LETTER

LYMPHOMA

Circulating tumor cells in Waldenström macroglobulinemia

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TO THE EDITOR:

Waldenström macroglobulinemia (WM) is a rare mature B-cell neoplasm defined by the association of a serum monoclonal IgM gammopathy and bone marrow (BM) infiltration by lymphoplasmocytic lymphoma [1]. Differential diagnosis with other indolent B-cell lymphomas, including marginal zone lymphoma (MZL), is sometimes difficult, due to overlapping clinico-biological features. Indeed, detection of a monoclonal IgM component is common in MZL, and the *MYD88* L265P mutation, a hallmark of WM, can also be detected in a proportion of MZL cases [2, 3], whereas splenomegaly and lymphocytosis, though more commonly associated with MZL, is sometimes observed in WM. Although WM patients with cytologically detectable circulating lymphoplasmocytes have been described, the exact proportion of WM patients who present with circulating tumor cells (CTCs) and the potential correlation with clinico-biological characteristics is not known. Molecular features may influence the presence and level of CTCs, notably *CXCR4* mutations that have been shown to increase medullar homing of *CXCR4*-mutated B-cells [4, 5]. This could be also relevant for prognosis, since the detection of CTCs in other lymphoid neoplasms (such as follicular lymphoma or myeloma) has been recently shown to confer a poorer prognosis [6, 7]. We therefore aimed in a large cohort of WM patients to evaluate the presence of detectable CTCs by flow cytometry (FCM) at diagnosis, their potential correlation with clinico-biological features and outcomes.

Among 270 patients diagnosed from 2000 to 2021 and followed up in Pitié-Salpêtrière hospital (Paris, France), 187 patients had peripheral blood (PB) FCM analysis performed at diagnosis and were included in this retrospective study. Criteria for diagnosis, treatment initiation and response followed the WHO classification and recommendations from the eleventh IWWM [8]. All patients underwent BM biopsy and IgM-MGUS cases were excluded. CTC counts were assessed by multiparameter FCM analysis (see details in Supplementary methods and Table S1), allowing for the detection of phenotypically aberrant clonal CD19+ B-cells harboring light-chain restriction in line with patient-specific IgM monotype. Briefly, FCM analyses were performed on erythrocyte-lysed blood samples. Several monoclonal antibodies panels were used regarding the period of analyses (Supplementary Table S1). After washing serum Ig, a stain-and-then-lyse direct immunofluorescence technique was used to evaluate all surface markers. Data were acquired on FACSCanto™ II cytometer (BD Biosciences) (with a target of 10, 000 CD19+ events stored), processed and analyzed on FACSDivaTM (BD Biosciences). Markers were evaluated in mature B cells identified as CD19+/SSC low cells. An example of gating strategy is described in details in Supplementary Fig. S1. CTC counts were expressed as absolute numbers in giga per liter (x10^9/L). Routine cytogenetic analyzes and allele-specific PCR for *MYD88* L265P and *CXCR4* S338X were performed for 133, 132 and 127/187 respectively (see Supplementary methods and ref. [9]), in bone marrow samples. Additionally, targeted NGS of 21 genes (*MYD88, CXCR4, TP53, MLL2, ARID1A, CARD11, CD79A, CD79B, SPI1, TNFAIP3, PRDM1, EZH2, IKZF3, NOTCH2, KLF2, NFKBIE, HIST1H1E, CREBBP, TBL1XR1, ETV6* and *IRF4)* was performed for 127 patients for whom DNA material was sufficient (see Supplementary Table S2 for genomic coordinates of targeted regions). The study was conducted in accordance with the declaration of Helsinki and with ethical approval from national (CNIL 2212382) and local (CPP Ile-De-France 05212014) ethics committees. Time to first treatment (TFT) was analyzed in asymptomatic patients, progression-free survival (PFS) in symptomatic patients and overall survival (OS) in the entire cohort as well as specifically in those of asymptomatic or symptomatic patients. Additional information regarding genetic and statistical analyses is available in Supplementary methods.

Main clinical and biological characteristics of the FCM cohort ($n = 187$) were not statistically different from those of the entire WM cohort and are summarized in Supplemental Table S3. Briefly, median age was 63 years (interquartile [IQR25-75], 54–71); median values for IgM levels and BM infiltration were 14.6 g/L (IQR, 7.8–24.5) and 31% (IQR, 20–58) respectively. Fourteen percent of patients presented with splenomegaly and 26% had elevated LDH. Absolute lymphocytosis, defined as total lymphocyte counts

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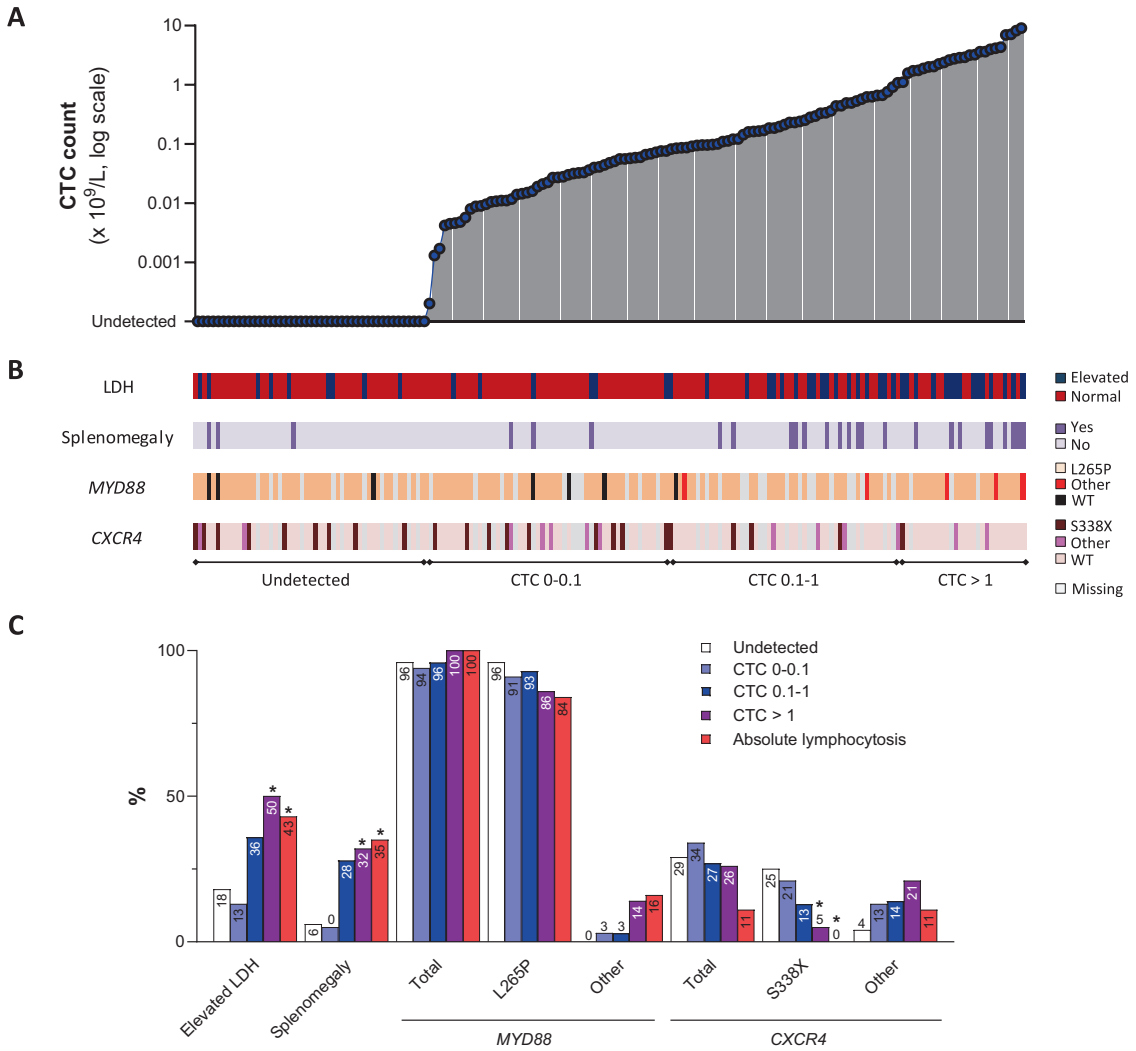

Fig. 1 Circulating tumor cells (CTC) counts and correlation with clinical and biological characteristics. A Distribution of CTC counts among the WM cohort, expressed in giga per liter (x10^9/L) and represented in exponential scale, and (**B**) their corresponding clinical (elevated LDH, splenomegaly) and molecular (*MYD88* and *CXCR4* mutations) features. Each bar or box represent a patient sample. **C** Proportion of patients with selected clinical (elevated LDH, splenomegaly) and molecular (*MYD88* and *CXCR4* mutations) features in different subgroups according to the absence of detectable CTCs (undetected; $n = 51$, white bar), CTC between 0–0.1 × 10^9/L ($n = 66$, light blue), 0.1–1 × 10^9/L ($n = 40$, dark blue), >1x10^9/L ($n = 30$, purple), and absolute lymphocytosis (defined by lymphocytes >4 × 10^9/L; $n = 23$, red). All patients with absolute lymphocytosis were included in the CTC > 1 × 10^9/L group. Percentages are indicated at the top of each bar. Statistical comparisons were performed between the selected group and the rest of the cohort. *$p < 0.05$. CTC circulating tumor cell, LDH lactate dehydrogenase, WM Waldenström macroglobulinemia, WT wild-type.

>4 × 10^9/L, was observed in 23 cases (12.5%). *MYD88* L265P mutations were detected in 120/132 (90%) by AS-PCR and NGS and *CXCR4* mutations in 35/127 (28%) by AS-PCR and/or NGS (see Supplemental Table S3 and S4 for details and list of mutations identified by targeted NGS). Specifically, *CXCR4* mutations were frameshift and nonsense in respectively 13/35 (37%) and 22/35 (63%), and 21/35 (60%) affected S338 amino-acid. With a median follow-up of six years (IQR, 3.5–12.5), 76% (n = 141) of the cohort required treatment. Main reasons for therapy initiation are summarized in Supplementary Table S5. First-line (1 L) treatment consisted mainly in chemo-immunotherapy with rituximab (67%) or chemotherapy alone (27%). No patient was treated with a BTK inhibitor in 1 L. Median time to first treatment (TFT) for asymptomatic patients was 79.7 months; 5-year progression-free survival (PFS) and overall survival (OS) were 59% and 91% respectively (Supplementary Fig. S2). Among the 187 patients with blood FCM analysis at diagnosis, 136 (72.5%) had detectable CTCs, including 67/99 (67%) asymptomatic and 69/88 (78%) symptomatic WM. Median CTC count was 0.11 × 10^9/L (range, 0.0003–9.1; IQR25-75, 0.027–0.76) representing 3% of total lymphocyte counts (range, 0.5–87) and 1.2% of total whole blood cells (WBC) (range, 0.2–61). Thirty-five percent (n = 66) had CTCs below 0.1 × 10^9/L, 21.5% ($n = 40$) between 0.1 and 1 × 10^9/L and 16% ($n = 30$) over 1 × 10^9/L, including 4% (7/187) with 4 × 10^9/L.

The CTC distribution in the population is shown in Fig. 1A. Characteristics of patients with and without detectable CTCs are presented in Supplemental Table S6. Briefly, patients with detectable CTCs more often presented with hyperviscosity syndrome (10 vs. 0%, $p = 0.02$), had higher BM infiltration (median, 35 vs. 22%, $p = 2.10^{-4}$), and showed a trend for more frequent need for therapy at diagnosis (51 vs. 31%, $p = 0.05$). CTCs levels between asymptomatic and symptomatic WM patients were also

Table 1. Correlation between main parameters and circulating tumor clone size.

Variable		Mean (10^9/L)	95% CI	*P*
Genetic parameters				**P***
del6q	No/Yes	0.89/0.46	0.50–1.27/0.07–0.85	0.11
del17p	No/Yes	0.79/1.09	0.46–1.12/0.00–2.71	0.70
MYD88 mutation	No/Yes	0.02/0.77	0.00–0.05/0.45–1.10	**0.001**
L265P	No/Yes	1.33/0.67	0.00–3.07/0.37–0.96	0.42
Other	No/Yes	0.71/3.17	0.38–1.05/0.00–7.70	0.20
CXCR4 mutation	No/Yes	0.87/0.38	0.48–1.26/0.02–0.74	0.06
S338X	No/Yes	0.86/0.13	0.50–1.23/0.00–0.29	**0.0003**
Non-S338X	No/Yes	0.85/0.79	0.45–1.24/0.00–1.79	0.92
ARID1A mutation	No/Yes	0.89/0.15	0.50–1.28/0.15–0.30	**0.0005**
TP53 mutation	No/Yes	0.90/0.15	0.50–1.29/0.00–0.40	**0.001**
MLL2 mutation	No/Yes	0.71/0.60	0.28–1.15/0.00–1.69	0.86
SPI1 mutation	No/Yes	0.73/3.55	0.40–1.07/0.00–8.90	0.25
Clinical parameters				**P***
Sex	Male/Female	0.52/0.73	0.25–0.79/0.34–1.12	0.36
Hyperviscosity syndrome	No/Yes	0.56/0.56	0.35–0.77/0.00–1.46	0.99
Adenopathy	No/Yes	0.54/0.63	0.30–0.77/0.19–1.07	0.70
Splenomegaly	No/Yes	0.40/1.61	0.23–0.56/0.55–2.66	**0.03**
Cytopenia	No/Yes	0.43/0.80	0.22–0.64/0.37–1.24	0.12
Biological parameters		**Parameter estimate**	**R square**	**F test**
IgM spike	Continuous	0.008	0.006	0.31
Bone marrow infiltration	Continuous	0.012	0.05	**0.01**
Hemoglobin	Continuous	−0.10	0.03	**0.03**
Platelets	Continuous	−0.0014	0.015	0.12
LDH	Continuous	0.59	0.04	**0.02**
B2-microglobulin	Continuous	0.016	0.001	0.27
Outcomes		**Hazard-ratio (95% CI)**		**P****
Time to first treatment[§]		1.138 [1.03–1.26]		**0.01**
Progression free survival^		1.002 [1.00–1.19]		0.98
Overall survival		1.064 [0.83–1.36]		0.62

**t* test, **, proportional hazard regression model. [§], in asymptomatic patients. ^, in symptomatic patients. The relationship between CTC count and other continuous biological characteristics was assessed using linear regression models. CTC count was used as a continuous explanatory variable in proportional hazard models of survival after checking the validity of the underlying assumption with the Grambsch and Therneau test.
Significant *P* and F test values below 0.05 are indicated in bold.
CI confidence interval, *del* deletion.

different (median, 0.04 vs. 0.13×10^9/L, $p = 0.02$). Splenomegaly was also more frequent although this did not reach statistical significance (16% vs. 6%, $p = 0.09$). There was no difference between the two groups regarding the prevalence of *MYD88* and *CXCR4* mutations or recurrent cytogenetic abnormalities. No significant difference was observed in terms of TFT, PFS and OS (Supplementary Fig. S2).

Since the range of distribution of CTC counts was large, we then assessed their correlation and impact as a continuous variable (Table 1), to highlight potential associations that were not identified by our initial dichotomous (with vs. without detectable CTC) approach. Higher CTC counts, as a continuous variable, correlated with increased prevalence of splenomegaly ($p = 0.02$), elevated LDH ($p = 0.01$), higher BM infiltration percentage ($p = 0.01$) and lower hemoglobin level ($p = 0.04$) at diagnosis. This translated into a significantly shortened TFT ($p = 0.01$, HR 1.138 [95%CI, 1.03–1.26]) in asymptomatic patients, meaning that the risk of first treatment increased by 13.8% for a one-point increase (in 10^9/L) in CTC. However, no significant impact of higher CTC counts was observed on PFS or OS in univariate analysis and on TFT in multivariate analysis (Supplementary Table S7). Among genetic abnormalities, *CXCR4* S338X mutations were significantly associated with lower CTC counts ($p = 0.0003$) (Table 1 and Fig. 1B). Interestingly, other (non-S338X) *CXCR4* variants did not impact CTC counts ($p = 0.92$). In this cohort, *ARID1A* and *TP53* mutations were also associated with lower CTC counts; however these results should be taken with caution given the low number of mutated patients. No correlation between CTC counts and any other gene mutation (including *MYD88* L265P) or recurrent cytogenetic abnormalities was observed (Table 1). Concordant data were also observed when comparing the different subgroups of patients with no detectable CTCs, CTCs 0–0.1, 0.1–1 and >1, as illustrated in Fig. 1C. The positive association of CTC presence and counts with splenomegaly and elevated LDH raises the question of differential diagnosis with other lymphoma subtypes, notably splenic MZL (SMZL).

Looking specifically at patients with absolute lymphocytosis ($n = 23$; median CTC counts of 2.9×10^9/L [range, 1.7–9.1]) (Fig. 1C), eight (34%) had splenomegaly and 10 (43%) elevated LDH (vs. 10% and 22% in the rest of the cohort, $p = 0.001$ for both comparisons). *MYD88* mutations were identified in 19/19 (100%) (L265P, 16; other, 3), while *CXCR4* mutations were observed in 2/19 (10.5%) (S338X, 0; other, 2) and del6q in 4/21 (19%), which was not statistically different from the rest of the cohort. This, along with the absence of *NOTCH2* and *KLF2* mutations (among 107 patients successfully screened), recurrent in SMZL [3], suggests cases were not SMZLs misdiagnosed as WM.

Our study has some limits inherent to its retrospective nature. Although FCM techniques have improved over years with a rate of CTC detection slightly higher in the most recent period, this only concerns very small clones $<0.1 \times 10^9$/L (Supplementary Table S1) and it should be noted that the median rate and distribution of CTCs did not significantly differ during the study period. We believe analysis of CTC populations as a continuous variable also limits this potential bias. Certain key markers such as CD25 and LAIR1 [10] were not used in our work and future studies will be needed to define which panel with which antibodies is most sensitive for the detection of CTCs. Main cytogenetic/molecular features were not available for the entire cohort. However, we did not observe any difference between the groups of patients with or without cytogenetic/molecular data, with regard to usual clinico-biological characteristics, and the median CTC count and its distribution (Supplementary Table S8).

We identified in this large monocentric cohort of WM patients that most patients (73%) at diagnosis harbor detectable CTCs. In most cases, low counts were observed, in line with the absence of absolute lymphocytosis generally observed in WM. To our knowledge, this is the largest study reporting systematic evaluation by FCM of detectable CTCs in WM, and the first to correlate these data with extensive cytogenetic and molecular features. Several studies have reported the detection of the *MYD88* L265P mutation by DNA sequencing in PB cells, indirectly suggesting the presence of CTCs, in up to 98% of WM cases, though with highly variable sensitivity (range, 39–98%) depending on the technique used [11, 12]. Collectively, this data suggests that CTCs are very frequent in WM at diagnosis and could therefore allow phenotypic description of the tumor population in PB, for the vast majority of patients. However, it should not replace BM biopsy for diagnosis.

We also showed that the proportion of patients with detectable CTCs was significantly higher in symptomatic WM patients and that CTC levels were associated with a shorter TFT in asymptomatic patients. The presence of increasing levels of CTCs was associated with other specific features, notably the presence of splenomegaly and elevated LDH, which could represent a gray zone group of patients between SMZL and WM, with cytogenetic and molecular features similar to other WM patients, but a clinical presentation more closely resembling SMZL.

With regards to correlation with intrinsic biology, we identified a striking association between the presence of *CXCR4* S338X mutation and significantly lower rate of CTCs, not observed with other *CXCR4* variants. This is consistent with recent in vitro studies showing that there is a genotype- phenotype correlation among *CXCR4* variants, with *CXCR4* S338X associated with the strongest chemotaxis impairment [13]. These data could also partly explain the poorer response and PFS on ibrutinib observed in this group of patients [14, 15], in whom the BM niche may play a protective role, counteracting ibrutinib activity. Although the levels of CTCs did not impact PFS and OS in our cohort treated with 1 L chemo-immunotherapy, this might be explored in prospective studies and in the context of BTK inhibitors.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author, DRW, upon reasonable request.

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AUTHOR CONTRIBUTIONS

CBG, ES, MLT, MB and DRW designed the research, analysed data and wrote the manuscript. CBG, ES, PM, EC, FNK, CB, FD, MLT, MB and DRW performed experiments. CBG, PM, VM, NG, AG, IB, VL, SC, MB and DRW recruited patients. All authors reviewed and approved the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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