Introduction

Type II mixed cryoglobulinaemia (MC) syndrome is a systemic vasculitis mainly mediated by immune complexes [1]; hepatitis C virus (HCV) infection is strongly associated with MC, and it is considered the triggering factor of the disease, when present [2]. Rheumatoid factor (RF)-positive B-cell clonal expansion leading to cryoglobulin production represents a key pathogenic event in type II MC and may be an important therapeutic target as demonstrated by recent preliminary studies with B-cell depletion strategies [3–5]. The typical clinical triad of MC syndrome is characterized by fatigue, arthralgias and purpura [1], while nephritis is one of the most important clinical features affecting the mortality of the MC patients [6]. In addition, persistent viral infection stimulates the B-cell clones proliferation suggesting the role of HCV in the pathogenesis of both the MC syndrome and the related lymphomagenesis [7–9]. About 5% of type II MC patients develop a malignant B-cell lymphoproliferative disorder (LPD) [6, 10], but risk factors for such evolution are still unknown.

The end point of this study was to investigate whether bone marrow (BM) B-cell clonal expansion may influence the clinical pattern of MC syndrome. The issue of BM clonal B-cell expansion may influence the disease duration was 6 yrs (range 1–26) and the mean follow-up after BM analysis was 2.65 yrs (S.D. = 1.33). Peripheral neuropathy was present in 33 patients (60%), nephritis in 14 (25.4%), skin ulcers in 14 (25.4%) and lymphoma or atypical lymphoproliferative disorder (LPD) in 17/55 (30.9%). Anti-HCV antibodies were found in 43/55 patients (78.2%). BM B-cell expansion was evaluated by a semi-nested PCR amplification of the V–D–J region of the IgH genes.

Results. A clonal B-cell expansion in the BM was found in 33/55 (60%) patients, while a polyclonal pattern in 22/55 (40%). A BM pattern of clonal B-cell expansion increased the risk of nephritis of about 10 times [odds ratio (OR) = 10.11, CI95%1.52–67.31], if compared to a polyclonal pattern. In contrast, the risk of skin ulcers was decreased in BM clonal cases (OR = 0.09, CI95%-0.02–0.49). Overt lymphomas did not emerge from patients with BM monoclonal expansion (without clinical or histopathological features of lymphoproliferation; or with LPD) in a short-term, consistent with the finding that monoclonality was associated with nephritis and not with an underlying, not recognized lymphoma.

Conclusion. BM clonal B-cell expansion is associated with nephritis in MC syndrome. Particular B-cell clones may be preferentially expanded and may play a pathogenic role in MC nephritis.

Key words: Mixed cryoglobulinaemia, B-cell, Bone marrow, Nephritis, Lymphoma, Clonality.

Patients and methods

Patients

Stored BM biopsy specimens and bone marrow blood samples from 55 patients with type II MC syndrome, all referred to the Rheumatology Clinic of the University of Udine, Italy, were analysed (Table 1). These were 42 women (76.4%) and 13 men (23.6%), with a mean age of 61 yrs (median 64, range 24–82). The mean disease duration was 7.9 yrs (median 6, range 1–26). Patients’ follow-up after the collection of the BM biologic samples was available in 46 patients and ranged from 4 to 66 months [mean ± S.D.: 32 ± 16 months]. Four patients had Sjögren’s syndrome according to the American–European consensus criteria [11]. Thirty-three patients had peripheral neuropathy (60%; sensory and/or motor peripheral nerve disturbances, confirmed by electrophysiological study), 14 had renal involvement (25.4%; reduced glomerular filtration rate corrected for age in 9/14; and biopsy-proven glomerulonephritis in 10/14) and 14 had skin ulcers (25.4%). LPDs were observed in 17 out of 55 patients (30.9%). In particular, an atypical BM non-malignant LPD, as defined previously [8, 12], was found in eight patients. Among the remaining nine patients, four had a B-cell lymphoplasmocytoid lymphoma, all with BM infiltration, four with a salivary mucosa-associated lymphoid tissue (MALT) lymphoma (two of them had Sjögren’s syndrome with MC syndrome) with BM infiltration in 2/4 and one with a nodal marginal zone/mono/lymphocytoid lymphoma with BM infiltration. Sera obtained at the time of MC diagnosis were positive for anti-HCV antibodies in 43 out of 55 patients (78.2%) [by enzyme-linked immunosorbent assay (ELISA)] (HCV 3.0; Ortho Diagnostic System, Raritan, NJ, USA) and by recombinant-based immunoblot assay (Chiron RIBA 2nd generation; Ortho Diagnostic System), as well as for HCV-RNA [13] (Table 1). HCV genotype was also studied [9]. Serum cryoglobulins were determined as described [7], and classified according to Broquet et al. [14]. All the patients signed an informed consent related both to the invasive
procedure (BM biopsy) and to the inclusion in the study (personal data management and genetic analyses).

The time of viral exposure was not known. HCV genotype 1 was present in 19/43 (44.2%), genotype 2 in 13/43 (30.2%), genotype 3 in 4/43 (9.3%), genotype 5 in 1/43 (2.3%); in the remaining six patients the HCV genotype was not determined. Previous hepatitis B virus infection was documented in the seven patients, with six being antiHBsAg antibody-positive and only one patient being serum HBsAg positive and antiHBsAg antibody-negative: four of these patients showed concomitant HCV infection.

No patient had been treated with anti-CD20 monoclonal antibody therapy before BM sampling. Nineteen patients (19/43, 44.2%) had been previously treated with interferon for HCV without virus clearance in any case.

**Histopathological analysis**

All of the BM biopsy specimens were evaluated by a reference pathologist. BM specimens were fixed in 10% buffered formalin for 24 h, decalcified in EDTA for 2 h, and embedded in Paraplast or Erbaplast at 57°C. Three micrometre-thick sections were cut and stained for routine histological study, with haematoxylin and eosin, Giemsa, periodic acid-Schiff and Gomori silver impregnation for reticulin fibres. Immunophenotypic analysis was performed with anti-B-cell and anti-T-cell antibodies, and by the alkaline phosphatase-anti-alkaline phosphatase technique [15]. The histological diagnoses were formulated according to the Revised European-American Lymphoma Classification [16]. The atypical non-malignant LPDs were defined as previously described [8, 12].

**Molecular analysis of B-cell BM pattern**

BM B-cell expansion was evaluated in BM needle aspiration samples by semi-nested polymerase chain reaction (PCR) using an upstream primer directed to the third framework variable (V) region of the IgH gene and downstream primers directed to the joining (J) region [7, 17]. Samples were tested in duplicate, and the results were confirmed in repeated experiments. PCR products were analysed on 10% polyacrylamide gels stained with ethidium bromide. A clonal B-cell expansion was defined by the presence of discrete, reproducible narrow band(s) within the predicted size range, while a polyclonal pattern by a ladder of bands with similar intensities or by the presence of weakly dominant bands, not become reproducible in repeated amplification.

**Other molecular and functional studies**

Four cases of this series with HCV-related MC syndrome, two with lymphoma without nephritis and two with nephritis without lymphoma or LPD, all showing a clonal pattern of B-cell expansion in the BM, were further analysed, as reported (patients 1, 2, 3 and 8 in reference [9], corresponding to patients 1, 2, 3 and 12 in reference [18]). The IgM component of cryoprecipitate was purified and characterized by peptide mass fingerprinting. Results were integrated with an NCBI IgBlast data bank search and with the corresponding DNA sequences of the VDJ heavy and light chain immunoglobulin (Ig) rearranged genes of the BM clonally expanded B-cells (patients number 1, 2, 3, and 12 in reference [18]). The possible reactivity of the monoclonal RF-positive IgM also against HCV-genotype-specific NS3 protein was evaluated by ELISA in the same four patients, as reported (patients number 1, 2, 3, 8 in reference [9]). In this study, the results of the previous studies [9, 18] were re-evaluated by considering the renal or lymphomatous involvement in the corresponding patient. Sequence and functional analyses in additional cases were beyond the scope of this study.

**Statistical analysis**

The Shapiro–Wilk test was used to assess the normality of data distribution. To test clinical and serological differences related to BM B-cell expansion, we used chi-square or Fisher’s exact test for categorical variables, and the t or Mann–Whitney test for quantitative variables after we have verified the assumptions.

Stepwise logistic regression was used to assess whether BM B-cell clonal expansion (choosing as reference category the polyclonal pattern) may predict MC syndrome manifestations and MC-associated lymphoma. Age, sex, HCV infection, disease duration, serum cryoglobulin and C4 levels and RF were chosen as covariates to be included in the model. Data were analysed with SPSS software 13.1 version. Results were considered statistical significant when \( P \leq 0.05 \). The design of the work was approved by local Institutional Review Board.

**Results**

**Bone marrow pattern of clonality**

Clonal BM B-cell expansion was found in 33/55 (60%) patients (group 1) with one or two dominant bands noticed in 32/33 and three reproducible dominant bands in the remaining case, while a polyclonal pattern was documented in the remaining 22 patients (40%; group 2). The two groups were not significantly different for age, sex distribution, disease duration, HCV positivity (Table 2). In the 43 patients (78.2%) with HCV infection, the HCV genotype was also not significantly different between groups.

Among the 19 patients previously treated with interferon, all being HCV-RNA-positive at the time of BM analysis, 14 (73.7%) showed a BM clonal expansion and 5 a polyclonal pattern. No pre-interferon BM sample was available to verify the effect of the antiviral therapy on B-cell BM expansion in these cases.

**Clinical correlations**

**Nephritis.** Renal involvement was recorded in 14 patients; clonal BM B-cell expansion was found in 12/14 (85.7%). The difference between the rate of nephritis among the patients with BM clonal pattern and the patients with BM polyclonal pattern was statistically significant (12/33 vs 2/22, respectively; \( P = 0.023 \), chi-square test) (Table 2). When excluding the patients with lymphoma and BM involvement, the difference between the rate of nephritis among the patients with BM clonal pattern and the patients with BM polyclonal pattern remained statistically significant (11/27 vs 2/22, respectively; \( P = 0.013 \), chi-square test) (Fig. 1A).

Stepwise logistic regression showed that nephritis was associated with clonal BM B-cell expansion [odds ratio (OR) = 10.11, CI95% 1.52-67.31] and with age [OR = 0.93, CI95% 0.86-0.99]. Thus, having a clonal BM B-cell expansion increases nephritis risk 10 times with respect to a BM polyclonal pattern. Furthermore, a higher age implies a lower nephritis risk.

**Neuropathy.** Peripheral sensory and/or motor neuropathy was documented in 33 patients; clonal BM B-cell expansion was observed in 16/33 patients (48.5%). The difference between the rate of neuropathy among the patients with BM clonal expansion and the patients with BM polyclonal pattern was statistically significant (16/33 vs 17/22; \( P = 0.033 \), chi-square test) (Table 2). Stepwise logistic regression also showed that neuropathy was associated with BM B-cell expansion [OR = 0.21, CI95% 0.06-0.78]. Thus, having a clonal BM B-cell expansion decreases neuropathy risk with respect to a BM polyclonal pattern. However, when excluding the patients with lymphoma and BM involvement, the difference was not statistically significant (15/27 vs 17/22, Chi-square test) (Fig. 1B).
Skin ulcers. Skin ulcers were documented in 14 patients; BM monoclonal pattern of clonality was observed in 4/14 patients (28.6%). The difference between the rate of skin ulcers among the patients with BM clonal population and the patients with BM polyclonal pattern was statistically significant (4/33 vs 10/22; \( P = 0.005 \), Chi-square test) (Table 2), and this result was confirmed also after excluding from statistical analysis patients with lymphoma and BM infiltration (3/27 vs 10/22; \( P = 0.007 \), chi-square test) (Fig. 1C).

Stepwise logistic regression also showed that skin ulcers were associated with BM B-cell expansion (OR = 0.09, CI_{95%}=0.02–0.49). Thus, having a clonal BM B-cell expansion decreases the risk to have skin ulcers.

Other MC syndrome manifestations. No statistically significant differences between the two groups were found as concerns the other MC-related manifestations, such as purpura, arthralgias, fatigue, sicca syndrome, or Sjögren’s syndrome, also after excluding from statistical analysis patients with BM lymphomatous clonal involvement (Chi-square test, Table 2). Among the four Sjögren’s syndrome patients, one patient had BM B-cell clonal expansion (a case with stage IV MALT lymphoma), while three patients had BM polyclonal pattern (Table 2).

Bone marrow clonality and lymphoproliferative disorders. Seventeen out of 55 patients (30.9%) had a LPD (or a frank lymphoma). Eight patients showed an LPD, while nine patients had an overt lymphoma. In the first group, 6/8 patients had a BM B-cell clonal expansion, while 7/9 patients with lymphoma showed a BM B-cell clonal population. The two patients with lymphoma without BM clonal expansion were the only lymphoma patients without BM neoplastic infiltration and presented a salivary MALT lymphoma stage IE. The difference between the rate of LPDs was not statistically significant (Table 2).
The stepwise logistic regression also did not show any significant association of BM pattern of clonality with LPDs.

**Bone marrow clonality and laboratory features MC-related.** No statistically significant differences were shown in the two groups of patients for what concerns serum cryoglobulin concentration, RF and serum C4 levels. The stepwise logistic regression also did not show any significant association of BM pattern of clonality with MC-related laboratory features (Table 2).

Gene usage by expanded clones and reactivity of clonal cryoglobulinemic IgM. A monoclonal BM B-cell expansion was found in three out of the four HCV-positive patients studied (patients number 1, 2, 3 in reference 9; patients 1 and 3 with lymphoma, and patient 2 with nephritis). The remaining case with nephritis was oligoclonal (patient 8 in reference [9]). The Ig gene rearrangement resulted as follows: heavy chain V1-2/D2-15/J4m plus light chain V3-15/J1 and heavy chain V3-7/D3-22/J4 plus light chain V3-15/J1 for the two lymphoma patients; heavy chain V1-69/D3-22/J4 plus light chain V3-20/J1, and heavy chain V4-59 and light chain V3-20, V3-15, V1-2 for the two patients with nephritis [9, 18]. Notably, while monoclonal IgM isolated from the cryoprecipitate showed RF reactivity in 4/4 (as expected), anti-HCV NS3 protein reactivity was strong in only two cases, i.e. the two cases with lymphoma, while in the remaining two nephritic cases without lymphoma the anti-NS3 reactivity was very weak or absent.

**Bone marrow clonal expansion and lymphoma development.** Follow-up was available in 16 of the 19 patients showing a BM B-cell clonal population without clinical or histopathological features of lymphoproliferation, i.e. frank lymphoma or LPD. None of these 16 patients developed an LPD or an overt lymphoma in a period of 6-61 months of follow-up (mean ± s.d.: 29.9 ± 16.4 months), after the collection of the BM sample analysed, and during a mean disease duration of 9.4 yrs.

In addition, no lymphoma development was noticed also in the six patients with BM monoclonal pattern and with LPD, in a period from 7 to 60 months (mean ± s.d.: 34 ± 17.6 months) of follow-up after BM analysis, and during a mean disease duration of 8.5 yrs.

The stepwise logistic regression did not show any significant association of BM pattern of clonality with lymphoma development.

**Discussion**

MC syndrome is an immuno-complex-related systemic vasculitis characterized by skin involvement, peripheral neuropathy, glomerulonephritis and a number of other manifestations [1]. It is associated with a higher incidence of lymphomas, generally B-cell lymphomas [10]. HCV is the well-known infection underlying most MC cases [2]. Renal failure due to glomerulonephritis, chronic hepatitis with cirrhosis, widespread vasculitis and B-cell NHL are the main causes of death of MC patients [6].

This is the first study where the pattern of B-cell clonal expansion in the BM has been studied in a large population of patients with MC, and related to the clinical and serological disease manifestations. With the limitation of the retrospective design of the present study, our results suggest that the different patterns of B-cell expansion observed in the BM are associated with different clinical pictures of the MC syndrome. Renal involvement resulted associated with BM B-cell clonal expansion, while skin ulcers and peripheral neuropathy appeared inversely related to the BM clonal pattern. However, when statistical analysis was performed by excluding the seven patients with lymphoma and BM infiltration (all with BM B-cell clonal expansion), the BM clonal population directly correlated with renal involvement, while it was inversely related only with skin ulcers, and not with peripheral neuropathy. When considering the key serological markers of MC (i.e. serum cryoglobulins, RF and C4 levels), no significant differences between patients with BM clonal and polyclonal pattern of B-cell expansion were noticed.

These findings highlight a relationship between the development of nephritis and the overexpansion of particular B-cell clones in MC syndrome, and are consistent with a key pathogenetic role of immune complexes in MC nephritis. Previous studies have demonstrated, in general, that the B-cell receptor repertoire expressed by B cell in MC syndrome is not random, V1-69/V3-20 heavy chain/δ-light chain gene combination being one of the most used [19]. The presence of a BM clonal population in MC patients with nephritis could be strictly related to the production of peculiar Ig and to the formation of particular immune-complexes, due to specific gene sequences (resulting not only from a biased Ig gene rearrangement, but also from additional sequence characteristics, e.g. due to random extra-nucleotide insertions or somatic mutations). The absence of significant differences in the serological markers of MC syndrome between the two subsets of BM B-cell expansion may then reflect a qualitative, rather than a quantitative biological difference between cryoglobulinemic patients. Notably, two HCV-positive patients of this series with glomerulonephritis were successfully treated with rituximab after BM analyses (patients number 1 and 3 in reference [5]), and bone marrow analyses were repeated after 6 months. The disappearance of BM dominant bands after treatment was noticed, though in the lack of BM B-cell depletion [20]. Previous studies showed the morphological disappearance of the BM monoclonal infiltrate in HCV-related MC patients responding to α-interferon [21], possibly due to the removal of the key antigenic trigger in this case. Thus, two very different treatment approaches targeting either the viral trigger or the autoreactive B cells may prove both effective in MC syndrome [22].

The inverse association between BM clonal expansion and skin ulcers may be consistent with immune complex-mediated damage not implying the preferential expansion of peculiar B-cell clones and/or with the simultaneous, relevant role of other pathogenetic mechanisms. While immune complex-mediated damage is again supported by the efficacy of B-cell depletion for both MC-related skin ulcers and neuropathy [3, 4], the role of T-cell-mediated neural and cutaneous damage has been also stressed [23, 24]. However, since neuropathy has been shown to be heterogeneous in MC syndrome [23], with a range of pathology extending from demyelination to necrotizing vasculitis, it cannot be definitely excluded that clonality may be associated with peculiar subsets of peripheral neuropathy in MC.

As expected, BM B-cell clonal expansion characterizes the patients with histological features of lymphoma localized in the BM, while the BM was uninvolved and polyclonal in 2/2 MC patients with salivary MALT lymphoma (stage IE). In contrast, when considering in a single group all the patients with BM histological features of abnormal lymphoproliferation (i.e. both LPD and lymphoma), a higher prevalence of B-cell clonal expansion, but not statistically significant, was documented (Table 2). This confirms that clonal expansion characterizes MC as a non-neoplastic, rather than a low-grade malignant B-cell disorder [7, 17].

The sequences of BM expanded clones and the functional properties of the IgM component isolated from the cryoprecipitate were evaluated in four cases with respect to the renal or lymphomatous involvement. Notably, the genes rearranged in the nephritic cases (VH1-69, VH4-59, V3-20) were the same usually employed by MC-related lymphomatous clones. This might imply that, even if a biased gene usage characterizes MC syndrome in
general, fine differences in antibody sequences and functional properties may mediate different clinical manifestations. Additional mechanisms (e.g. genetic polymorphisms or local tissue factors such as cytokines and BLyS) could also be involved [25, 26]. Secondly, cross-reactivity of the monoclonal IgM cryoglobulinaemic RF against HCV-NS3 antigen appeared much more evident in the lymphoma-related clones by very preliminary analyses. However, when also considering the lack of association between lymphoma and nephritis [10]; also noticed in the present series, data not shown), this issue is of major value and deserves additional research, since a deeper involvement of anti-HCV NS3 cross-reactivity for lymphoma development might be hypothesized.

Notably, a frank lymphoma did not emerge either from the patients who had a LPD or from the patients with a BM clonal expansion without histological features of LPD during a mean follow-up of almost 3 yrs after BM collection, and of about 9 yrs after MC syndrome diagnosis. These results confirm the link of BM clonality with nephritis rather than with an underlying lymphoma. Furthermore, they are again consistent with the concept that MC is a non-neoplastic disorder, despite the histopathological and immunophenotypic features of LPD in the BM, when present [7, 17]. Preliminary evidence that B-cell clonal expansion in the BM is not predictive of overt lymphoma evolution in the short-term is also provided in this study, in accordance with reported evidence of BM clonal cases not developing lymphoma after a long-term follow-up [7]. In a previous study, Vallat and colleagues [27] found 14/25 (56%) HCV-infected patients with type II MC showing a clonal B-cell expansion in the blood and/or liver: this percentage is very similar to BM clonal expansion reported in our series. In the study of Vallat et al. [27] 2/11 type II MC patients developed Waldenström’s macroglobulinaemia during follow-up. Ferri et al. [6] recently described a large population of 231 MC patients: 20 of them (8.6%) developed a B-cell lymphoma after an average of 6 yrs of follow-up. Thus, a longer follow-up is required in the present series to reach conclusions for the long-term prognosis for MC for what concerns lymphoma evolution in patients showing a different pattern of BM clonal expansion, since baseline BM biopsy at disease onset was not available. However, the median MC disease duration was 8.5 yrs in this study.

In conclusion, BM B-cell clonal expansion is associated with renal involvement in MC syndrome. Overexpansion of particular clones producing specific Ig may be then implicated for the development of MC nephritis, consistently with the efficacy of B-cell depletion in MC nephritis already observed [5, 28]. Nephritis and B-cell lymphoma appear ‘clonal’ features in the course of HCV-related MC syndrome, although chronic HCV infection may induce such manifestations by means of different pathogenetic mechanisms [22, 29].

**Rheumatology key messages**

- BM B-cell clonal expansion is a key feature of nephritis or lymphoma in MC syndrome.
- Overexpansion of particular clones producing specific Ig may be implicated in MC nephritis.
- BM B-cell clonal expansion seems to be not predictive of overt lymphoma evolution in MC syndrome.

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**References**


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