Safety and efficacy of rituximab in patients with hepatitis C virus related mixed cryoglobulinemia and severe liver disease

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ABSTRACT

The effectiveness of rituximab in HCV-related mixed cryoglobulinemia (MC) has been shown. However, the risk of an increase in viral replication limits its use in cirrhosis, a condition frequently observed in MC patients. In this prospective study, 19 HCV-positive patients with MC and advanced liver disease, who were excluded from antiviral therapy, were treated with rituximab and followed-up for 6 months. MC symptoms included purpura, arthralgias, weakness, sensory-motor polyneuropathy, nephropathy, and leg ulcers. Liver cirrhosis was observed in 15 out of 19 patients, with ascitic decompensation in 6 cases. A consistent improvement in MC syndrome was evident at the end-of-treatment (EOT) and end-of-follow-up (EOF-U). Variable modifications in both mean viral titers and ALT values were observed at admission, EOT, third month of follow-up and EOF-U (2.62x10^6, 4.28x10^6, 4.82x10^6 and 2.02x10^6 IU/mL and 63.6, 49.1, 56.6 and 51.4 IU/L, respectively). Improvement in liver protidosynthetic activity and ascites degree was observed at EOT and EOF-U, especially in more advanced cases.

This study shows the effectiveness and safety of rituximab in MC syndrome with advanced liver disease. Moreover, the depletion of CD20+B-cells was also followed by cirrhosis syndrome improvement in spite of the possibility of transient increases of viraemia titers.
INTRODUCTION

Hepatitis C virus (HCV) chronic infection is a major cause of end-stage liver disease and liver cancer worldwide. Much evidence has shown that HCV infection is associated with B-cell lymphoproliferative disorders (LPDs), such as mixed cryoglobulinemia (MC) and B-cell non-Hodgkin’s lymphoma (B-NHL). MC is the most investigated of these LPDs and strictly HCV-related with both epidemiological and pathogenetic data supporting such an association. As an obvious consequence, the possible usefulness of antiviral therapy has been investigated and its effectiveness proven. However, such therapy is burdened by a number of side effects and is often not tolerated or contraindicated. In addition, several studies strongly suggest that MC is frequently associated with severe liver disease. The usefulness and safety of rituximab (RTX) - a chimeric monoclonal anti-human CD20 antibody - in MC have been clearly shown in several studies involving patients with contraindications to antiviral therapy. RTX was shown to be highly effective in modifying the dynamics of B cells by deleting expanded clones and markedly improving MC syndrome in most cases. Such improvement is generally prolonged for >6 months. However, the possibility that viral replication and aminotransferase values may suffer an immunodepression-related increase limits its use in patients with advanced chronic liver disease (CLD). Consequently, very limited data exist about RTX treatment of patients with advanced CLD. In a preliminary study, we observed that RTX treatment was followed by improvement of both MC and cirrhotic syndromes in 2 patients. Such results prompted us to perform the present, prospective study involving a consistent number of patients with HCV-positive MC and advanced CLD, to better evaluate its safety and effectiveness.

PATIENTS AND METHODS
Patients

We prospectively studied 19 patients (13 women, 6 men, mean age 63 yrs, range: 37–77 yrs.) with HCV-related MC (type II in 17 cases and type III in 2) and advanced CLD who were administered RTX. Patients were consecutively recruited at the Department of Internal Medicine, Center for Systemic Manifestations of Hepatitis Viruses (MaSVE) of the University of Florence, Italy. Study inclusion criteria were the presence of HCV-positive MC syndrome with advanced CLD, previously excluded from antiviral therapy due to intolerance or contraindications. Exclusion criteria were all known causes of CLD other than HCV infection, with particular attention focused on the presence of HBsAg (hepatitis B surface antigen), and anti-HIV (anti–human immunodeficiency virus) antibodies. All patients provided informed consent in accordance with the Principles of the Declaration of Helsinki and approved by the University of Florence Ethics Committee.

The main demographic, clinical and virological characteristics of patients are outlined in Table 1. HCV infection was proven by detecting circulating anti-HCV antibodies (EIA-2 and RIBA-2, Ortho Diagnostic Systems, Raritan, NJ) and/or HCV RNA (AMPLICOR® HCV Test, v2.0. Roche Diagnostics, Alameda, CA). All patients had serum anti-HCV antibodies and all but one scored persistently serum HCVRNA-positive. HCV genotype (INNO-LiPA HCV II, Immunogenetics, Gent, Belgium) was 1b in 11 patients, 2a/2c in 6, and 4c/4d in one patient. The mean duration of HCV infection was about 169 months (range 75 - 366 months). The source of infection was unknown in 14 cases and most probably related to i.v. drug abuse in 2 and blood transfusion in 3. No patients were IgM anti-HBc or HBV-DNA-positive, but 3 patients were positive for total anti-HBc. These patients received Lamivudine prophylaxis during the study to avoid the risk of occult HBV reactivation\(^1\) and were tested monthly for serum HBV-DNA.

MC syndrome was diagnosed according to previously described criteria\(^2,3\). Serum cryoglobulin levels and characterization, levels of complement fractions, RF and auto-
antibodies were evaluated as described\textsuperscript{13-15}. The mean duration of MC was 88 months (range 24-172 months). The main MC symptoms are presented in table 1. The most relevant clinical manifestations were purpura in 17 patients and leg ulcers in 3, arthralgia and weakness in 15. Five patients had renal involvement and all but one experienced peripheral neuropathy.

The diagnosis of liver disease was performed according to standard (histological and/or clinical and ultrasound) criteria\textsuperscript{16,17}. Four patients had advanced chronic hepatitis: according to the Metavir score\textsuperscript{18} one patient scored A3F3, two patients A2F3 and one patient A1F3. A diagnosis of liver cirrhosis, according to clinical and ultrasound data, was made in the remaining 15 patients (Table 1).

**Comorbidities**

In addition to MC, the diagnosis of autoimmune hemolytic anemia (patients no. 5 and 10, Table 1) or idiopathic thrombotic thrombocytopenic purpura (patient no. 7) was made in 3 patients, according to standardized criteria\textsuperscript{18,19}.

**Treatment**

RTX was administered as previously described\textsuperscript{9,10,20-22} and consisted of intravenous injection of 375 mg/m\textsuperscript{2} BSA (Body Surface Area) once weekly over a 1-month period. Patients who already received low-dose corticosteroids (all but one patient, no. 14) continued this treatment. None of the patients had been previously treated with RTX.

The patients were followed before, at the end of treatment (EOT), at 3 months (3m.F-U) and 6 months after treatment (EOF-U) with an accurate evaluation of the main clinical, immunological and biochemical parameters corresponding to both the MC syndrome, the CLD and the viral infection.

**Evaluation of treatment response**
**MC syndrome/immunological data**

The main MC-related parameters were evaluated as previously described\(^9,23\). Overall, a complete clinical response was defined as improvement in all baseline clinical manifestations and a partial response as improvement in at least half of the baseline clinical symptoms. All other patients were classified as clinical non-responders. In regard to the main MC symptoms, extension of purpura was classified according to four main semi-quantitative grades: 0 (no purpura); + (limited or fluctuating involvement of the lower limbs); ++ (diffuse and persistent involvement of the lower limbs); +++ (diffuse and persistent involvement of the trunk and the lower limbs). Leg ulcer response was considered as: complete (when all the ulcers completely healed); “major” (reduction of at least 75% of the diameter and/or recovery of at least 75% of the ulcers); “minor” (reduction of 25-74% of the diameter of one or more ulcers and/or recovery of 25-74% of the ulcers); no response (less than 25% reduction in the diameter of one or more ulcers and/or recovery of less than 25% of the ulcers, or their worsening).

Arthralgia and neuropathic symptoms, including both paresthesias/pain and clinically evident motor deficit were measured through a patient-scored Visual Analog Scale (VAS) (range: 0-100) and, when possible, by electromyographic analysis. Treatment response was defined as: complete (disappearance of symptom/s); “major” (>50% VAS reduction); “minor” (25-50% VAS reduction); no response (<25% VAS reduction or worsening); relapse/progression (>50% VAS increase).

The possibility of a malignant lymphoproliferative disorder was evaluated by TC total body, bone marrow biopsy/aspirate, as well as by biohumoral data.

Renal function was evaluated according to serum creatinine and proteinuria/24h. A complete response was defined as the combination of normalization of renal function when abnormal (serum creatinine) and proteinuria ≤0.5g/day. A partial response was defined as stable or improved renal function and/or persisting ≥ 50% reduction of proteinuria.
response was defined as worsening of renal function not attributable to different causes and/or proteinuria increase or a reduction insufficient for the definition of complete or partial response.

**Hepato-virological evaluation**

The degree of liver function was evaluated with the help of the biohumoral liver function tests (LFTs) and the Child/Pugh score\(^24\); in addition, the degree of ascites (in the absence of infection or development of hepatorenal syndrome) was graded as follows: grade 1 (mild): only detectable by ultrasound examination; grade 2 (moderate): moderate symmetrical abdominal distension; grade 3 (large): marked abdominal distension\(^25\).

The determination and quantification of HCV-RNA in serum samples was performed by a very sensitive Real-time PCR technique that has been extensively described in previous studies\(^26,27\). In some cases, peripheral blood mononuclear cell (PBMC) samples were tested for the presence of isolated HCV infection in this compartment, as previously described\(^27\).

**Evaluation of liver ultrastructure**

Transjugular biopsies for microscopy were obtained in two patients (no. 12 and 14, table 1) with liver cirrhosis, both before treatment and 3 months later; another biopsy was obtained before treatment from a further patient (no. 19). The samples were fixed with 2% formaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, sonicated and embedded in epoxy resin. 1-2 µm thick sections were stained with alkaline toluidine blue and examined by light microscopy. Sections about 70 nm thick were stained with lead acetate and uranyl acetate and observed in a Jeol JEM 1010 electron microscope (Tokyo, Japan) at 80 kV.

**Analysis of reticulo-endothelial system (RES) function by Resovist-enhanced Magnetic Resonance Imaging (MRI) quantitative evaluation of liver parenchyma.**
Three patients with liver cirrhosis (no. 12, 14, 19) underwent MRI with a 1.5-T unit (Gyroscan ACS NT, Philips, Eindhoven, The Netherlands). Unenhanced examinations were repeated after slow administration of Resovist (SHU-555A, Bayer Schering Pharma, Berlin, Germany), a super-paramagnetic iron oxide (SPIO) contrast agent, as previously shown. A simple quantitative analysis of percentage signal intensity changes of cirrhotic liver parenchyma, in delayed phases (10 minutes acquisition), after contrast agent administration, was performed. The dose of 0.9/1.4 mL of SHU-555A was administered in patients whose body weight was below/over 60 kg (range 7.0-12.9 µmol iron/Kg). To obtain a wider window of enhancement the entire administration was prolonged to about 30-40 seconds. Patients were analyzed before treatment and after the third month of follow-up.

Psycho-physical evaluation

The psycho-physical state was evaluated by administration of the SF36 questionnaire. The SF36 questionnaire is a widely used instrument for measuring quality of life and physical functioning. It consists of 8 health subscales and 2 summary scores, the physical component summary (PCS) and the mental component summary (MCS).

Statistical Analysis

Data are expressed as the mean±SEM. Comparisons between baseline and end of follow-up values were analyzed using the paired Student t test and paired Wilcoxon test. All tests were two-sided at a 0.05 significance level. Analyses were performed by using the Stata v.9.0 (StataCorp LP, College Station, TX, USA) and True Epistat standard version (Epistat Services, Richardson, TX, USA) statistical packages.
RESULTS

All patients completed RTX therapy without notable side effects. The overall period of observation ranged from 6 to 48 months.

MC syndrome/immunological data

Analysis of the main MC-associated clinical aspects at EOF-U showed a complete response in 12 patients and partial response in 7. Table 2 shows the main clinical data gathered during the study. At EOF-U, complete disappearance of purpura was observed in 14 out of 17 cases: in the remaining 3 patients only a partial response was observed. Patient no. 3 responded early, but relapsed at the EOF-U. Pre-treatment leg ulcers (1 to 3) were present in 3 patients. The response was rapid and complete or major. Neuropathic pain was evident in all but one patients (Table 1). A complete or major response was evidenced at EOF-U in 11 patients, a minor response in 3, and no response in 4 patients. Paresthesias were present in all but one patients before treatment; a complete or major response was observed in 10 patients, a minor response in 4, and no response in the remaining 4. The electromyographic analysis, available in 5 patients, showed sensitive-motor neuropathy with aspects of an axonopathic-degenerative process involving the arms and legs in all patients. In 2 patients no consistent modifications were shown after RTX. Arthralgias were present in 15 patients before treatment and in all a complete or partial response was obtained. Five patients had renal involvement, confirmed by renal biopsy showing a cryoglobulinemic membranoproliferative glomerulonephritis in 2. Three patients showed a partial response, whereas the remaining patients had a complete one. The overall improvement of MC syndrome was also shown by the consistent reduction of the need for corticosteroid therapy that was progressively reduced in most patients, and completely interrupted in some cases. The EOF-U analysis of the main MC-associated biochemical parameters showed a consistent reduction of the cryocrit mean values (p < 0.01) (Figure 1). Complete cryocrit negativization was observed in 9, a cryocrit decrease >50% in 2, or between 25% and 50%
in 3 patients, whereas in 5 patients no consistent cryocrit reduction was shown, in spite of transient negativity at 3m.F-U in 2 cases. Finally, in patient no. 16 we observed an increase of cryocrit values in spite of a complete clinical response. A consistent reduction of IgM ($p = 0.02$) and RF values as well as an increase of complement C4 component levels were observed (Figure 1). Cytofluorimetric analysis showed a dramatic decrease of CD19+ peripheral blood cells starting early after anti-CD20 infusion ($p = 0.01$). This was followed by a partial reconstitution of cells starting at 6 months after EOT (Figure 2). No significant modifications of the T lymphocyte pool were observed during therapy and follow-up. RTX administration favorably influenced also autoimmune comorbidities (patient no. 5 and 10) with disappearance of the Coombs test positivity and a progressive improvement of hemoglobin values during the study. In the patient (no. 7) with idiopathic thrombotic thrombocytopenic purpura, platelet values progressively improved (from 14000/mm³ to 61000/mm³ at the EOF-U).

**Hepato-virological evaluation**

Concerning the hepato-virological data, serum HCV-RNA scored persistently positive before the study in all but one patient (no. 3). In HCV-RNA-positive patients, mean viremic levels were $2.62 \times 10^6$ UI/mL before RTX infusion, reached a mean value of $4.28 \times 10^6$ UI/mL at EOT, slightly increased at 3m.F-U ($4.82 \times 10^6$ UI/mL) and decreased to the pre-treatment mean levels at the EOF-U ($2.02 \times 10^6$ UI/mL) (Figure 2). Pre-treatment mean ALT value (63.6 IU/L) was not significantly modified by RTX therapy scoring 49.1 IU/L at EOT, 56.6 IU/L at 3m.F-U and 51.4 IU/L at EOF-U (Figure 2).

In contrast, serum albumin titers showed important modifications. For all patients, mean albuminemia values were 3.61 g/dL, 3.78 g/dL, 4.26 g/dL, and 3.91 g/dL at pre-treatment, EOT, 3m.F-U and EOF-U, respectively (data not shown). This improvement in albuminemia was especially observed in the six patients harboring decompensated cirrhosis and pre-treatment low values (Table 3). Improved hepatic function in patients with liver cirrhosis was
demonstrated by the improved Child-Pugh score observed in most patients with advanced disease (>A score) (Table 3). In these patients the score improved from 1 to 4 points in all but one patient who maintained the B7 score (Table 3). An impressive reduction in degree of ascites was observed in the 6 patients with decompensated cirrhosis (Figure 3). In 4 out of these 6 patients a complete, persistent disappearance of ascites, was observed from the EOT and 3m.F-U. The remaining 2 patients experienced a complete disappearance of ascites at 3m.F-U, with only ultrasound evidence of mild ascitic effusion at EOF-U. This improvement was followed by a progressive reduction of diuretics doses (antialdosteronic and furosemide, p = 0.05), the amount of albumin infusion per week (Figure 3), and the need for paracenteses (patients no. 4 and 8, who were previously treated at by-monthly intervals, did not need paracenteses during the study period). Of the 3 patients who received 20 g of albumin weekly during the pre-treatment period, only one needed albumin at the EOF-U (40 g monthly, patient no. 4). Such an impressive effect on the degree of ascites and need for albumin infusions in patients with decompensated cirrhosis did not appear to be justified by a corresponding improvement in proteinuria levels. In the 3 patients with proteinuria, the mean values were 1.072, 0.309, and 0.391 g/24h before treatment, at EOT and at EOF-U respectively (data not shown).

Interestingly, 2 patients (no. 16 and 18), who were excluded from the antiviral therapy before this study due to cytopenia, could be given viral eradication therapy after RTX treatment. Patient no. 16 (high viral load - >850000 IU/ml - HCV genotype 2a/c) had a history of ineffective antiviral treatments and, 3 months after RTX therapy, received standard treatment with pegylated interferon and ribavirin for 6 months. Patient no. 18, who was treatment-naïve and had similar viral characteristics (high viral load and HCV genotype 2a/c), stopped treatment after only 6 weeks due to the occurrence of severe psychiatric complications. Both patients experienced a sustained virological response. The analysis of HCV-RNA sequences in both peripheral and bone marrow mononuclear cells confirmed the viral eradication. In fact, HCV-RNA sequences were not detected in both uncultured and
mitogen-stimulated, cultured cells in blood samples obtained at 3 different times (3 and 6 months) after therapy interruption and at the last control visit (12th month post-treatment) (data not shown).

**Evaluation of liver ultrastructure**

In patients undergoing transjugular liver biopsy, portal areas hosted a rich infiltrate, mainly made by dendritic cells and lymphocytes with a few macrophages and plasma cells, and many bile ductules. Small foci of dendritic cells and lymphocytes were located among hepatocytes. The sinusoids were scarce. Kupffer and Ito cells had a normal aspect. No consistent modification in such aspects was seen after RTX administration (data not shown).

**Analysis of RES function by Resovist-enhanced MRI quantitative evaluation of liver parenchyma.**

Analysis of MRI data was not dissimilar from mean percentages reported in the literature\(^3\). In the 3 patients who underwent Resovist enhanced MRI, we did not find any significant change in the mean SI percentage of cirrhotic liver parenchyma pre and post-therapy either with the T2 (-43 vs. -41%) or with T2\(^*\) (-37 vs. 35%) sequence.

**Psycho-physical evaluation**

A consistent improvement in the patient’s perception of his/her health was shown by the SF36 questionnaire compiled before treatment and at different times during follow-up (data not shown).
DISCUSSION

MC is both an autoimmune and B-cell lymphoproliferative disorder. This suggested that RTX may represent an interesting alternative to traditional therapeutic approaches for MC patients, which often have limited efficacy and/or significant side effects. Several previous studies indicate that RTX may be successfully used for treatment of HCV-related MC. However, its efficacy, safety and cost/benefit in relation to different organ manifestations and duration of clinical response, have not been fully investigated.

In the present study, RTX efficacy was confirmed in patients with HCV-related MC and advanced CLD. This study also shows, for the first time, that B-cell depletion induced by RTX can lead to improved concomitant liver cirrhosis syndrome.

Regarding MC syndrome, effects of RTX treatment did not substantially differ from previous reports. RTX was mainly effective in inducing the disappearance or consistent regression of skin manifestations, such as purpura and leg ulcers. Less evident improvement appeared in peripheral neuropathy, especially considering electromyographic changes. Better results were reported in a recent work showing that RTX is effective and safe in the treatment of patients with MC-associated neuropathy. The limited number of patients undergoing electromyography before and after therapy in the present study makes it difficult to arrive at a definitive conclusion. In addition, subjective improvement in neuropathic pain and paresthesias, according to the VAS scale, was highlighted in the large majority of cases. Although the present study did not include patients with severe renal disease, the use of RTX led to consistent improvement in renal function. For almost all patients with active urinary sediment before treatment, this was significantly reduced or absent after treatment and in half of patients with proteinuria, a >50% reduction was observed. In addition, in view of the wide range of possible overlapping extrahepatic manifestations frequently observed in patients with chronic HCV infection the
contemporary resolution of both MC and autoimmune haemolytic anaemia or idiopathic thrombotic thrombocytopenic purpura we observed in 3 patients, is of great clinical interest.

Concerning the liver disease, previous data showed the absence of direct hepatotoxicity of the treatment. However, in HCV-positive patients, the risk of an immunodepression-related increase in viral replication, with the consequent possibility of worsening liver damage, justified the exclusion of patients with advanced liver disease from previous studies. Such an exclusion would greatly limit the use of RTX for MC. In fact, an association between HCV-positive MC and advanced liver disease was shown in the majority of available studies\textsuperscript{7,8,36}, although data from some geographical areas may be contrasting and the limitation of such an association to a specific MC type has been proposed\textsuperscript{37,38}. In addition, the use of RTX has been especially suggested when antiviral therapy is ineffective or contraindicated, and it is known that liver cirrhosis consistently limits the effectiveness of anti-HCV treatment or represents a contraindication in case of more advanced, decompensated cases. The results of the present paper clearly show that this drug may be effective and safe even in this type of patients. In fact, the mean ALT appeared slightly reduced in our total patients, even though, a moderate increase was observed in some patients. However, such an increase was not followed by a worsening of liver function, but rather by improvement, as shown by increased protein synthesis, mainly evident in patients with cirrhosis complicated by ascites at the time of enrolment. In the majority of these patients RTX treatment led to a significant decrease in Child-Pugh class. However, the presence of end-stage liver disease should always prompt a cautious clinical approach and careful clinical monitoring, due to patient variability and the possible presence of comorbidity. In this respect it is noteworthy that none of our patients had evidence of malignant evolution of the lymphoproliferative or liver disorder, nor history of spontaneous peritonitis or hepato-renal syndrome.

Improved cirrhotic syndrome following RTX infusion is interesting because of the controversial and complex relationship between hepatic and extra-hepatic disease in HCV-
related MC. The mechanisms for such a paradoxical positive effect are at present unknown and thus more specific studies are needed. The initial theory suggesting a role played by the impairment of Kupffer cell function secondary to their massive involvement in the clearance of large circulating immune-complexes in MC, with a consequent improvement following cryocrit value reduction\textsuperscript{7}, may be considered but, in the present study, we did not obtain supporting data. Liver RES function in some patients was quantitatively investigated by Resovist-enhanced MRI\textsuperscript{29}. This super-paramagnetic T2-contrast agent can label the macrophage system indicating both access to and clearance of the medium by this cell system. The effect of SPIO decreases in the cirrhotic liver because Kupffer cell phagocytic activity is reduced in these patients\textsuperscript{30,31}. As expected, in cirrhotic patients the lower SI induced by Resovist was less than in normal liver, but no evidence of a significant modification after RTX therapy was seen. The lack of consistent modifications of liver ultrastructure, and especially Kupffer cells, after successful therapy, was in agreement with the absence of differences when using Resovist-enhanced MRI. Better knowledge of the main role that B-cells play (directly and/or through the modulation of T-cell function) in the pathogenesis of both MC and liver disease would probably help to clarify the observed phenomena and potentially increase the chance of designing personalized protocols\textsuperscript{39,40}.

Another positive result of the present study was the possibility of reducing or interrupting previous treatments, namely the use of steroids, as well as the need for previous weekly albumin infusions and the frequent paracenteses in some patients resulting in a significant improvement in the patient's quality of life.

Furthermore, since HCV eradicating treatment should be considered the first-line therapeutic option in HCV-related MC - to be performed as early as possible\textsuperscript{5,6,35} -, but many MC patients cannot undergo this therapy because of contraindications or intolerance, it is expected that the RTX-driven improvement in patients' overall clinical condition (i.e., improved hemochrome values, healing of leg ulcers, reduction of Child-Pugh class), may make it possible, for some patients, to successfully undergo the antiviral therapy\textsuperscript{41,42}. 
Accordingly, in the present study, two patients who were previously excluded from antiviral therapy, could achieve viral eradication. One of these patients even had history of previous, ineffective antiviral therapy performed in a less severe phase of disease, suggesting that B-cell depletion has a beneficial effect on viral eradication. Further dedicated studies will ascertain this interesting working hypothesis.

In conclusion, our study shows the safety and effectiveness of RTX as treatment of HCV-related MC even in patients with concomitant severe liver disease. The observation of an improvement of cirrhotic syndrome following B-cell depletion strongly suggests the interest for further studies investigating the role - directly or indirectly - played by the B-cell compartment in the HCV-related liver damage.

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Author’s Contributions

A.P. designed and performed research, wrote the paper; L.R. performed research; P.C. analyzed data and wrote the paper; S.C. performed research, provided vital analytical tools and wrote the paper; P.R. performed research, provided vital analytical tools and wrote the paper; F.V. performed research; U.A. performed research; C.G. analyzed data; M.M. performed research; P.M. performed research; M.M.C provided vital analytical tools and performed research; A.B. provided vital analytical tools and performed research; G.L.
analyzed data and designed research; A.L.Z. designed and performed research, analyzed data and wrote the paper.

The authors have no conflicts of interest to declare.
REFERENCES


Table 1. Main laboratory and clinical characteristics of the 19 patients with MC and severe CLD

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<th>Sex</th>
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<th>MCS duration (m)/cryoglobulin type</th>
<th>HCV genotype</th>
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<td>173</td>
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<td>137 / II</td>
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<td>P(++)</td>
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</tr>
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<td>24 / II</td>
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<td>2a/2c</td>
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<tr>
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<td>2a/2c</td>
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<td>30 / II</td>
<td>1b</td>
<td>Yes</td>
<td>P(+)</td>
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pt.: patients; yrs: years; m: months; CLD: chronic liver disease; MCS: mixed cryoglobulinemia syndrome; M: male; F: female; n.d.: not determined; CH: chronic hepatitis; C: cirrhosis; P: purpura; N.A.: not applicable
<table>
<thead>
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<th>N° patient</th>
<th>Gender</th>
<th>Skin ulcers</th>
<th>Purpura</th>
<th>Neuropathic pain</th>
<th>Paresthesias</th>
<th>Nephritis</th>
<th>Arthralgias</th>
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<td></td>
<td>(months after EOT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
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<td>M.R.(&lt;6m)</td>
<td>M.R.(&lt;6m)</td>
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<td>n.a.</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
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<td>M.R.(EOT)</td>
<td>m.r</td>
<td>M.R.(&lt;1m)</td>
<td>n.a.</td>
<td>C.R.(&lt;1m)</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>C.R.(&lt;1m)</td>
<td>M.R.*(&lt;3m)</td>
<td>N.R.</td>
<td>N.R.</td>
<td>P.R.</td>
<td>P.R.</td>
</tr>
<tr>
<td>4</td>
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<td>M.R.(&lt;3m)</td>
<td>M.R.(&lt;6m)</td>
<td>M.R.(&lt;6m)</td>
<td>P.R.</td>
<td>C.R.(&lt;1m)</td>
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<tr>
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<td>M.R.(&lt;6m)</td>
<td>C.R.(&lt;1m)</td>
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<td>M.R.(&lt;3m)</td>
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<td>P.R.</td>
<td>n.a.</td>
</tr>
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<td>C.R.(&lt;1m)</td>
<td>n.a.</td>
<td>P.R.</td>
</tr>
<tr>
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<td>M.R.(&lt;3m)</td>
<td>M.R.(&lt;6m)</td>
<td>m.r.</td>
<td>C.R.(&lt;2m)</td>
<td>C.R.(&lt;1m)</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>n.a.</td>
<td>M.R.(&lt;3m)</td>
<td>M.R.(&lt;6m)</td>
<td>m.r.</td>
<td>n.a.</td>
<td>P.R.</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
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<td>M.R.(&lt;3m)</td>
<td>N.R.</td>
<td>N.R.</td>
<td>n.a.</td>
<td>P.R.</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>n.a.</td>
<td>m.r</td>
<td>M.R.(&lt;6m)</td>
<td>N.R.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>n.a.</td>
<td>m.r</td>
<td>C.R.(&lt;1m)</td>
<td>m.r.</td>
<td>n.a.</td>
<td>P.R.</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>n.a.</td>
<td>n.a.</td>
<td>C.R.(&lt;1m)</td>
<td>C.R.(&lt;1m)</td>
<td>n.a.</td>
<td>C.R.(&lt;1m)</td>
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<tr>
<td>14</td>
<td>M</td>
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<td>M.R.(&lt;6m)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
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<tr>
<td>15</td>
<td>M</td>
<td>M.R.(&lt;1m)</td>
<td>M.R.(&lt;6m)</td>
<td>M.R.(&lt;6m)</td>
<td>M.R.(&lt;6m)</td>
<td>C.R.(&lt;6m)</td>
<td>P.R.</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
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<td>N.R.</td>
<td>n.a.</td>
<td>C.R.(&lt;1m)</td>
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<td>F</td>
<td>n.a.</td>
<td>M.R.(&lt;6m)</td>
<td>M.R.(&lt;6m)</td>
<td>m.r.</td>
<td>n.a.</td>
<td>P.R.</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>C.R.(&lt;1m)</td>
<td>m.r.</td>
<td>N.R.</td>
<td>N.R.</td>
<td>n.a.</td>
<td>C.R.(&lt;1m)</td>
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<tr>
<td>19</td>
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<td>n.a.</td>
<td>M.R.(EOT)</td>
<td>N.R.</td>
<td>C.R.(&lt;1m)</td>
<td>n.a.</td>
<td>P.R.</td>
</tr>
</tbody>
</table>

EOT: end of treatment; C.R.: complete response; M.R.: major response; n.a.: not applicable; m.r.: minor response; P.R.: partial response; m.: month/s; months after EOT: timing of response after the end of treatment

*This patient had a relapse sixth months after EOT
Table 3. Modifications of the main hepato-virological data induced by rituximab therapy in the 6 patients with decompensated cirrhosis

<table>
<thead>
<tr>
<th>N° pt.</th>
<th>Treatment phases</th>
<th>Total protein (g/dL)</th>
<th>Albumin (g/dL)</th>
<th>ALT (IU/L)</th>
<th>HCV-RNA (IU/mLx10^3)</th>
<th>Prothrombin Activity (%)</th>
<th>Child-Pugh</th>
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<td>6.5</td>
<td>2.52</td>
<td>46</td>
<td>350</td>
<td>64</td>
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<td>2.95</td>
<td>65</td>
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<td>78</td>
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<tr>
<td></td>
<td>3 m. F-U</td>
<td>6</td>
<td>3.1</td>
<td>20</td>
<td>n.d.</td>
<td>71</td>
<td>B-7</td>
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<tr>
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<td>6 m. F-U</td>
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<td>3.1</td>
<td>61</td>
<td>650</td>
<td>80</td>
<td>B-7</td>
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<td>3.9</td>
<td>180</td>
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<td>6 m. F-U</td>
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<td>3.6</td>
<td>82</td>
<td>n.d.</td>
<td>64</td>
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<td>6 m. F-U</td>
<td>5.8</td>
<td>3.9</td>
<td>46</td>
<td>n.d.</td>
<td>59</td>
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<td>3.53</td>
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<td>950</td>
<td>100</td>
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<td>3.9</td>
<td>31</td>
<td>n.d.</td>
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<td>6.7</td>
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<td>n.d.</td>
<td>63.5</td>
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<td>3.13</td>
<td>15</td>
<td>720</td>
<td>72.67</td>
<td>B-7</td>
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</table>

pt.: patients; T.0: data before Rituximab infusion; EOT: end of treatment; 3 m. F-U: third month of post-therapy follow-up; 6 m. F-U: sixth month of post-therapy follow-up; n.d.: not determined
Legends to the figures.

**Figure 1.**
Behaviour of mean values of the main immunological data of the 19 MC patients before rituximab therapy and at the end of follow-up (6th month after therapy interruption).

**Panel A:** IgM mean values (g/L); **Panel B:** C4 component of complement mean values (g/L); **Panel C:** cryocrit mean values (%); **Panel D:** Rheumatoid Factor (RF) mean values (IU/mL).

**Figure 2.**
Pattern of HCV-RNA, ALT and CD19 mean values of the 19 HCV-positive MC patients during the study. RTX: rituximab infusions; EOT: end of treatment; ALT: alanine aminotransferase.

**Figure 3.**
Modifications of pre-treatment therapeutic measures and ascites severity in the six patients with MC and decompensated cirrhosis.

**Panel A:** Mean doses of Kanrenone (mg/die) before rituximab therapy (baseline) and at 6 months of follow-up after therapy interruption (EOF-U);

**Panel B:** Mean doses of Furosemide (mg/die), before treatment and at 6 months of follow-up (EOF-U);

**Panel C:** Mean doses of Albumin infusion (g/month) before treatment and at 6 months of follow-up (EOF-U);

**Panel D:** modifications of ascites severity degree according to Arroyo et al.\textsuperscript{25} observed before rituximab (Baseline), at the end of therapy (EOT), at 3 months of follow-up (3m.F-U), and at 6 months of follow-up (EOF-U).
Figure 1

A. IgM

*\( p = 0.02 \)

B. C4

C. Cryocrit

*\( p < 0.01 \)

D. RF
Figure 2

HCV-RNA (IU/mL x 10^6)

ALT (IU/L)

CD 19+

RTX

Follow-up (months)

*p = 0.01
Figure 3

A: Kanrenone

Baseline 6m. F-U

mg/die

B: Furosemide

Baseline 6m. F-U

mg/die

*p = 0.05

C: Albumin infusion

Baseline 6m. F-U

gr/month

D: Ascites

Pz. 4 Pz. 5 Pz. 7 Pz. 8 Pz. 14 Pz. 15

Baseline 3m. F-U EOT EOF-U
Safety and efficacy of rituximab in patients with hepatitis C virus related mixed cryoglobulinemia and severe liver disease

Antonio Petrarca, Luigi Rigacci, Patrizio Caini, Stefano Colagrande, Paolo Romagnoli, Francesco Vizzutti, Umberto Arena, Carlo Giannini, Monica Monti, Paolo Montalto, Marco Matucci-Cerinic, Alberto Bosi, Giacomo Laffi and Anna Linda Zignego