Improved (4 Plus 2) Rituximab Protocol for Severe Cases of Mixed Cryoglobulinemia: A 6-Year Observational Study

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Abstract

Background: In a prospective, single-center open study, we evaluated the very long-term effects of rituximab (RTX) administered to patients with severe mixed cryoglobulinemia (MC). **Methods**: RTX was administered to 31 patients with MC (type II in 29 cases and type III in 2) with diffuse membranoproliferative glomerulonephritis (16 cases), peripheral neuropathy (26) and large skin ulcers (7). All but 4 patients had serum anti-hepatitis C virus antibodies. RTX was administered at a dose of 375 mg/m 2 , according to a '4 + 2' protocol (days 1, 8, 15 and 22 plus 1 dose 1 and 2 months later). No other immunosuppressive drugs were added. Response was evaluated over a very long-term follow-up (mean 72.47 months, range 30–148). **Results**: Complete remission of pretreatment active manifestations was observed in all cases of purpuric lesions and non-healing vasculitic ulcers, and in 80% of the peripheral neuropathies. Cryoglobulinemic nephropathy significantly improved during follow-up, starting from the 2nd month after RTX (serum creatinine from 2.1 ± 1.7 to 1.5 ± 1.6 mg/dl, $p \leq 0.05$; 24-hour proteinuria from 2.3 ± 2.1 to 0.9 ± 1.9 g/24 h, $p \leq 0.05$). Improvement of cryoglobulinemic serological hallmarks, such as cryocrit and low complement C4, were observed. No clinically relevant side effects were recorded. Re-induction with RTX was carried out in 9 relapsed patients after a mean of 31.1 months (12–54), again with beneficial effects. The survival rate was 75% at 6 years and the probability of remaining symptom-free for 10 years without any therapy was of about 60% after a single '4 + 2' infusion cycle, while the probability of living symptomfree 5 years after relapsing was 80% if given the same treatment. **Conclusion**: In this open, prospective study, RTX appeared to be very effective and safe in the treatment of the most severe cases of MC.

Introduction Mixed cryoglobulinemia (MC) is a systemic vasculitis characterized by multiple organ involvement due to the vascular deposition of immune-complexes, mainly mixed IgG–IgM cryoglobulins and complement [1–3] . These cryoprecipitable immune-complexes are the result of B-cell clone proliferation, triggered by chronic hepatitis C virus (HCV) infection in most cases [4] . Rituximab (RTX), an anti–human CD20 antibody has been shown to be highly effective in deleting expanded B-clones and improving MC syndrome [5–13] and other autoimmune conditions [14–17] in a majority of cases. However, no data are presently available on the very longterm efficacy and safety after RTX administration in MC, nor on the possible indications of maintenance therapy. In this prospective, single center open study, we focused on the very long-term effects of RTX administration in a large cohort of severe MC patients who were administered the '4 + 2' infusion protocol [5, 14] . This intensive treatment was associated with long-

lasting remission in most patients, despite the absence of maintenance therapy with immunosuppressive agents. The '4 + 2' infusion protocol has also been associated with a reduction in the need for corticosteroid therapy by the 2nd month, followed by discontinuation within 6 months in more than 50% of cases (including up to 90% of patients with nephritic involvement), and within 12 months in the remaining patients. Methods Patients We prospectively studied 31 patients (18 women, 13 men; mean age 59.8; range 36–80 years) with MC (type II in 29 cases and type III in 2) who received RTX. Twenty-one patients were intolerant (n = 12) or resistant (n = 9) to other therapies (including PEGinterferon with (n = 11) or without ribavirin (n = 6), corticosteroids, mycophenolate mofetil, cyclosporine). If the patients were already receiving corticosteroids when starting RTX, they were allowed to continue prednisone at a maximum dose of 25 mg/day. Corticosteroids were slowly tapered until complete discontinuation in 6 months. Immunosuppressants were discontinued at least 12 weeks before the first dose of RTX. Ten subjects received RTX as front-line therapy (5 due to severe bone marrow lymphocyte infiltration). RTX was administered intravenously at a dose of 375 mg/m 2 on days 1, 8, 15, and 22 as previously described [5, 14] . Two more doses were administered 1 and 2 months later ('4 + 2' infusion protocol). Four naïve patients (including 3 nephritic subjects) and 1 who was resistant to other therapies also received 3 pulses of 500 mg of methylprednisolone together with the first course of RTX, followed by oral prednisone 0.5 mg/kg/day tapered until discontinuation in 8 weeks. No other immunosuppressive drugs was administered. None but one of the patients received anti-viral therapy (a male who showed an increase in viral load and alanine aminotransferase (ALT) 16 months after RTX). All patients provided informed consent in accordance with the principles of the Declaration of Helsinki and in conformity with the regulations of Piedmont Region for the use of off-label drugs in rare diseases. MC was diagnosed on the basis of previously described criteria [18] . Serum cryoglobulin levels and characterization, levels of complement components, rheumatoid factor (RF), and autoantibodies were evaluated as described elsewhere [18] . The mean duration of disease was 115 months (range 24–240 months) and the mean follow-up after RTX was 72.47 (30–148) months: 30–51 months for 8 patients, 51–75 for 6 patients, 75–115 for 10, and >115 for 6. One patient, with a history of drug abuse, was lost to follow-up after 60 months. Sixteen patients had biopsy-proven renal involvement and 26 experienced peripheral neuropathy. The other most relevant clinical manifestations were purpura in 16 patients and leg ulcers in 7, arthralgia in 26, and gastrointestinal vasculitis in 1 case. HCV infection was proven by detecting circulating anti-HCV antibodies and/or HCV RNA (AMPLICOR HCV Test, version 2.0; Roche Diagnostics). All but 4 patients had serum anti-HCV antibodies. HCV genotype was 1a in 1, 1b in 15, 2a/2c in 8, and 3a in 3 patients. With regard to constitutional and non-renal symptoms, complete clinical response (CR) was defined as a complete remission of all baseline clinical manifestations, and partial response (PR) was defined as a substantial (and measurable) improvement of every baseline clinical symptom. The degree of purpura was classified according to 4 main semiquantitative grades as follows: 0 (no purpura), 1+ (limited or fluctuating involvement of the lower limbs), 2+ (diffuse and persistent involvement of the lower limbs), 3+ (diffuse and persistent involvement of the trunk and the lower limbs). Leg ulcer resolution was considered in the following manner: complete (when all the ulcers completely healed), major (a reduction of >75% of the diameter and/or recovery of >75% of the ulcers), minor (a reduction of 25–74% of the diameter of >1 ulcer and/or recovery of 25–74% of the ulcers), no response (<25% reduction in the diameter of > 1 ulcer and/or recovery of <25% of the ulcers, or worsening). A patient-scored Visual Analog Scale (VAS; range 0–100) was applied for arthralgia. Neuropathic symptoms, including paresthesia/pain and clinically evident motor deficiency, were measured both by VAS and by electrophysiological examination. Electrodiagnostic studies were performed before and every 6 months during follow-up using standard electromyographic equipment. Motor nerve conduction studies were performed by supramaximal percutaneous nerve stimulation. Compound muscle action potential (CMAP) was recorded by using surface electrodes. Motor conduction velocity, distal motor latencies, and amplitude

of CMAP (baseline to negative peak) were measured on the peroneal nerve on both sides. Sensory nerve conduction velocity and amplitude sensory nerve action potential (SNAP) were measured in the sural nerve on both sides. SNAPs were recorded by using surface electrodes.

led out by total body computed tomography, bone marrow biopsy (performed in 11 patients), as well as by using biochemical data. Renal function was evaluated by serum creatinine and creatinine clearance, while urinary abnormalities by 24-hour proteinuria and evaluation of microscopic hematuria. Renal involvement was confirmed by percutaneous biopsy. Complete renal response (CRR) was defined as a combination of normalization of serum creatinine of previously impaired renal function, proteinuria 30% (or decrease of creatinine clearance by >10%) that was not attributable to different causes and/or persistent proteinuria. Circulating B cells in the peripheral blood were investigated by CD20+ and CD19+ B cells that were analyzed by flow-cytometry at baseline, months 1, 2 and every other month thereafter. We examined changes in T-cell homeostasis following RTX-induced Bcell depletion in 5 patients. Analysis included flow-cytometry studies at baseline (before the first RTX infusion), and at months 3, 6, and 9. Whole blood samples obtained in EDTA in the morning were stained with monoclonal antibodies against CD45 (APC 100 eBioscience Bender Medsystems, Calif., USA), CD3 (FITC eBioscience Bender Medsystems, Calif., USA), CD4 (PC7 Beckman Coulter, Calif., USA), CD19 (Pacific Blue TM , Beckman Coulter, Calif., USA), CD20 (PE Beckman Coulter, Calif., USA), CD25 (PerCP-eFluor 710 eBioscience/Bender Medsystems, Calif., USA), FOXP3 (PE Staining Set, eBioscience Bender Medsystems, Calif., USA), CD28 (PE Staining Set, eBioscience Bender Medsystems, Calif., USA), CD8 (PB Staining Set, eBioscience Bender Medsystems, Calif., USA), HLA-DR (PERPC-710, eBioscience Bender Medsystems, Calif., USA).

Statistical Analysis

For the comparison of variables at baseline and follow-up, Student's t test was used for normally distributed parameters, and the non-parametric Mann–Whitney test was used for non-normally distributed parameters. Correlations were calculated and significance was determined by Fisher's test. Multivariable logistic regression analysis was used to identify any independent predictors of flare. Kaplan–Meier hazard plots were constructed for survival time and for time free from disease. The Prism (GraphPad Software, Calif., USA) and SPSS (IBM Corporation, N.Y., USA) software programs were used for these analyses. $p < 0.05$ was considered statistically significant.

Results

The main clinical and laboratory data gathered during the study are shown in figure 1 a, b and c and table 1 . An analysis of the main MC-associated biochemical parameters showed a statistically significant reduction of the cryocrit mean values (at 1, 2, 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 months). At 3 months, complete cryoglobulin disappearance was observed in 18 patients. Trace amounts of cryoglobulin (mean value 1 ± 0.7%) were redetected in 15 patients after 36 months. A statistically significant increase in C4 levels was observed starting the first month after RTX administration ($p = 0.021$). RF significantly decreased at 1 month, whereas IgG values remained stable during follow-up. Mean pretreatment ALT values (44.7 IU/l) were stable and HCV RNA serum load resulted decreased after RTX therapy ($p = 0.04$; online suppl. fig. 1S; for all online suppl. material, see www.karger.com/ doi/10.1159/000445841). Cytofluorimetric analysis showed a dramatic decrease in CD19 and CD20 peripheral blood cells starting early after anti-CD20 infusion. As a consequence of the '4 + 2' infusion protocol, partial reconstitution was observed in the following months, but 24 months after RTX treatment, CD20 cells still remained lower than baseline (fig. 1 c). As shown in online supplementary figure 2S, upon detection of B-cell depletion, we observed a 9-fold increase in circulating Treg (CD4+CD25+FOXP3+) and a 7.5- fold decrease in activated T CD8+ cells over 12

months. Overall, CR was achieved in 65% of the patients and PR was reached in 32% of patients assessed at the last follow-up visit. One patient became RTX-resistant due to the appearance of anti-chimeric antibodies. Constitutional symptoms, skin ulcers, purpura, arthralgia, weakness, paraesthesia, and fever disappeared or improved. In detail, complete remission of pretreatment active manifestations was observed in all cases of skin purpuric lesions and non-healing vasculitic leg ulcers. At 3 weeks, complete disappearance of purpura was observed in 15 of 16 cases; response was observed after 1.5 months in the remaining patient. Pretreatment leg ulcers were present in 7 patients. The response was complete or major in all patients. Representative features of skin ulcer response are shown in online supplementary figure 3S. Neuropathic pain or paresthesia was evident in all but 2 patients, and electromyographic analysis, which is available for all patients, showed sensitive motor neuropathy with aspects of an axonopathic degenerative process involving the arms and legs of all patients (online suppl. table 1S). Complete or major response was seen in 85% of subjects for paresthesia, 74% for burning feet, and 89% for weakness. Arthralgia was present in 26 patients before treatment; however, CR or PR was obtained for all patients. Sixteen patients had renal involvement, which was confirmed by renal biopsy showing cryoglobulinemic membranoproliferative glomerulonephritis. CRR or PRR was observed in 75 and 19% of cases at the end of follow-up, respectively. Cryoglobulinemic nephropathy significantly improved during follow-up starting from the 2nd month after RTX (serum creatinine from 2.1 ± 1.7 to 1.5 ± 1.6 mg/dl, $p \leq 0.05$; 24-hour proteinuria from 2.3 ± 2.1 to 0.9 ± 1.9 g/24 h, $p \leq 0.05$; fig. 1 b). The representative features of renal response in a severe patient with MC are shown in online supplementary table 2S. The present cohort of RTX-treated patients showed a 75% survival rate at 6 years (fig. 2 a). There were 6 deaths (all due to cardiovascular causes – mean age of patients 75.3) after a median of 55 months after their first RTX cycle. Time free from disease after the first RTX treatment and after the 2nd cycle is shown in figures 2 b and c, respectively. The probability of remaining symptom-free for 10 years without any therapy was about 60% after a single '4 + 2' infusion cycle. Nine patients needed a 2nd cycle after a mean of 31.1 months (12–54). The condition relapsed in 4 of these 9 patients thus requiring a 3rd cycle after a mean of 36.6 months (28–60), again with beneficial effects. Overall improvement of MC was also shown by the consistent reduction in the need for corticosteroid therapy by the 2nd month, which was then discontinued within 6 months in 17 cases, including 14 nephritic patients, and within 12 months in the remaining patients. Low titer anti-RTX antibodies were detected in 3 cases and at high titer in 1 patient (940 AU/ml, nv <12) who had previously had a 7-year response to a single course of RTX, and who then received a 2nd cycle of RTX due to relapsing proteinuria. No lymphocyte depletion was detected in this case, and no further CR to RTX was observed. She was successfully treated with a 9-month course of abatacept. As far as the cumulative data are concerned, multivariate analysis showed that patients with multi-organ involvement (log-rank, $p = 0.001$) and cryocrit >4% (log-rank, $p = 0.0001$) had significantly worse survival as compared to the remaining patients, as well as a higher relapse rate.

Side Effects

No acute or delayed severe side effects were seen. Drug-related bradycardia (<50 beats/min) was observed in 2 cases and was managed by reducing the infusion rate. Arterial pressure dropping to 100/60 mm Hg was observed in 2 cases and was also managed by reducing the infusion rate. Five cases of urinary tract infections were recorded. An increase in HCV RNA was noted in 1 patient. ALT levels measured simultaneously was normal (ALT 16 UI/l, VN<35) Bilirubin levels were also normal (total bilirubin 0.8 mg/dl, direct bilirubin is 0.3 mg/dl).

Discussion

Considering its complex pathogenesis and clinical polymorphism, the treatment of MC syndrome is particularly challenging, and the optimal therapeutic strategy for HCV-associated MC nephritis is still undefined [1] . The possible implications of the HCV in the pathogenesis of a majority of MC patients prompted researchers to develop approaches for HCV eradication in order to control the disease [1].

Aggressive immunosuppressive therapy remains indicated for patients with acute, severe disease. Cyclophosphamide, together with steroids and often plasma exchange, is the most frequently used cytotoxic drug in the vasculitic flares of MC. The use of other immunosuppressants such as cyclosporine, azathioprine, and mycophenolate mofetil to treat MC remains anecdotal. Besides the concerns surrounding the use of immunosuppressants in a virus-triggered disorder, especially when disease is characterized by a relapsing course requiring prolonged immunosuppression, some patients have complained of one or more of the several side-effects of conventional immunosuppressive drugs [6] . A number of studies suggest that clinical remission of cryoglobulinemic vasculitis can be achieved by RTX, a human/mouse chimeric monoclonal antibody, which specifically reacts with a CD20 antigen [19–36] . However, the appropriate position of RTX in the treatment strategy of MC remains controversial. Based on retrospective studies mainly examining heterogeneous cohorts (including both patients with severe vasculitis who were non-responders to antiviral therapy and IFN-naïve subjects), it has been suggested that the combination of antiviral and RTX therapy exerts a synergistic effect [26, 37–39] . A prospective, non-randomized cohort study showed that as compared to patients receiving PEG IFN+RBV [38] , patients treated with combined antiviral drug and RTX therapy required less time to reach clinical remission and had a higher rate of cryoglobulin clearance, and that 50% of patients achieved sustained virological response. These results have been confirmed by another group [33]. However, administering combined therapy is generally poorly tolerated. In naïve patients with severe clinical manifestations, RTX should be given first, and antiviral therapy added once the efficacy and safety of RTX have been assessed [40] . The results of our study show that RTX can be a therapeutic option for patients with MC, with a favorable, very long-term safety and efficacy profile. Indeed, patients whose manifestations are not appropriate for (or insufficiently controlled by) antiviral or conventional immunosuppressive treatment may respond to RTX [5, 7] . This is usually the case of patients with progressive renal failure, digital ischemia, gastrointestinal vasculitis or severe neuropathy. Two randomized, controlled trials were recently carried out on mixed cryoglobulinemic patients who failed, or were not eligible for, antiviral therapy. These trials compared RTX to conventional immunosuppressive treatment (corticosteroids, cyclophosphamide, azathioprine, or plasma exchange). The results emphasized that, at least in the short term, the superiority of RTX was undeniable with fewer or comparable adverse effects [34, 35] . With regard to this latter aspect, a French group reported that patients with high cryocrit levels may experience severe flares of vasculitis within 2 days of RTX infusion, particularly if the rheumatoid arthritis scheme (2 infusions of 1 g/day 2 weeks apart) is used [23] . We did not observe such reactions, and in clinical practice, slow administration of the lymphoma dose (375 mg/m 2) over 12–24 h, or administration of half a dose per day in 2 consecutive days given together with pre-medication with steroids, anti-histamine drugs and paracetamol reduces the risk of such reactions [5, 8, 24] . Following a previous pilot study [41] , the preliminary results of a phase II, single arm, multi-center study with low-dose RTX, that is, 250 mg/m 2 given twice at 1 week intervals, was recently published [32] . Twenty-seven patients were enrolled, but CR was evaluable in 19. The 13 patients who did not reach the end of follow-up at 12 months (8 because of relapse and 5 due to lack of response) were given additional therapy. Only 6 patients completed the 12-month follow-up. The CR rate for nephropathy (as well as neuropathy) was 39%. This scheme, which was designed for patients thought to be too compromised to receive a standard dose [41] , is not recommended for cryoglobulinemic nephritis [7] . A common limitation

of open and controlled studies is that data on the long-term effects of RTX – especially when administered alone – are lacking. Our study deals with a very long-term observation of patients with severe MC, using the '4 + 2' infusion protocol, which provides more prolonged B depletion compared to conventional RTX schemes (lymphoma or rheumatoid arthritis protocols). The main indications for RTX included not only severe worsening of renal function, mononeuritis multiplex, widespread skin ulcers [5] , but also life-threatening conditions or fulminant presentations, including peripheral necrosis of the extremities, for which plasma exchange and immunosuppressive drugs were considered the only therapeutic tools until a few years ago. Of note, ALT values were stable and HCV RNA serum load were found to be lower after RTX therapy (online suppl. fig. 1S). Glomerulonephritis and skin ulcers usually improve within 3 months after the beginning of therapy. Complete healing takes longer. Both sensitive and motor neuropathy improved within 5 months after RTX with a stable or improved electromyography picture. To date, the duration of response has been difficult to define due to the lack of long-term follow-up data in all reported studies. Short-term relapse, within 3–4 months, was reported in a minority of patients, while long-term response lasting more than 1 year has been reported as the most frequent outcome. Our strategy, that is, administering 2 more infusions of RTX 1 and 2 months after the standard 4-week course, definitely reduces or delays relapses. Maintenance therapy with RTX, for instance, with a single RTX infusion every 4 or 6 months as currently used in rheumatoid arthritis (1,000 mg) or ANCA-associated vasculitis (500 mg), may be taken into consideration in subjects with severe nephritis or untreatable gastrointestinal manifestations. However, in our experience, relapses occurred in one-third of the patients and a full course of re-treatment with RTX proved to be effective again for more than 30 months, suggesting that a strategy maintenance therapy might result in overtreatment. When focusing on the renal clinical outcome, a review of the literature showed a very heterogeneous response to RTX. Our study represents the largest single-center cohort of patients with MC and renal involvement prospectively treated with RTX. We observed a rate of any response (CR + PR) as high as 95% at the end of follow-up. We retrieved 11 studies [6, 10–13, 16, 18–22] with clearly reported renal outcomes (mean follow-up 13.6 months). The median overall renal response rate (including CR and PR) was 60%. The median CR rate was 57%. By focusing on studies published in the last 10 years, we saw that the median overall response was 73% (range 32–100) with a follow-up of 12–36 months. One could speculate that the intensified protocol (4 weekly infusions plus 2 further infusions at 1 and 2 months) might play a key role in maintaining sustained B-cell depletion and the consequent improvement in renal clinical outcomes. In fact, selective depletion of IgMproducing B cells represents the basis for RTX treatment in MC, a disorder in which the autoimmune process can become independent of the triggering virus and may take on a predominant role in the pathogenesis of the disease. RTX acts downstream of the disease trigger more selectively than the conventional immunosuppressive treatments [5, 8] . In this context, MC-associated nephritis represents a unique condition of immune-mediated disorder in which RTX specifically targets the nephrotoxic Ig-producing cells. Finally, RTX has been reported to restore B-cell homeostasis and reverse Th1/ Th2 imbalance by decreasing clonal VH1–69 memory B cells and by re-assessment of T-cell repertoire [22] . In a sub-cohort of our RTX-treated patients with MC, we noticed that at the onset of B-cell depletion, patients displayed sharp increases in CD4+CD25+FOXP3+ cells in the peripheral blood. Similar results have been found by Saadoun et al. [42] , who showed that the administration of low-dose interleukin-2 in patients with HCV-induced vasculitis was followed by an increase in the percentage of CD4+, CD25 (high), forkhead box P3 (FOXP3+) Tregs. A previously unexpected mechanism of RTX resistance was represented in our experience by the development of anti-chimeric antibodies. The search for antiRTX antibodies is probably needed whenever lack of depletion of B lymphocytes following RTX is observed. Considerable progress is being made in the treatment of HCV. The new direct antiviral agent has been proved to have great antiviral efficacy (>90% cure) and a good tolerance profile [43–45] . This approach may possibly modify both the incidence of vasculitis and the algorithm used

in the early stages of the disease. However, besides the uncertainty regarding drug pharmacokinetics and safety in patients with established renal failure, it seems unlikely that a pure antiviral agent (not possessing the immunomodulatory effects of interferon) may interfere with the pathogenesis of MC vasculitis and effectively impact the development of the immune-mediated injury when the immune disorder is triggered. Therefore, RTX will probably maintain a role in the treatment of the most severe forms of cryoglobulinemic vasculitis.

Limitations

The main limitation of our study resides in its open, non-blinded nature. This is counterbalanced by the sample size and follow-up, that is, by the largest single-center cohort of patients with kidney involvement and by the longest post-RTX observation ever described.

Conclusion

RTX has definitely changed the therapeutic approach of cryoglobulinemic vasculitis, and ultimately the natural history of the disease itself. The cumulative probability of survival in patients with cryoglobulinemic nephritis was less than 60% at 5 years in 2 different large cohorts of patients with cryoglobulinemic vasculitis [46, 47] . Most deaths, especially in the pre-RTX era [46] , were due to liver failure and infections. Despite comparable severity, the present cohort of RTX-treated patients showed a 5-year survival rate of 75%, and a 60% probability of remaining symptom-free for 10 years without any therapy after a single '4 + 2' infusion cycle, while the probability of remaining symptom-free 5 years after relapsing was of 80% if similarly treated. The only cause of death we observed was a cardiovascular event, indicating that RTX is not associated with an increased prevalence of severe infections or liver failure, at least in our cohort. Ideally, B-cell depletion provides the immune system with a new chance for proper regulation of emerging autoreactive B-lymphocytes and restoration of tolerance.

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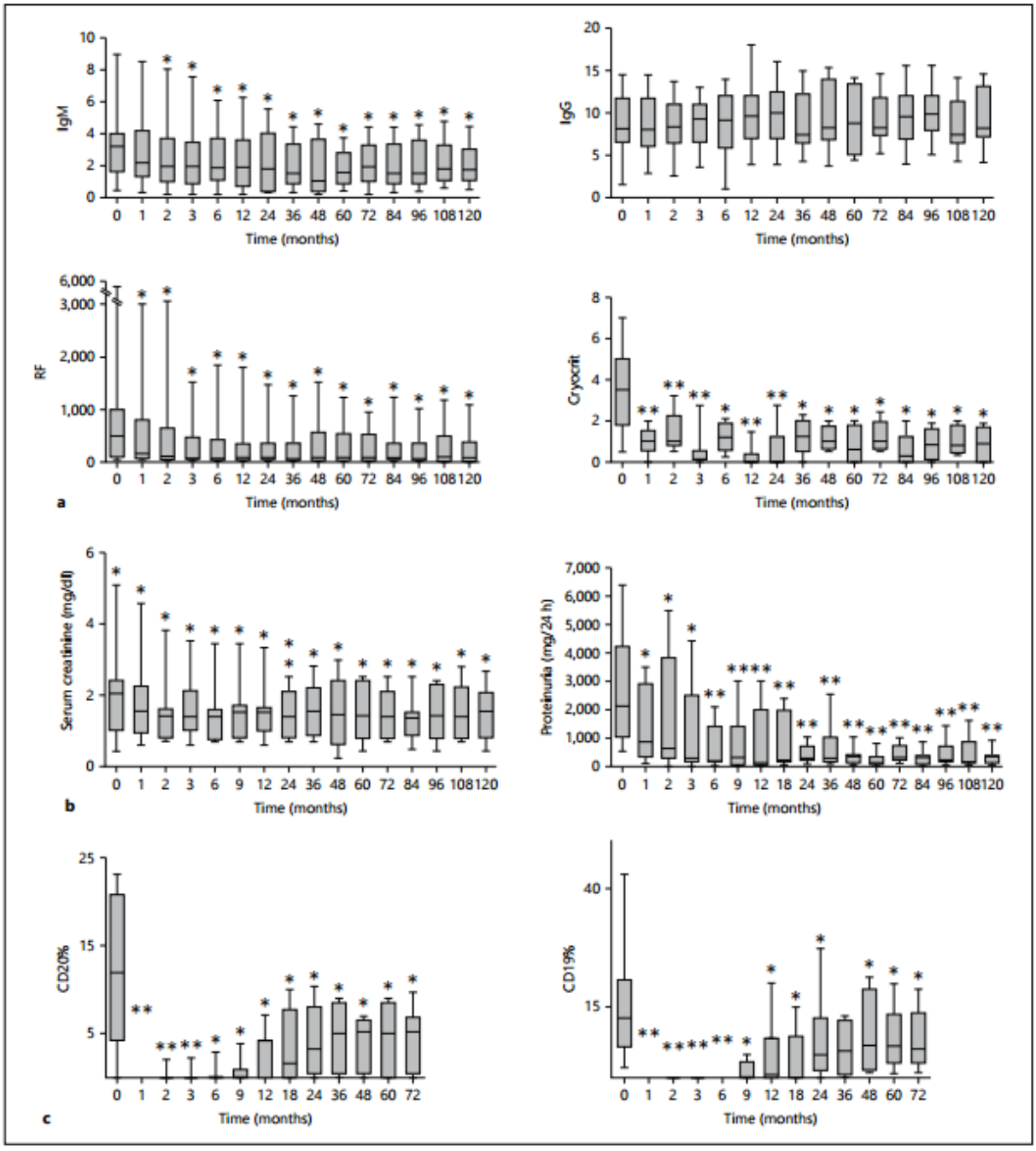

Fig. 1. a Serologic profile of the patients with severe MC treated with RTX. IgM, IgG, RF and cryocrit were evaluated at 0, 3, 6, and 12 months and then yearly. * $p < 0.05$; ** $p < 0.01$. b Serum creatinine and 24-hour proteinuria were evaluated at 0, 3, 6, 9, and 12 months and then yearly. * $p < 0.05$; ** $p < 0.01$. c Circulating B cells in the peripheral blood were investigated by the detection of CD20+ and CD19+ B cells and analyzed by flow-cytometry at baseline, months 1, 2 and every other month thereafter. * $p < 0.05$; ** $p < 0.01$.

	Baseline	Month 6	Month 12	End of follow-up (mean 72.47±18.3)	p value*
Age, years, mean ± SD	59.8±11.3	–	–	–	–
Sex, M/F	13/18	–	–	–	–
Type of cryoglobulinemia, n (%)	29 (94)				
Type II	2 (6)				
Type III					
HCV positive, n (%)	27 (87)				
HCV genotype**					
1a	1 (4)				
1b	15 (55)				
2a/2c	8 (30)				
3a	3 (11)				
Membranoproliferative glomerulonephritis, n (%)	16 (52)				
Peripheral neuropathy, n (%)	26 (84)	–	–	–	–
Large skin ulcers, n (%)	7 (23)	–	–	–	–
Serum creatinine, mg/dl	2.1±1.7	1.6±1.7	1.5±2.0	1.6±2.2	0.031
Proteinuria, g/24 h	2.3±2.1	0.9±1.5	0.6±1.9	0.8±0.7	<0.01
Cryocrit, %	4.6±1.1	1.2±0.7	0.6±0.9	0.9±1.1	0.037
ESR, mm/h	67±11.3	31±12.4	29±11.7	26±15.4	0.041
C3, mg/dl	125.7±60.8	131.2±56.6	139.3±57.2	142.7±62.6	NS
C4, mg/dl	5.6±7.4	15.3±7.9	16.7±9.0	20.3±8.0	0.021
RF, UI/ml	533±1,245	222±342	175±256	198±322	0.017
IgM, mg/dl	557±126	211±79	241±95	178±102	0.026
ALT, IU/l	44.7±13.5	41.3±10.0	39.7±12.6	40.6±11.9	NS
HCV RNA, IU/ml × 10^5	5.7±3.5	3.1±2.1	2.2±1.1	1.6±1.6	0.04

Values are expressed as mean ± SD; ESR = Erythrocyte sedimentation rate; NS = not statistically significant.
* For comparison of variables at baseline and follow-up, Student's t test was used for normally distributed parameters, and the nonparametric Mann–Whitney test for non-normally distributed parameters.
** Percent are computed on HCV+ patients only.

Table 1. Laboratory findings at baseline and after therapy with RTX (at 6, 12 months, last follow-up visit)

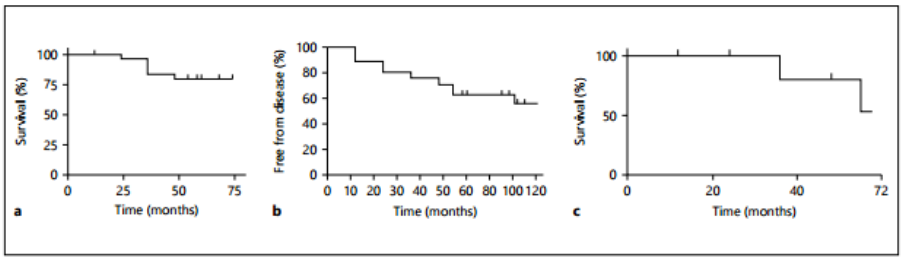

Fig. 2. a Kaplan–Meier curve showing survival after treatment with RTX. The present cohort showed a survival rate of 75% at 6 years. b , c Kaplan–Meier curve showing time free from disease after the first RTX treatment and after the 2nd cycle, respectively. Censured data refer to patients who died due to other reasons rather than MC, specifically cardiovascular cause (6 patients with a mean age 75.3 ± 6.9 died after a median 55 months since RTX therapy).