Lymphoma-like monoclonal B-cell lymphocytosis in a patient population: biology, natural evolution and differences from CLL-like clones

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Running title: Clinical lymphoma-like MBL: biology and outcome
Abstract

High-count monoclonal B-cell lymphocytosis (MBL) with a chronic lymphocytic leukemia (CLL)-phenotype is a well-known entity, featuring 1-4% annual risk of progression towards CLL requiring treatment. Lymphoma-like MBL (L-MBL), on the other hand, remains poorly defined and data regarding outcome are lacking. We retrospectively evaluated 33 L-MBL cases within our hospital population and compared them to 95 subjects with CLL-like MBL (C-MBL). Diagnoses of L-MBL were based on asymptomatic B-cell clones with Matutes score <3, B-cells <5.0 x 10³/µl, and negative computerized tomography scans. We found that median B-cell counts were considerably lower compared to C-MBL (0.6 vs 2.3 x 10³/µl), and remained stable over time. Based on immunophenotyping and immunogenetic profiling, most L-MBL clones did not correspond to known lymphoma entities. A strikingly high occurrence of paraproteinemia (48%), hypogammaglobulinemia (45%) and biclonality (21%) was seen, these incidences being significantly higher than in C-MBL (17%, 21% and 5%, respectively). Unrelated monoclonal gammopathy of undetermined significance was a frequent feature, as the light chain type of 5/12 paraproteins detected was different from the clonal surface immunoglobulin. After 46 months median follow-up, 2/24 patients (8%) had progressed towards indolent lymphoma requiring no treatment. In contrast, 41% of C-MBL cases evolved to CLL and 17% required treatment. We conclude that clinical L-MBL is characterized by pronounced immune dysregulation and very slow or absent progression, clearly separating it from its CLL-like counterpart.

Keywords: monoclonal B-cell lymphocytosis, chronic lymphocytic leukemia, non-Hodgkin’s Lymphoma, MGUS, immunophenotyping
Introduction

Monoclonal B-cell lymphocytosis is defined as a small monoclonal B-cell population (B-cells <5.0 x 10^3/µl) present in the peripheral blood of persons without B-symptoms (eg. weight loss and night sweating), autoimmune phenomena, lymphadenopathy or organomegaly. Three types of MBL can be distinguished based on the expression of CD5 and CD23: CLL-like MBL (C-MBL), by far the most frequent form, with CD5 and CD23 positivity, atypical CLL-like MBL (CD5+, CD23-) and non-CLL-like MBL (CD5-) [1]. However, recent data have shown that an indolent form of mantle cell lymphoma exists, which may account for a substantial percentage of MBL cases with a CD5+, CD23- phenotype [2]. It is therefore more logical and practical to group this subtype together with the non-CLL-like population, under the common name of non-Hodgkin lymphoma-like MBL (L-MBL) [3].

The outcome of C-MBL mainly depends on the B-cell count at diagnosis. When B-cell counts are below 0.5 x 10^3/µl, also termed low-count MBL, progression is rare. These clones are typically found when screening healthy subjects (‘screening’ MBL) [4]. High-count C-MBL, frequently found when investigating lymphocytosis (‘clinical’ MBL), involves a risk of evolution to CLL requiring therapy of about 1-4% per year [5-8]. However, the 0.5 x 10^3/µl cutoff has only been validated for C-MBL, and thus cannot be readily applied to L-MBL.

While the characteristics of C-MBL are well documented, L-MBL remains largely unexplored. Studies focusing on small non-CLL-like clones are scarce, and most do not mention medical imaging as a way of ruling out B-cell non-Hodgkin lymphoma (B-NHL) and/or include subjects with B-cell counts above 5.0 x 10^3/µl [9-14]. We aimed to accurately define and characterize a number of clinical L-MBL cases, thus gaining a better insight into the biology of L-MBL. We reviewed clinical, immunophenotypic and immunogenetic characteristics at diagnosis, and evaluated the natural history of these patients. A comparative analysis with C-MBL subjects was also performed.
**Methods**

**Study population**

The study was approved by the Ethics Committee of the University of Brussels. We retrospectively analyzed the flow cytometric database of our laboratory at the University Hospital of Brussels between 2000 and 2016. Cases of L-MBL were identified using the criteria for MBL as defined by Shanafelt et al [1]. Cases with a Matutes score ≥3 were excluded. All clinical and radiological data available in the patient’s data sheet, were examined. Patients with a diagnosis of B-NHL, or in whom computerized tomography scanning (chest and abdomen) was not performed, were omitted. We excluded all cases with important cytopenia (hemoglobin <10g/dl, platelets <100 x 10^3/µl or absolute neutrophil count <0.8 x 10^3/µl) that could not be explained by another pathological condition in the patient, unless a BM specimen had clearly shown that cytopenia was not caused by important lymphocytic invasion. C-MBL patients identified in the same time frame were also studied. All met the criteria for MBL and had B-cell clones with Matutes score ≥3.

**Immunophenotyping**

Three-color flow cytometric analysis was performed using standard methods [15] on a Beckman Coulter XL (before April 2009) or FC500 instrument (after April 2009; Beckman Coulter, Miami, FL) using the following multicolor antibody stainings: CD45-phycocerythrin cyanin 5.1 (PC5)/CD3-fluorescein isothiocyanate (FITC)/CD19-phycocerythrin (PE); CD19-PC5/kappa-FITC/Lambda-PE; CD19-PC5/immunoglobulin(Ig)M-FITC/IgD-PE; CD19-PC5/IgG-FITC/IgA-PE; CD19-PC5/isotype control(IC)-FITC/IC-PE. When a B-cell clone was detected (i.e. a skewed κ/λ ratio of >3 or <0.3, or abnormal clustering in κ or λ) the following antibody stainings were added: CD19-PC5/CD5-FITC/CD23-PE; CD19-PC5/CD20-FITC/CD10-PE; CD19-PC5/FMC7-FITC/CD22-PE; CD19-PC5/CD103-FITC/CD25-PE; CD19-PC5/CD79b-FITC/CD11c-PE; CD19-PC5/CD24-FITC/CD138-PE; CD19-PC5/CD5-FITC/CD38-PE and CD19-PC5/CD43-FITC. For each measurement, 2.5 x 10^3 B-cell events were collected. Fluorospheres (Dako, Glostrup, Denmark) and SPHERO Calibration Particles (Spherotech,
Lake Forest, Illinois, USA) were used to monitor the fluorescence detection voltages and the fluorescence linearity, respectively. The SPHERO particles were also used to harmonize the Beckman Coulter FC500 with the XL instrument, leading to identical results on both machines.

**Serum immunoglobulin analysis**

Serum protein electrophoresis was performed by capillary electrophoresis (Capillarys II, Sebia, Norcross, GA, USA). When a paraprotein was detected, immunofixation electrophoresis (Hydragel, Sebia) was used for isotype identification. Hypogammaglobulinemia was defined as a decrease in the gamma zone below 8 g/l (locally defined reference range). Cases with a paraprotein together with normal or increased gamma globulin levels were considered uncertain of having hypogammaglobulinemia, and were removed from data analysis.

**Histopathology and immunohistochemistry**

Bone marrow trephine biopsy specimens were routinely embedded in paraffin, and the original diagnostic sections were used for this study. The sections were stained with hematoxylin and eosin to evaluate morphologic features. Staining for CD79a was performed in all biopsies. CD20, CD45 and CD3 antibodies were used in most cases, at the discretion of the pathologist.

**Analysis of IGHV gene rearrangements**

DNA-PCR amplification and sequencing of IGHV gene rearrangements was performed following an established protocol, as previously described [16]. The international immunogenetics information system (IMGT) database and the IMGT/V-QUEST tool were used to analyze the sequence data. Somatically mutated sequences with >3% deviation from the germline sequence were considered significantly mutated [17].

**Statistical analysis**
Nonparametric methods were applied because most parameters were not normally distributed. Median value and range were determined for each parameter. To assess differences in continuous data, the Wilcoxon signed-rank test was used for paired samples, and the Mann-Whitney U test for independent samples. The Chi-squared test was used to compare nominal variables in independent samples.

Results

Patient characteristics

By reviewing our flow cytometry records, 33 patients could be classified as having L-MBL (Table 1). None presented with B-symptoms or autoimmune phenomena. Lymphadenopathy and splenomegaly were undetectable by clinical examination, and absent on computerized tomography scans in all patients. Most L-MBL cases were referred for a hematological workup because of mild lymphocytosis, anemia or presence of a paraprotein. A negative hepatitis C serology was seen in all cases with available data (11/33). In the same time period, 95 C-MBL patients were identified. Basic laboratory and clinical characteristics of both groups are shown in Table 2. In addition, we identified five patients with both a L- and C-MBL clone. These were not included in either cohort, and are discussed separately. In five L-MBL patients a hemoglobin value <10 g/dl was present at diagnosis but could be clearly attributed to a persistent infectious process, and normal values were seen before start of the infection. For the same reason, high maximum platelet, leukocyte and neutrophil counts were noted in our study population (Table 2). One patient had an absolute neutrophil count <0.8 x 10³/µl which was accredited to coexistent chronic myelomonocytic leukemia.

Serum immunoglobulin analysis

A paraprotein could be detected by serum protein electrophoresis in 16/33 L-MBL patients (Table 1). In most cases the size of the monoclonal peak was too small for accurate quantification. Immunofixation was successfully performed in 10/16 patients with a monoclonal gammopathy, and detected twelve paraproteins. In 5/12 paraproteins (5/10 patients) the light chain did not match the surface immunoglobulin (sIg) on the
monoclonal B-cells. Four of these unrelated paraproteins were of the IgG isotype. In the five remaining patients with available data, both the paraprotein heavy and light chain were of the same type as the sIg. Monoclonal gammopathy was significantly less prevalent in C-MBL cases (Table 2). The paraprotein light chain was different from the sIg light chain in six out of thirteen C-MBL patients with available immunofixation data. A discordance in the heavy chain isotype was observed in four of the other seven C-MBL cases with available data. Each of these had a B-cell clone with IgMD kappa expression on the surface, and an associated IgG kappa paraprotein.

Out of 22 L-MBL cases with available data, ten had hypogammaglobulinemia which was significantly more than in C-MBL (Table 2).

Bone marrow examination
A BM biopsy was available in 12 patients. In 6 cases there was no clear infiltrate detectable; all had a B-cell count <1.0 x 10^3/µl. An interstitial infiltration of scattered cells could be seen in 5/6 other cases, and in one patient a paratrabecular pattern of invasion was observed (Fig. 1). All six patients with clear invasion presented with >1.0 x 10^3/µl B-cells. Flow cytometric data analysis could demonstrate BM infiltration by the monoclonal B-cell population in all 14 patients with available data, with a maximum invasion of 20% relative to the nucleated cell population.

Genetic analysis
Thirteen monoclonal Ig rearrangements were found in 12 patients with available data (Table 1). Eleven clones (85%) used IGHV3- and IGHV4-family genes, and an equal proportion (11/13) had significantly mutated IGHV genes.

Flow cytometric analysis at diagnosis and during follow-up
Thirty-six L-MBL clones were detected in 33 patients (Table 1). At diagnosis, the median B-cell count was 0.6 x 10^3/µl which was significantly lower than in C-MBL patients (Fig. 2a). The percentage of monoclonal B-cells among the total B-cell population was 80% (median value; range 13-100%). Twenty-one monoclonal populations (64%) presented with
kappa light chain restriction. Two clones had coexpression of μ and γ heavy chains, and sIg was not detectable in one case. An immunophenotype not specific for one of the WHO lymphoma entities was observed in 28/36 cases (78%). Eight clones had a more distinct lymphoma phenotype. Three resembled the phenotype of splenic B-cell lymphoma/leukemia unclassifiable (SLLU), encompassing hairy cell leukemia-variant and splenic red pulp lymphoma, overlapping entities with similar immunophenotypic features [18]: CD25- and CD103+ or a combination of CD11c, bright CD22 and coexpression of μ and γ heavy chains. A mantle cell lymphoma-like phenotype (CD5+, CD23-, CD11c- and sIgMD+) was seen in two cases. Two clones had a follicular lymphoma-like phenotype (CD10+), while one was suggestive of a LPL phenotype (IgM+, IgD-, CD25+ and CD38+).

Due to the retrospective nature of this study, it was not possible to perform additional molecular testing in order to better characterize these cases.

Flow cytometric follow-up data were available in 19 patients with a median follow-up time of 41 months. B-cell counts at follow-up were not significantly different from the values at diagnosis, contrasting with results in C-MBL (Fig. 2b). All clones persisted, and two L-MBL patients presented with more than 5.0 x 10^3/µl B-cells at follow-up.

**Biclonal cases**

Eight out of 133 MBL (6%) patients had a second clone. Combinations of C-MBL and L-MBL were most common (n=5), and three patients had a double L-MBL clone. Subjects with two C-MBL clones were not seen. From these data we can derive that biclonality was present in 8/38 (21%) patients with a L-MBL clone and in 5/100 (5%) patients that harbored a C-MBL clone, which proved statistically different (p-value 0.004). Immunogenetic analysis was performed in two biclonal cases and usage of two different IGHV genes was demonstrated in both.

**Clinical follow-up**

Twenty-four L-MBL patients could be followed in time, with a median duration of 46 months (range 1-108 months). Twenty-one cases presented with a stable disease course, remaining without symptoms, clinically palpable lymph nodes or splenomegaly (Table 1).
Two patients (8%) progressed to lymphoma. At three years from diagnosis, patient #26 showed lymphocytosis with \(>5.0 \times 10^3/\mu l\) B-cells, and developed mild splenomegaly and lymphadenopathy. Because of age and a symptom-free presentation, treatment was not given. She died from acute heart failure four months after progression. A substantial B-cell infiltration of BM and lymph nodes was revealed by the autopsy. Immunohistological examination of the spleen disclosed a diffuse invasion of the red pulp leading to a SLLU diagnosis. Patient #3 evolved towards B-cell lymphocytosis above \(5.0 \times 10^3/\mu l\) while also developing axillary lymphadenopathy with indolent behavior. Up until present (3.5 years after progression) the patient is still untreated and in excellent condition, withholding the clinician from lymph node biopsy. The monoclonal gammopathy of undetermined significance (MGUS) detected in patient #9 (with light chain different from the sIg on the B-cells) progressed to multiple myeloma 17 months after diagnosis. Because of multiple comorbidities, curative treatment was not considered. In the C-MBL group, incidences of both progression and therapy-requiring disease were significantly higher (Table 2).

**Discussion**

MBL is a rather common finding amongst healthy individuals. Several large screening studies have disclosed a prevalence ranging from 0.6% to more than 20% in persons older than 60 years, depending on the sensitivity of the used technique [1]. In healthy blood donors, a prevalence of approximately 7% was observed [19]. The current consensus is a prevalence of 3-5% for persons older than 40 years [20]. For L-MBL, the prevalence varies around 1-2% in the overall population [21, 22]. Few data exist for high-count/clinical MBL. Rawstron et al. found 309 cases of C-MBL in 2228 subjects referred for lymphocytosis (13.9%), but the prevalence of clinical L-MBL was not mentioned in this study [5]. In our patient cohort, we found an L-MBL prevalence that was roughly three times lower compared to C-MBL, although this is biased due to the fact that 40 presumed L-MBL cases were omitted because of missing imaging studies.

A second MBL clone was detected in 21% of our L-MBL patients, in line with a previous report [23]. Important immunophenotypic differences between both clones were seen in all these cases, suggesting an unrelated origin. Two such patients in whom
immunogenetic analysis was performed, showed usage of two different IGHV genes, thereby reinforcing this hypothesis. Flow cytometric detection of two concurring CLL-like clones has rarely been reported [22, 24, 25], and was also not observed in our patient group. We presume that it is more difficult to identify because both clones will have an identical immunophenotype in most cases. However, by using molecular techniques, oligoclonality was detected in almost one-quarter of low-count C-MBL cases [26]. In contrast, CLL is mostly monoclonal [27]. It is postulated that, in the oncogenesis of CLL, one C-MBL clone achieves dominance over the others and progresses to clinical CLL [28]. Whether this also occurs in L-MBL is a topic for further study.

We detected a paraprotein in almost half of the L-MBL cases, which is considerably more frequent than in other B-cell lymphoproliferative disorders such as C-MBL (17%), CLL (4-20%) [29, 30] and B-NHL (17-28%) [31-33]. More striking was the discordance between the isotype of the paraprotein and the sIg on the clonal B-cells. From our data we can deduce that in nearly one-quarter of L-MBL patients a paraprotein is present with a Ig light chain that differs from the sIg, indicating coexistent MGUS. The prevalence of MGUS in the general elderly population has been estimated at 3.2% [34], and could therefore only account for about one eighth of the incidence in our L-MBL cohort. The true prevalence of MGUS in L-MBL could be even higher since paraproteins with light chains identical to the sIg might also be unrelated, but this would require sequencing analysis. Few data exist concerning the coincidence of MGUS and B-cell lymphoproliferative disorders because the relationship between the isotype of the paraprotein and the sIg has rarely been studied. Hansen DA et al. reported on this subject for HCL and CLL [35]. From their data we can deduce an MGUS prevalence of 8.5% and 6.5% in HCL and CLL, respectively. The latter contrasts with a more recent study, demonstrating a 100% light chain isotype concordance in 27 CLL patients with paraproteinemia [30]. In 63 clinical MBL patients, an unrelated MGUS was seen in two cases (3%) [14]. For one patient of our cohort it was clear that the paraproteinemia derived from a plasma cell clone, because of progression towards multiple myeloma with identical Ig isotype. This association between MBL and plasma cell dyscrasia has recently been documented. Using molecular techniques, a B-cell clone was found to be present in
16% of myeloma patients [36]. However, the association was less clear for MGUS, where only 2% had a detectable MBL. Genetic analysis showed that, in all these cases, the B-cell clone was unrelated to the plasma cell malignancy. It is speculated that both plasma cell and B-cell clones arise from cells developing in an underlying microenvironmental niche that promotes clonal evolution. The high frequency of biclonality and MGUS in L-MBL indeed suggests an oligoclonal diversification process. A possible explanation comes from the study of oligoclonality in C-MBL that has shown an antigen-initiated process in some subjects [28]. Of note, when comparing heavy chain isotypes between the paraprotein and the sIg, we noticed a perfect agreement in the non-MGUS L-MBL group, but a discordance in the C-MBL group for over half of the patients. The latter possibly reflects the potency of isotype-switching by CLL cells [37], or it might yet be another manifestation of oligoclonality.

The high prevalence of hypogammaglobulinemia in our C-MBL (21%) and especially L-MBL population (45%) is another remarkable finding, and could explain the elevated risk of infections associated with MBL [38]. A previous report found an occurrence of 29% in MBL patients [39], as opposed to another study reporting only 1% [40]. Differences in the phenotype (L-MBL/C-MBL) and the absolute B-cell count may contribute to these dissimilarities but this information was not available in the aforementioned studies. Hypogammaglobulinemia in other B-cell malignancies is rarely seen, except in CLL where it is common. Studies on CLL have put forward a diminished pool of normal CD5-negative B-cells as an explanation [41]. However, we could not find a correlation between the remainder of normal B-cells and the presence of hypogammaglobulinemia (data not shown). Alternatively, the nonclonal B-cells may be functionally inhibited by their clonal counterpart.

Ten out of 36 L-MBL clones had an immunophenotype that matched a WHO lymphoma entity with a (semi-)specific phenotype such as SLLU, LPL, follicular or mantle cell lymphoma, demonstrating the phenotypic heterogeneity among L-MBL. The majority however had a non-specific expression of surface antigens. By means of exclusion, one could postulate that these were either splenic marginal zone lymphoma (SMZL)-like or LPL-like clones. However, the immunogenetic profile did not match
either. None of the analyzed cases carried a clone with usage of the IGHV1-2*04 gene, and only two clones had a IGHV gene that was not significantly mutated. Both features are common in SMZL, being present in 31% and 51% of the cases, respectively [17]. As was already suggested by Xochelli et al., these non-CLL-like clones appear to have a better immunogenetic match to SLLU than to SMZL [12]. Also, histological examination of the spleen, available in one of our patients with a non-specific L-MBL, revealed a diffuse infiltration of the red pulp, diagnostic of SLLU rather than SMZL. The MYD88 L265P mutation, strongly associated with LPL [42], was detected in only 1/14 L-MBL cases (data not shown). In a recent report, this genetic abnormality was also found in 10/53 patients with a marginal zone-like clonal lymphocytosis (also involving clones > 5.0 x 10^3/µl) [43]. Although these data seem to suggest that most L-MBL clones are unrelated to either SMZL or LPL, they do not exclude it. An explanation can be derived from studies analyzing the Ig repertoire in C-MBL. Herein, investigators have disclosed a different immunogenetic profile in low-count C-MBL compared to CLL. Unmutated IGHV genes for example, present in 40-50% of CLL, were only seen in 30% of C-MBL. Likewise, the IGHV1-69 gene, present in 10-20% of CLL was not detected in C-MBL. Based on the genetic differences between both entities, it is hypothesized that only specific subgroups of low-count C-MBL progress to CLL [25]. Finally, it is conceivable that some L-MBL clones derive from an occult mucosa-associated lymphoid tissue lymphoma. Previous studies, however, have shown that this is a rather rare finding in patients with non-CLL-like lymphocytosis [12].

With regard to the natural course of L-MBL, Nieto et al. reported absent progression in 13 healthy subjects, with an average clone size of 0.2 x 10^3/µl, after one year of follow-up [23]. In 102 asymptomatic patients with a marginal zone-like B-cell clone, progression was seen in 17 cases [12], but this study also included patients with B-cell counts above 5.0 x 10^3/µl, making it difficult to compare data. In our cohort, we have shown that the clone size remained small in the majority of the patients, as reflected by very stable median B-cell counts. Conversely, C-MBL patients showed progressive B-cell counts in a significant number of patients (Fig. 2b). This finding could well explain the three-fold difference in B-cell counts between both groups at diagnosis (Fig. 2a). We did
not see any clone disappear during follow-up, contrasting with the study of Fazi et al. showing a non-detectable clone after three years in 33% of persons with a CD5-negative screening MBL [4]. However, the clone size in these cases was much smaller than in our cohort, and might explain its transient nature. During the first three years of follow-up, our C-MBL group presented with a 4.2% annual risk of evolution to CLL requiring therapy, comparable to what was reported by an Italian group [7], but considerably more frequent than in other studies [5, 6, 8]. Because of its natural course, annual follow-up by a hemato-oncologist is put forward as a guideline for management of clinical C-MBL. For clinical L-MBL, follow-up every 6-12 months is advised, although data regarding outcome were nonexistent until now [1]). Based on our results, documenting very indolent behavior, such a stringent follow-up may not be indicated.

In summary, we have shown that L-MBL patients often present with multiple B-cell clones and/or unrelated paraproteins, possibly reflecting an antigen-driven oligoclonal diversification process. Taken together with the high incidence of hypogammaglobulinemia, it suggests a dysregulated immune system in these subjects. The frequent finding of seemingly unrelated MGUS in L-MBL patients is intriguing as to its origin. It may prompt looking for clonal plasma cell populations, or utilizing more sensitive flow cytometry methods in order to detect smaller B-cell clones. Finally, the outcome of L-MBL appears very favorable compared to C-MBL, but from our data we could not extract criteria for patient follow-up. Prospective studies with larger cohorts will need to address this issue.

**Author Contributions**

SVM and KJ designed the study. SVM analyzed the data, and wrote the paper. BH and RS performed diagnoses. WR performed immunophenotyping analyses. MB and BM were responsible for molecular data analysis, and HDR for interpretation of pathologic findings. KJ supervised the study. BH, WR, MB, BM, HDR, RS and KJ reviewed drafts of the paper and approved the final version.
Compliance with ethical standards

Conflict of interest  The authors declare that they have no conflict of interest.

References


Figure legends

Fig. 1 Bone marrow pathology. Patterns of BM infiltration by mainly small B-cells displaying (a) an interstitial pattern of infiltration (CD79a antibody), and (b) a paratrabecular pattern of infiltration (CD20 antibody)

Fig. 2 B-lymphocyte counts in MBL. (a) B-cell counts at diagnosis. The whisker boxes show the extreme values (vertical bars), the low and high quartiles (rectangles) and the median values (horizontal bar); outliers are depicted with “+”; (b) B-cell counts in MBL cases with FU flow cytometric data. Results are expressed as median counts. nL-MBL=19, nC-MBL=35; median follow-up time was 41 and 30 months respectively *Statistically significant differences