Mast cell density and its clinical relevance in Waldenström’s macroglobulinemia

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Abstract
The presence of numerous mast cells (MCs) mixed with tumor cells in the bone marrow (BM) is a hallmark of the diagnosis of Waldenström’s macroglobulinemia (WM). MCs have been shown to support lymphoplasmacytic cell growth, but there is thus far no demonstration of the prognostic impact of BM MC density in WM. We investigated BM MC density by sensitive and specific digital quantification, allowing the analysis of a large area infiltrated by BM tumor cells. A total of 65 WM patients were investigated, including 54 at diagnosis and 11 at relapse. Tryptase and CD20 immunohistochemistry staining was performed on contiguous sections of deparaffinized BM trephine biopsies. After numerization of each section, the BM surface area was manually marked out.
excluding the bone framework and adipocytes to limit the analyses to only hematopoietic tissue. MCs were assessed using a digital tool previously used to quantify immune-cell infiltrates on tumor-sections. Deep next-generation sequencing and allele-specific PCR were used to explore the MYD88 and CXCR4 mutational status. MC density was heterogeneous among the WM patients. An optimal MC density threshold (> 56 MC.mm\(^{-2}\)) was defined according to ROC curve analysis of overall survival. A higher MC density (> 56 MC.mm\(^{-2}\)) was associated with greater BM involvement by WM lymphoplasmacytic cells and less hepatosplenic involvement (\(p = 0.023\)). Furthermore, MC density significantly correlated with a higher ISSWM score (\(p = 0.0003\)) in symptomatic patients. Patients with a higher MC density showed shorter median OS (56.5 months vs. nonreached, \(p = 0.0004\)), even in multivariate analysis after controlling for other predictive variables, such as age, ISSWM score, and CXCR4 mutational status. In conclusion, MC density can be accurately measured in WM patients using a specific digital tool on well-outlined hematopoietic tissue surfaces. High MC density is associated with aggressive features and a poor clinical outcome, emphasizing the need for further investigation of the involvement of MCs in the pathophysiology of WM.

**KEYWORDS**
mast cells, tumor biology, Waldenström’s macroglobulinemia

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### 1 | INTRODUCTION

Waldenström’s macroglobulinemia (WM) is characterized by lymphoplasmacytic infiltration of the bone marrow (BM), along with the presence of a serum monoclonal IgM. MYD88 L265P and CXCR4 mutations have been reported in > 90% and ≈ 25% of WM cases, respectively [1–6]. The MYD88 L265P mutation results in the constitutive activation of NFκB by Bruton’s tyrosine kinase and is thought to be a clonal driver mutation [1]. The CXCR4 mutations are mostly nonsense or frameshift mutations that occur most often in the S338 position. They result in truncation of the cytoplasmic portion of the receptor for the chemokine CXCL12, similarly to CXCR4 mutations seen in WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome. Such truncation leads to impaired internalization of the receptor after ligation and results in prolonged activation [5]. CXCR4\(^{WHIM}\) mutations are subclonal and associated with greater BM involvement, more symptomatic disease, and higher genomic complexity [6–8].

One historical hallmark for WM histological diagnosis is the presence of numerous mast cells (MCs) mixed with the tumor cells in the BM [9]. MCs are myeloid-derived cells that are widely disseminated throughout all tissues and act as sentinels of the surrounding environment. They store and can release a wide spectrum of biologically active mediators after activation, leading to their central role in allergic diseases. The presence of numerous MCs in human tumors is well described and their pleiotropic molecule production is thought to explain their pro-oncogenic roles (recently reviewed in [10,11]). In hematological malignancies, MCs have been shown to be associated with progression and poor (or even good) prognoses in various Hodgkin’s and non-Hodgkin’s lymphomas [12–19]. In WM, MCs have been shown to support lymphoplasmacytic cell growth through CD154/CD40 signaling [20]. MC density in WM has already been explored by optical microscopy, showing a median of 49 MC.mm\(^{-2}\) (0.4–149.5), in contrast to a median of 14 MC.mm\(^{-2}\) (3.8–31.4) found in healthy subjects [21]. In this series, MC density was shown to significantly increase after treatment for nonresponders, to remain stable for minor responders, and to significantly decrease for major responders.

There has thus far been no clear demonstration of the clinical relevance nor prognostic impact of BM MC density in WM. We thus investigated BM MC density by sensitive and specific digital quantification, allowing the analysis of a large area of the BM infiltrated by tumor cells, to assess its clinical relevance in WM.

### 2 | MATERIALS AND METHODS

**2.1 | Patients and samples**

Sixty-five patients with available BM trephine biopsy for WM at the Clermont-Ferrand and La Pitié-Salpêtrière University Hospitals were included between 1998 and 2012 in accordance with the Declaration of Helsinki. All included patients presented with WM as defined by the international guidelines [2–4], that is, BM infiltrated by an immunoglobulin M-producing clonal lymphoplasmacytic lymphoma. Fifty-four of the included patients had never been treated before the BM trephine biopsies, whereas 11 had already received treatment for WM. The median follow-up from BM trephine biopsy was 69 months (1–133 months). Fifty-three of 65 patients became symptomatic during the follow up after the BM trephine biopsy and were treated
| Table 1  | Patient characteristics (A) general population and (B) according to MC density |
|----------|--------------------------------------------------------------------------------|
|          | All patients | Low MC density | High MC density | p-value |
| No. of patients | 65 | 27 | 38 |        |
| Treatment naive, N (%) | 54 (84) | 26 (96) | 28 (74) | 0.02 |
| Previously treated, N (%) | 11 (16) | 1 (4) | 10 (26) |        |
| Sex ratio (% male) | 63 | 52 | 71 |        |
| Age at diagnosis, mean [range], years | 60 [36–81] | 57.5 [36–79] | 65.4 [52–81] | 0.0026 |
| ISSWM (n = 43) |          |                |                |        |
| low risk, N (%) | 18 (42) | 10 (67) | 3 (10) | 0.0007 |
| intermediate risk, N (%) | 13 (30) | 3 (20) | 15 (54) |        |
| high risk, N (%) | 12 (28) | 2 (13) | 10 (36) |        |
| Clinical presentation |          |                |                |        |
| Adenopathy, N (%) | 24 (37) | 11 (41) | 13 (34) | 0.59 |
| Hepatosplenomegaly, N (%) | 15 (23) | 9 (33) | 6 (16) | 0.023 |
| Hyperviscosity, N (%) | 10 (15) | 4 (15) | 6 (16) | 1 |
| Neuropathy, N (%) | 9 (14) | 5 (19) | 4 (11) | 0.472 |
| Fever, N (%) (n = 51) | 3 (6) | 1 (6) | 2 (6) | 1 |
| Weight loss, N (%) (n = 49) | 13 (27) | 3 (18) | 10 (31) | 0.498 |
| Cryoglobulinemia, N (%) (n = 39) | 14 (36) | 6 (35) | 8 (36) | 0.667 |
| Mutational status |          |                |                |        |
| MYD88 L265P, N (%) | 49/55 (89.1) | 17/21 (81) | 32/34 (94.1) | 0.19 |
| CXCR4 MUT, N (%) | 15/53 (28.3) | 6/19 (31.6) | 9/34 (26.5) | 0.76 |
| Biology |          |                |                |        |
| Hb level ≤ 11.5 g/dl, N (%) | 39 (60) | 12 (44) | 27 (71) | 0.031 |
| Hb, mean [range], g/dl | 10.8 [5.4–15.2] | 11.7 [6.3–15.2] | 10.2 [5.4–15] | 0.02 |
| Platelet count < 100 X 10^9/L, N (%) | 6 (9) | 0 | 6 (16) | 0.037 |
| Platelet count, mean [range], X 10^9/L | 224 [18–439] | 247 [115–439] | 208 [18–422] | 0.11 |
| β2 microglobulin > 3 mg/L, N (%) (n = 50) | 25 (50) | 9 (41) | 16 (57) | 0.254 |
| β2 microglobulin, mean [range], mg/L (n = 50) | 3.33 [1.4–9.8] | 3 [1.4–6.3] | 3.6 [1.7–9.8] | 0.12 |
| Monoclonal component > 70 g/L, N (%) | 2 (3) | 0 | 2 (5) | 0.507 |
| Monoclonal component, mean [range], g/L | 28.4 [2.3–68.9] | 28.2 [7–66.4] | 28.5 [2.3–68.9] | 0.95 |
| Histopathological characteristics |          |                |                |        |
| Lymphoplasmocytic infiltration > 60% | 22 (34) | 5 (19) | 17 (45) | 0.0352 |
| Diffuse tumor pattern | 18 (28) | 3 (11) | 15 (40) | 0.0132 |

*bold values are for statistically significant data*

According to the Athens criteria [4]. Thirty of the 53 symptomatic patients (56.6%) received chlorambucil and/or rituximab monotherapy; 23/53 (43.4%) received chemoimmunotherapies with a backbone of cyclophosphamide and fludarabine or bendamustine. The patient characteristics are shown in Table 1A.

### 2.2 Evaluation of MC density

Immunostaining was centrally performed at the Clermont-Ferrand pathology laboratory using a monoclonal mouse anti-human CD20 antibody (clone L26; Dako, Carpinteria, CA, USA, 1/100 dilution for 1 h) to stain WM tumor cells and a monoclonal mouse anti-human tryptase antibody (clone AA1; Dako, 1/1000 dilution for 24 min) to stain MCs. Anti-CD20 and antitryptase immunostaining was performed on two contiguous sections of BM trephine biopsies to assess the tumor infiltration characteristics and compare them to the MC infiltration pattern.

All computer-based analyses were conducted using the “Immunoscore module” from Definiens Developer XT, which has already been used to quantify immunological infiltrates within tumors [22]. After digitization, each antitryptase immunostained section was...
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FIGURE 1 Sensitive and specific detection of tryptase-positive cells with the immunoscore module. (A) BM detected with the anti-tryptase Ab. Cells marked in brown are MCs. The background signal is shown in the right panel. (B) MC detection with the immunoscore module. Cells marked in red are the detected MCs. The background signal (not detected as MCs) is shown in the right panel manually marked out. Bone framework, adipocytes, and technical artifacts were excluded to limit the analyses to only hematopoietic tissue. We then defined the optimal thresholds for horseradish peroxidase signal detection to increase sensitivity while retaining specificity (Figure 1). The MC density was then automatically recorded as the number of tryptase-positive cells per mm² of the selected BM tissue surface area. For each BM section, we systematically reviewed random zones to verify whether the detection threshold was appropriate and avoid background noise.

For four patients, MC density was evaluated on two to four consecutive available BM trephine biopsies using the same protocol.

2.3 Molecular analyses for MYD88 and CXCR4 mutational status

Tumor DNA was available for 55/65 patients (84.6%), including 45/54 untreated (83.3%) and 10/11 previously treated (90.9%) patients. Fifty-three samples were assessed by deep next-generation sequencing for MYD88 and CXCR4 mutations, as previously described [23], allowing better assessment of the percentage of allele variants with 1% sensitivity. Two samples were assessed by allele-specific PCR for the MYD88 L265P mutation only.

2.4 Statistical analyses

The therapeutic requirements and time to reach the endpoints were defined according to published recommendations [4,24,25]. Statistical analyses were performed using SPSS Statistics v22 (IBM), PRISM v8.0 (Graphpad), and/or R software [26]. The Pearson Chi-square, two-sided Fisher, Mann-Whitney, and Kaplan-Meier tests with Log-rank and Cox multivariate models were applied to the data as appropriate. MC density was first treated as a quantitative parameter. Then, a sensitivity analysis was conducted to categorize MC density according to its statistical distribution (i.e., median and interquartile range) and then ROC curve analysis, applying Youden’s index, to determine the optimal threshold based on overall survival (OS).

3 | RESULTS

3.1 The distribution of MC density and its correlation with deep tumoral infiltration

The MC density ranged from 6.7 to 487 MC.mm⁻² (mean 106.1 MC.mm⁻²; median 79.9 MC.mm⁻²). The optimal threshold was defined to be 56 MC.mm⁻² by ROC curve analysis based on OS, pinpointing two populations with a high versus low MC density (Figure 2A).

A high MC density correlated with BM tumor involvement (i.e., lymphoplasmocytic infiltration > 60% of the BM surface; \( p = 0.0352 \)) and a diffuse tumor pattern (\( p = 0.0132 \)).

3.2 The association of MC density with clinical and biological features

The clinical and biological features of the patients are shown in Table 1B according to their MC density. There was a statistical association between MC density and the ISSWM prognosis score (\( p < 0.001 \)) (Figure 2B). Patients with a high MC density had significantly less hepatosplenic involvement (\( p = 0.023 \)) but higher anemia (< 115 g.L⁻¹; \( p = 0.031 \)) and more thrombocytopenia (< 100 g.L⁻¹; \( p = 0.037 \)). High MC density was also statistically associated with an older age at diagnosis (65.4 years [52–81] vs. 57.5 years [39–69]; \( p = 0.0026 \)) and previous treatment for the disease (\( p = 0.02 \)).

3.3 The association of MC density with the MYD88 nor CXCR4 mutational status

The MYD88 L265P mutation was detected in 49 of the 55 tested patients (89.1%), including 39/45 (86.7%) who were treatment naïve and 10/10 (100%) who had been previously treated. Fifteen of the 53 tested patients (28.3%) carried a WHIM-like mutation in CXCR4, including 13/43 (30.2%) who were treatment naïve and 2/10 (20%) who had been previously treated (details of the CXCR4 mutations are shown in Table 2). All CXCR4 mutations were observed in MYD88L265P-mutated WM. MC density did not correlate with MYD88 nor CXCR4 mutational status but MYD88 WT patients showed a trend toward a lower MC density (40.9 vs. 90.6 for MYD88 L265P patients, \( p = 0.0742 \); size effect 0.61 [−0.24; 1.45]).
3.4 Impact of MC density on survival

High MC density was associated with a poor clinical outcome, shown by shorter OS (56.5 months vs. not reached in patients with low MC density; \( p = 0.0004 \)) (Figure 3A). OS was still significantly shorter for patients with high MC density in multivariate analysis after controlling for other predictive variables, such as age and ISSWM score in first-line symptomatic patients and CXCR4 mutational status, with MC density as a categorical (with a 56 MC.mm\(^{-2}\) cutoff) (Figure 3B) or continuous variable (Figure 3C). MC density had no impact on time to treatment initiation, strength of response, progression-free survival, nor time to next treatment.
TABLE 2  Details of CXCR4 mutations in this series

| Nucleotide change | Amino acid change | Variant allele frequency |
|-------------------|-------------------|-------------------------|
| c.1000C > T       | Arg334Ter         | 41                      |
| c.976dupC + c.1013C > A | Leu326Pro + Ser338Ter | 1.5 + 12               |
| c.1013C > A      | S338X             | 2                       |
| c.1013C > G      | S338X             | 45                      |
| c.1013C > A      | S338X             | 7                       |
| c.1013C > G      | S338X             | 25                      |
| c.1013C > A      | S338X             | 1                       |
| c.1013C > G      | S338X             | 3                       |
| c.1017dupT       | V340C             | 2                       |
| c.1025_1026delCT | T342R             | 34                      |
| c.1013C > A      | S338X             | 24                      |
| c.1021delT       | S341P             | 12                      |
| c.1013delC       | S338Y             | 31                      |
| c.1013C > G      | S338X             | 4                       |
| c.968_969delGG    | G323V             | 31                      |

FIGURE 4  Sequential BM trephine biopsies of four patients. (*biopsies at subsequent relapse)

3.5  Evaluation of MC density at successive timepoints

We evaluated MC density at various timepoints for four patients by examining one to four BM trephine biopsies during the clinical course of the disease (Figure 4). MC density increased upon relapse for all four patients.

4  DISCUSSION

Interactions between lymphoplasmacytic cells and the microenvironment, including MCs, are known to support their survival and proliferation. This study is the first to evaluate MC density using a sensitive and specific semi-automated tool on large fields of well-outlined hematopoietic tissue of the BM of Waldenström patients.

The distribution of MC density varied widely among patients. BM MC density in various hematological malignancies has been previously optically studied [21,27–29]. Our results, based on this higher performing approach, are in line with the median of 49 MC mm⁻² (0.4–149.5) previously reported for 19 WM patients [21]. Some of our WM patients showed a very high MC density (up to 487 mm⁻²); such a high MC density has already been reported in cases of myelodysplastic syndrome using optical methods (median of 214 MC mm⁻² [50–507 mm⁻²]) [30], illustrating the reliability of our method. Interestingly, while previously MC density evaluations have been optically studied with a focus toward tumor infiltrates and its periphery, we choose to evaluate MC density at all hematopoietic areas with this digital solution, in order to deliver a more impartial and accurate analysis. Namely, this allows us to accurately evaluate MC density for patients with interstitial infiltrate (even with paratrabecular aggregates) – that is, with lymphoid infiltrate being, by itself, uneasy to define.

We provide statistical evidence that MC density is associated with greater BM tumor involvement (i.e., infiltration > 60% of the BM surface) (p = 0.0352) and a diffuse tumor pattern (p = 0.0132), in accordance with the literature on the pathological description of WM [9,21,30,31], emphasizing the place of MCs as a diagnostic tool of the disease. Neither of these two histological criteria were associated with the (1) clinical presentation, (2) biological aggressivity, nor (3) outcome of the WM patients. Thus, MC density appears to be the only histopathological criterion to have such prognostic value.

MC density was statistically associated with an older age at the diagnosis of WM, relapse status, and a higher ISSWM score. In addition, we evaluated MC density at various timepoints for a selected group of four patients during the clinical course of the disease: MC density appeared to increase upon subsequent relapse, suggesting that MCs accumulate during WM and may be involved in disease relapse. MC density should be sequentially evaluated during the clinical course of WM (before and after treatment and at subsequent relapses) to clearly assess the impact of treatment(s) on MCs and/or the involvement of MCs in relapses.

MC density was associated with several features of more aggressive disease, such as anemia and thrombocytopenia, which are consistent with an association between high MC density and greater tumor involvement in the BM. These data, and the previously described bilateral communication between WM tumor cells and MC [20], suggest that (1) MCs may help to segregate lymphoplasmacytic cells in BM and/or (2) the WM tumor cells are directly responsible for the increase in MC density. These bilateral interactions are yet to be explored.

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MC density was a pejorative prognostic factor for OS in our series. We evaluated age, relapsing disease, ISSWM score, and CXCR4 mutational status by multivariate analysis, as these criteria can clearly affect OS. MC density was still the only criterion that affected OS, when considering it as a categorical or continuous variable after controlling for age, ISSWM score, and CXCR4 mutational status in first-line patients.
The proportion of patients carrying MYD88 and CXCR4 mutations in our series was as expected [1,5]. MC density was not associated with MYD88 nor CXCR4 mutational status in our series but the number of patients may have been insufficient to show such an association. Interestingly, WT MYD88 patients showed a trend toward a lower MC density, arguing for a possibly different involvement of MCs in the pathophysiology of WT MYD88 WM. This, however, has yet to be evaluated in an independent series.

All treated patients in our series received classical therapy with alkylating agents or chemoimmunotherapy. Currently, targeted therapies are increasingly used in this setting, namely, Bruton’s tyrosine kinase inhibitors, such as ibrutinib, and the BCL2 inhibitor venetoclax, which is in clinical development. The clinical relevance of MC density and its evolution under such therapy still needs to be explored.

MC density can be accurately measured in WM patients using a specific digital tool on a well-outlined hematopoietic tissue surface. High MC density is associated with several aggressive features, BM tumor burden, and poor clinical outcome. Further studies are necessary to precisely characterize the crosstalk between MCs and tumor cells in WM and evaluate the impact of WM therapies on MCs.

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AUTHOR CONTRIBUTIONS

RL, SP, AL, AT, BL, TT, FP, and OT performed the research. RL, SP, YL, VL, BP, OH, RG, FP, and OT designed the research study. FP, AT, JOB, FC, RL, SP, AL, AT, BL, TT, FP, and OT performed the research. RL, SP, YL, VL, Lawrence Marliot, and Laetitia Pere for their technical assistance.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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