). However, data on the treatment of patients with cryoglobulinemic glomerulonephritis are scarce. Consistent with our results, 2 other studies showed a loss of proteinuria in sustained viral responders treated with IFN plus ribavirin (37, 38). Kidney injury could result from the fixation of circulating immune complexes containing HCV antibodies, HCV antigens, and complement on glomerular capillaries in the mesangium (39). In a recent study, Rossi *et al*. (40) showed an improvement in serum creatinine levels and a decrease in renal injury in 3 patients, with persistence of glomerular lesions on the second renal biopsy after antiviral treatment withdrawal. This suggests that deposition of immune

complexes leads to definitive glomerular lesions, therefore, early treatment with antiviral agents is needed before definitive sclerosis occurs.

Although interferon treatment has sometimes been associated with a worsening of immunological outcome of cryoglobulin-associated peripheral neuropathy, in our study we observed, during therapy, an overall response rate of 50% (complete plus partial responses). Only one patient worsened (5%) and therapy was withdrawn. Though some patients relapsed and only 7 cases (32%) obtained a complete response, these results confirm the safety and the efficacy of combination therapy in HCV subjects with peripheral neuropathy.

In the last five years, several papers reported data on the efficacy of rituximab, an anti-CD20 monoclonal antibody, in patients with HCV-MC active vasculitis (41, 42) and in some life-threatening manifestation of MC such as abdominal vasculitis (43). It appears that rituximab is very efficacious against cryoglobulin production and its clinical consequences (*i.e.*, inflammatory vascular lesions). However, those studies did not allow conclusions on the efficacy of this therapy on peripheral neuropathy and nephropathy (44, 45). Since the virological response seems not to be closely related with clinical response, and since some patients showed persistent clinical symptoms despite the clearance of HCV-RNA, the anti-CD20 treatment could be very useful in the virological responders with persistent clinical symptoms, to eliminate the surviving B lymphocyte clones responsible of cryoglobulin production. A recent paper indicates the efficacy and safety of the anti-CD20 treatment even in advanced liver cirrhosis: despite a transient increase in the HCV-RNA concentration, the liver function showed a improvement (46). Even the use of anti-CD20 treatment in association with antiviral therapy (47) has been recently explored in HCV-MC and the results indicate a increase of the virological response rate and a decrease of the relapse. These surprising results seem to be associated with the higher efficacy of anti-CD20 to eliminate the monoclonal B-cell population present in the peripheral blood of MC patients. However, other authors did not find any difference in the virological response rate, perhaps for a different treatment schedule (48, 49). Both studies indicate a better immunological and clinical response rate, therefore, the innovative association of antiviral and anti-CD20 treatment seems well tolerated and more effective than Peg-IFN plus ribavirin in severe HCV-MC.

In conclusion, our data with Peg-IFN document a lower response rate than that observed in the most large clinical trials with HCV chronic hepatitis, but the presence of comorbidities and older age should be taken in consideration. Peg-IFN plus ribavirin seem a safe and effective therapy in hepatitis C mixed cryoglobulinemia patients, but the association with anti-CD20 should be taken into consideration especially for the cases with severe disease. Further clinical research is needed to prove whether the addition of the new antiviral drugs such as boceprevir (50) or telaprevir (51), that have been recently shown to improve the rate of SVR in patients with a chronic HCV genotype 1 infection, are also able to increase the rate of virological response and reduce the relapse rate of patients with MC.

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