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ORIGINAL PAPER

Prognostic impact of *MYD88* and *CXCR4* mutations assessed by droplet digital polymerase chain reaction in IgM monoclonal gammopathy of undetermined significance and smouldering Waldenström macroglobulinaemia

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Summary

Waldenström macroglobulinaemia (WM) is characterized by recurrent somatic mutations in *MYD88* and *CXCR4* genes. However, limitations arise when analysing these mutations in IgM monoclonal gammopathy of undetermined significance (MGUS) or smouldering WM (SWM) given the lower tumour load. Here, we used droplet digital polymerase chain reaction (ddPCR) to analyse *MYD88* L265P and *CXCR4* S338* mutations (C1013G and C1013A) in unsorted bone marrow (BM) or cell-free DNA (cfDNA) samples from 101 IgM MGUS and 69 SWM patients. ddPCR was more sensitive to assess *MYD88* L265P compared to allele-specific PCR, especially in IgM MGUS (64% vs 39%). *MYD88* mutation burden correlated with other laboratory biomarkers, particularly BM infiltration ($r = 0.8$; $p < 0.001$). *CXCR4* C1013G was analysed in *MYD88*-mutated samples with available genomic DNA and was detected in 19/54 (35%) and 18/42 (43%) IgM MGUS and SWM cases respectively, also showing correlation with BM involvement ($r = 0.9$; $p < 0.001$). ddPCR also detected 8 (38%) and 10 (63%) *MYD88*-mutated cfDNA samples in IgM MGUS and SWM respectively. Moreover, high BM mutation burden (≥8% *MYD88* and ≥2% *CXCR4*) was associated with an increased risk of progression to symptomatic WM. We show the clinical applicability of ddPCR to assess *MYD88* and *CXCR4* in IgM MGUS and SWM and provide a molecular-based risk classification.

David F. Moreno and Mónica López-Guerra these authors contributed equally in this work.

Dolors Colomer and Carlos Fernández de Larrea these authors jointly supervised this work.

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KEYWORDS
CXCR4, droplet digital PCR, IgM MGUS, MYD88, Waldenström macroglobulinaemia

INTRODUCTION

Waldenström macroglobulinaemia (WM) is a B-cell neoplasm characterized by a lymphoplasmacytic infiltration in the bone marrow (BM) and the presence of a serum IgM monoclonal protein (M-protein).[1] WM is preceded by two asymptomatic stages named IgM monoclonal gammopathy of undetermined significance (MGUS) and smouldering WM (SWM). The presence of highly recurrent somatic mutations is another key feature of WM. For instance, whole-genome sequencing has identified mutations in *MYD88* and *CXCR4* genes in up to 90% and 27% of WM patients respectively.[2,3] Another recurrent alteration well described is del(6q), which was identified in up to 50% of WM patients using fluorescent *in situ* hybridization.[4,5] The identification of abnormal B cells in IgM MGUS with a mutational and phenotypical background similar to those found in later stages of the disease strongly supports this evolutionary model.[6–8]

Allele-specific polymerase chain reaction (AS-PCR)-based methods to detect *MYD88* L265P using unsorted or $CD19^+$ selected BM samples showed that the mutation prevalence varied from 54% to 87% in IgM MGUS and 86% to 93% in WM respectively.[9,10] On the other hand, AS-PCR and Sanger sequencing in $CD19^+$ selected BM samples have identified 17% and 43% of *CXCR4* mutations in IgM MGUS and untreated WM respectively.[11] Similarly, another study reported a prevalence of up to 33% of *CXCR4* mutations in IgM MGUS using Sanger sequencing.[12] Regarding targeted next-generation sequencing (NGS), *CXCR4* mutations were identified in 9% and 23% in IgM MGUS and WM respectively.[13]

This knowledge of the genomic landscape has given insights to better elucidate the impact on disease progression from asymptomatic stages. For instance, a higher risk of progression was observed in *MYD88*-mutated IgM MGUS[14] and wild-type (wt) *MYD88* SWM patients.[15] Regarding *CXCR4* mutations, a worse outcome was described in *CXCR4* mutated SWM patients,[13] while another study reported no significant difference.[16]

Although data in WM have shown highly reproducible results using different technologies, data in IgM MGUS and SWM are more variable due to the low tumour burden, the heterogeneous BM infiltration, or the availability of BM samples in asymptomatic patients.[9–19] Thus, there is a need to find more precise techniques to measure disease burden in this group of patients. In addition, considering that the reproducibility of the prognostic studies might be affected by the technology used to assess *MYD88* and *CXCR4* mutations, the impact of the mutational status of early disease stages on the risk of progression to symptomatic WM remains an ongoing field of investigation.

Therefore, we wondered whether using more precise methods to detect somatic mutations in asymptomatic IgM monoclonal gammopathies could overcome technical diagnostic issues in samples with low tumour burden, thereby improving prognostic risk models. In this sense, droplet digital PCR (ddPCR) technology provides an absolute quantification of nucleic acid target sequences, thus being useful to detect small clones. ddPCR can achieve higher sensitivity, precision and reproducibility compared to the standard AS-PCR.[20] ddPCR has been used for *MYD88* L265P detection using genomic DNA from $CD19^+$ selected, unsorted BM samples, and cell-free DNA (cfDNA) of WM patients.[21,22] More recently, ddPCR has also been applied to assess *CXCR4* S338* mutations in symptomatic WM patients.[23] However, *MYD88* and *CXCR4* mutations in cfDNA samples from asymptomatic patients have not yet been systematically evaluated.

We thus analysed *MYD88* and *CXCR4* mutations leveraging ddPCR technology in a cohort of patients with IgM MGUS and SWM, and identified more accurate markers of disease progression. The findings of this study provide further insights into the genomic landscape of IgM MGUS and SWM.

METHODS

Patients

From 1985 to 2020, 201 patients that met the search criteria for 'IgM monoclonal gammopathy' were identified in the monoclonal gammopathies database of the Hospital Clínic of Barcelona. Diagnosis was based on the Second International Workshop on Waldenström Macroglobulinaemia and the Mayo Clinic criteria.[1,24] Patients with less than 10% BM involvement and the presence of immunophenotypical findings of lymphoplasmacytic lymphoma were categorized as SWM. Patients diagnosed with 'IgM-related disorders' were not included in this study. The patients provided informed consent for sample collection in the biological samples bank of the Hospital Clínic of Barcelona in accordance with the Declaration of Helsinki. The study was approved by the institutional review board.

Sample collection

In this study, 240 BM and plasma samples from 170 patients categorized as IgM MGUS ($N = 101$) and SWM ($N = 69$) were

included. They were the main cohort of analysis. Additional samples from patients with symptomatic WM ($N = 31$) were used as positive controls for experimental analysis. BM samples were available in the entire cohort (101 IgM MGUS, 69 SWM, and 31 symptomatic WM patients), while plasma samples were only available in 39 patients (21 IgM MGUS, 16 SWM and two symptomatic WM patients). BM and plasma samples were collected at diagnosis (IgM MGUS and SWM), and before treatment initiation (symptomatic WM). The distribution of paired samples according to each diagnosis is graphically depicted in Figure 1. Processing of the samples is detailed in the supporting information (File S1).

Flow cytometry analysis

BM samples treated with ethylenediaminetetraacetic acid (EDTA) were evaluated by multiparametric flow cytometry. Briefly, three eight-colour monoclonal antibody (mAb) combinations were used for lymphocyte immunophenotyping (Table S1). Data acquisition was performed with a BD FACSCanto II flow cytometer and analysed using FACSDiva software (BD Biosciences, San Jose, CA, USA).

Allele-specific polymerase chain reaction assay for *MYD88* L265P

MYD88 L265P mutation analysis was performed on DNA from unsorted BM samples using AS-PCR technology (qBiomarker Somatic Mutation PCR Assay, MYD88_85940; Qiagen, Germany). Amplification Refractory Mutation System technology was used for allele-specific amplification. Diffuse large B-cell lymphoma cell line OCI-LY3 DNA was used as positive control, as described previously.[9]

Droplet digital polymerase chain reaction assays for *MYD88* L265P and *CXCR4* S338* mutations

ddPCR assays for *MYD88* L265P and *CXCR4* mutations were commercially available (Bio-Rad, Hercules, CA, USA). The two *CXCR4* mutations analysed were: c.1013C>G p.Ser338Ter (*CXCR4* C1013G) and c.1013C>A p.Ser338Ter (*CXCR4* C1013A). Briefly, DNA samples were tested in duplicate. We used the QX200 Droplet Digital PCR System (Bio-Rad) to generate and individually analyse each droplet. Data were then analysed in the QuantaSoft Software 1.0 (Bio-Rad). We assessed *CXCR4* mutations in *MYD88*-mutated cases, as nearly all *CXCR4* mutations occur in *MYD88*-mutated patients.[3] When analysing *CXCR4* mutations, we first assessed the C1013G mutation. Then, we analysed the C1013A mutation only in samples that were negative for the C1013G. This approach was chosen to take advantage of the availability of genomic DNA or cfDNA, the exceptional cooccurrence of both *CXCR4* mutations in the same sample, and the clinical-oriented applicability of ddPCR. Detailed description is available in the supplementary information (S1).

Statistical analysis

Pearson correlation or Spearman tests were used to analyse correlation between tumour burden and laboratory biomarkers. The Fisher exact test was used to analyse categorical data. Mutation burden (*MYD88* and *CXCR4*) was fitted as a continuous variable into a Fine and Grey regression model to analyse the impact on the risk of progression to symptomatic WM. Progression to symptomatic disease criteria was defined according to previous consensus recommendations.[25,26] To construct a practical model, we later categorized the mutation burden according to an X-tile approach obtaining cut-point subsets. Plots were calculated based on the cumulative incidence function (CIF) (Figure S1). All statistical analyses were performed using Stata version 16 (StataCorp LLC, College Station, TX, USA). Detailed description is available in the supplementary information (S1).

RESULTS

Baseline patient characteristics

One-hundred seventy patients (88 male/82 female; median age, 75 years) with available sample material, diagnosed with IgM MGUS (101 patients) and SWM (69 patients) were selected for the current study. Thirty-eight (22%) patients were diagnosed before the year 2000, 31 (18%) patients between

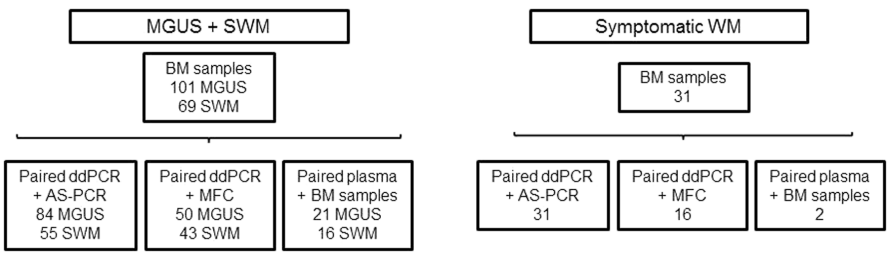

FIGURE 1 Sample processing methodology. AS-PCR, allele-specific polymerase chain reaction; BM, bone marrow; ddPCR, droplet digital polymerase chain reaction; MGUS, monoclonal gammopathy of undetermined significance; MFC, multiparameter flow cytometry; SWM, smouldering Waldenström macroglobulinaemia; WM, Waldenström macroglobulinaemia.

2000 and 2010, and 101 (59%) after 2010. The main clinical and biological patient characteristics are summarized in Table 1.

MYD88 L265P detection by allele-specific versus droplet digital polymerase chain reaction in bone marrow

We first analysed the *MYD88* L265P mutation prevalence in genomic DNA from unsorted BM samples using both AS-PCR and ddPCR techniques. We did both AS-PCR and ddPCR analyses in 84 IgM MGUS and 55 SWM patients at diagnosis. In IgM MGUS, the *MYD88* L265P mutation was detected in 33 (39%) patients using AS-PCR, but ddPCR was more sensitive, detecting the mutation in 54 (64%) patients ($p < 0.001$). Similarly, in SWM, *MYD88* L265P was detected in 40 (73%) patients using AS-PCR, while ddPCR identified the mutation in 45 (82%) patients ($p < 0.001$) (Figure 2A). Analysing *MYD88* mutation burden distribution, we observed that the *MYD88* L265P mutation was not detected in 50% of cases by AS-PCR when the variant allelic frequency of *MYD88* L265P assessed by ddPCR was lower than 1%. This finding was supported by the 0.0259% limit of detection of ddPCR after serial dilutions of a *MYD88* L265P sample in wt DNA (Table S2). To evaluate the higher sensitivity of ddPCR compared to AS-PCR, we analysed 50 out of the 84 IgM MGUS cases who had their immunophenotype analysis available at the time of DNA collection. In this group, 30 out of 50 (60%) and 19 out of 37 (51%) patients were positive for the *MYD88* mutation by ddPCR and AS-PCR respectively. In samples without detectable clonal B cells by flow cytometry, *MYD88* L265P was detected in 15 (50%) and 7 (33%) cases using ddPCR and AS-PCR respectively. In the case of SWM, 27 out of 32 (84%) and 23 out of 28 (82%) patients were positive for the *MYD88* mutation by ddPCR and AS-PCR respectively. All 27 (100%) SWM patients who had clonal B cells were also positive for the mutation by ddPCR, and 22 (96%) by AS-PCR. Thus, the agreement of clonal populations observed by ddPCR and flow cytometry was 62% (Cohen's k 0.3; $p = 0.016$) in IgM MGUS and 91% (Cohen's k 0.6; $p < 0.001$) in SWM.

TABLE 1 Baseline characteristics of the patients with asymptomatic IgM monoclonal gammopathies

Baseline characteristics	$N = 170$
Median age, years (IQR)	75 (65–84)
Sex, female (%)	82 (48)
Diagnosis (%)	
IgM MGUS	101 (59)
SWM	69 (41)
M-protein size (g/l), median (IQR)	12.1 (6–15)
Bone marrow involvement (% total celullarity)	17 (11–28)
Albumin (g/l), median (IQR)	43 (41–45)
Haemoglobin (g/l), median (IQR)	134 (121–145)
Platelet count (10^3/μl), median (IQR)	236 (184–287)
β2-microglobulin (mg/dl), median (IQR)[a]	2.3 (1.8–2.9)

Abbreviations: IQR, interquartile range; M-protein, serum monoclonal protein; MGUS, monoclonal gammopathy of undetermined significance; SWM, smouldering Waldenström macroglobulinaemia.

[a]Available in 145 patients.

MYD88 L265P mutation burden in bone marrow

The median *MYD88* mutation burden obtained by ddPCR was 1.13% [interquartile range (IQR) 0.42–2.78] in IgM MGUS ($n = 62$) and 5.36% (IQR 2.49–11.00) in SWM ($n = 54$). We also analysed a group of symptomatic WM cases as positive controls ($n = 31$) and the median *MYD88* mutation burden was 11.00% (IQR 5.61–18.49). *MYD88* mutation burden was significantly higher in successive disease stages (IgM MGUS versus SWM, $p < 0.001$; IgM MGUS, SWM versus symptomatic WM, $p < 0.001$). The distribution plots are shown graphically in Figure 2B. We then compared *MYD88* mutation burden assessed by ddPCR to standard laboratory biomarkers. We observed that the mutation burden as a continuous variable correlated with the serum M-protein size ($r = 0.3$; $p = 0.001$), the serum IgM concentration ($r = 0.4$; $p < 0.001$), the infiltration of the BM by morphology ($r = 0.7$; $p < 0.001$), and the percentage of BM clonal B cells by flow cytometry ($r = 0.8$; $p < 0.001$) (Figure 2C).

Analysis of *CXCR4* C1013G and C1013A mutations by droplet digital polymerase chain reaction in bone marrow

In addition, we analysed the two most prevalent *CXCR4* mutations in WM (C1013G and C1013A) using ddPCR. The limit of detection of 0.0399% was established after a serial dilution of a *CXCR4* S338* C1013G sample in wt DNA (Table S3). Four symptomatic WM BM samples previously known to harbour the *MYD88* L265P mutation were used as controls. Amongst them, three cases harboured the C1013G variant with a mutation burden of 1.5%, 4.8% and 7.6%, while the *MYD88* L265P mutation burden was 20.5%, 8.1% and 5.1% for each case respectively. The fourth case had the C1013A variant with a mutation burden of 17.2%, while the *MYD88* L265P was 18.5%. We then analysed the presence of *CXCR4* mutations in asymptomatic IgM monoclonal gammopathy patients who were *MYD88*-positive and had available genomic BM DNA (54 IgM MGUS and 42 SWM patients). By ddPCR, *CXCR4* C1013G was positive in 19 (35%) and 18 (43%) patients with IgM MGUS and SWM respectively. The median *CXCR4* C1013G mutation burden distribution in IgM MGUS was 0.4% (IQR 0.3–1.4), which was similar to that in SWM (0.4%, IQR 0.2–12). Overall, the distribution of the mutation burden suggested a subclonal pattern for *CXCR4* mutations. As a

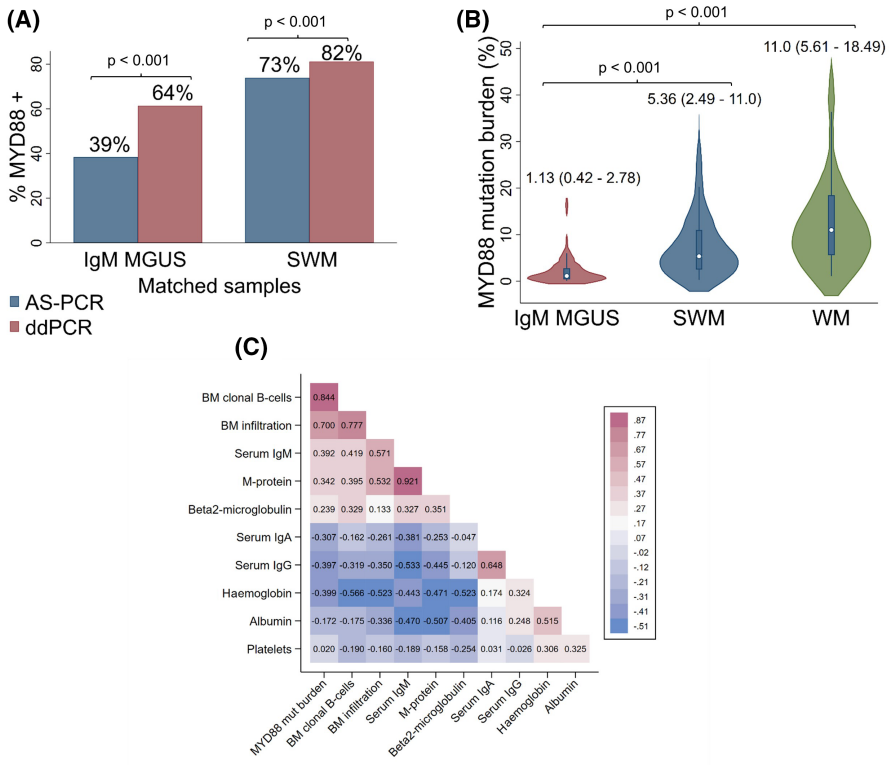

FIGURE 2 *MYD88* L265P analysis in the bone marrow of IgM monoclonal gammopathy patients. (A) Comparison between AS-PCR and droplet digital PCR (ddPCR) to detect *MYD88* L265P in IgM monoclonal gammopathy of undetermined significance (MGUS) and smouldering Waldenström macroglobulinaemia (SWM). (B) *MYD88* mutation burden distribution in IgM MGUS, SWM and symptomatic Waldenström macroglobulinaemia (WM). (C) Heatmap correlation plot of *MYD88* mutation burden and other common laboratory biomarkers in IgM monoclonal gammopathies. Each number shows the Pearson correlation coefficient. AS-PCR, allele-specific polymerase chain reaction; BM, Bone marrow; ddPCR, droplet digital polymerase chain reaction; M-protein, serum monoclonal protein; MGUS, monoclonal gammopathy of undetermined significance; SWM, smouldering Waldenström macroglobulinaemia; WM, Waldenström macroglobulinaemia.

continuous variable, *CXCR4* C1013G showed positive correlations with BM infiltration assessed by morphology (r = 0.4; $p < 0.001$), *MYD88* mutation burden assessed by ddPCR (r = 0.6; $p < 0.001$), and BM clonal B cells assessed by flow cytometry (r = 0.9; $p < 0.001$). *CXCR4* C1013A was identified in only five *MYD88*-mutated patients not harbouring the C1013G mutation, two corresponding to IgM MGUS cases and three with SWM, all of them with less than 2% mutation burden. Figure 3 shows the mutation burden distribution along samples that had both *MYD88* and *CXCR4* mutations.

Evaluation of *MYD88* L265P by droplet digital polymerase chain reaction in cell-free DNA

ddPCR has been reported to be a reliable technology to detect *MYD88* L265P in cfDNA samples with high tumour burden.[21] Here, we attempted to demonstrate that ddPCR could additionally be used to detect *MYD88* mutation in the cfDNA of patients with a low tumour burden. In cfDNA samples from IgM MGUS patients, *MYD88* mutation was detected in eight out of 21 (38%) with a mutation burden median distribution of 0.54% (IQR 0.20–1.32). In the case of SWM patients, ddPCR detected the mutation in 10 out of 16 (63%) patients with a median distribution of 1.78% (IQR 0.24–7.26). In two cases of symptomatic WM, *MYD88* mutation was detected in both cases (1.84% and 3.12% of mutation burden). Overall, the minimum *MYD88* mutation burden in cfDNA was 0.20%, and the maximum was 7.26% (Figure 4A). As a biomarker, *MYD88* in cfDNA positively correlated with the serum M-protein size (n = 39; r = 0.3; p = 0.047), the serum IgM concentration (n = 37; r = 0.4; p = 0.016), the BM infiltration assessed by morphology (n = 39; r = 0.4; p = 0.015), and the *MYD88* BM mutation burden as assessed by ddPCR (n = 35; r = 0.5; p = 0.001) (Figure 4B–D). Regarding *CXCR4* mutations assessed in cfDNA, we were only able to detect the C1013G variant in one sample from a SWM patient (1.16% mutation burden), which was also positive for that mutation in BM (13.1%) and for *MYD88* L265P in both cfDNA (0.84%) and BM (13.36%).

Prognostic impact of *MYD88* and *CXCR4* mutations assessed by droplet digital polymerase chain reaction in bone marrow

With a median follow-up of six years (IQR 3–9 years), progression to symptomatic WM was observed in 23 (14%)

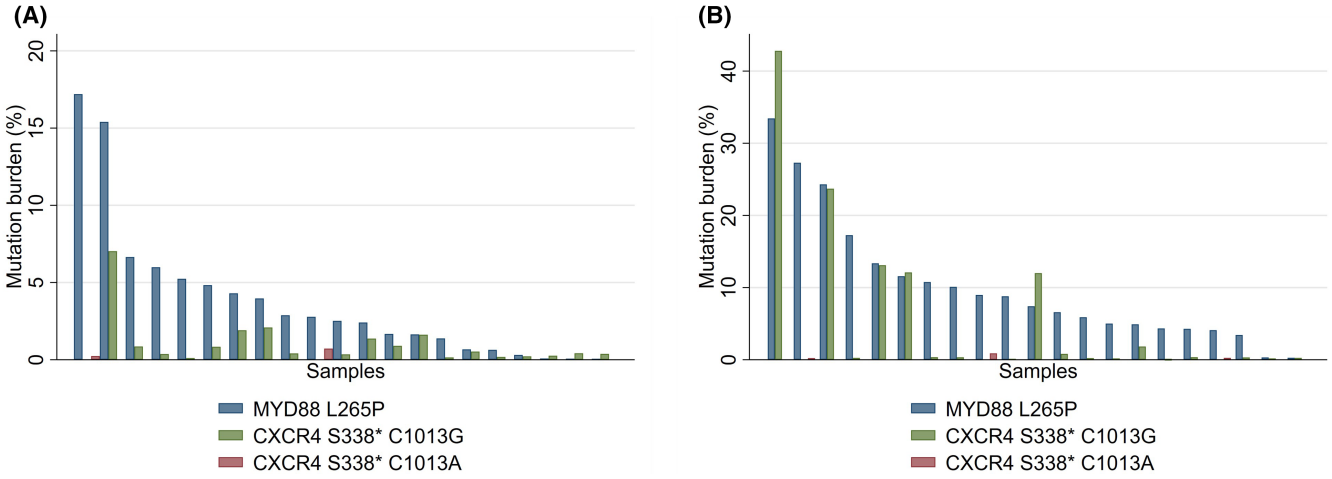

FIGURE 3 *CXCR4* mutations burden (%) distribution in *MYD88* L265P-positive bone marrow samples. (A) *CXCR4* S338* C1013G and C1013A in IgM MGUS. (B) *CXCR4* S338* C1013G and C1013A in smouldering Waldenström macroglobulinaemia (SWM).

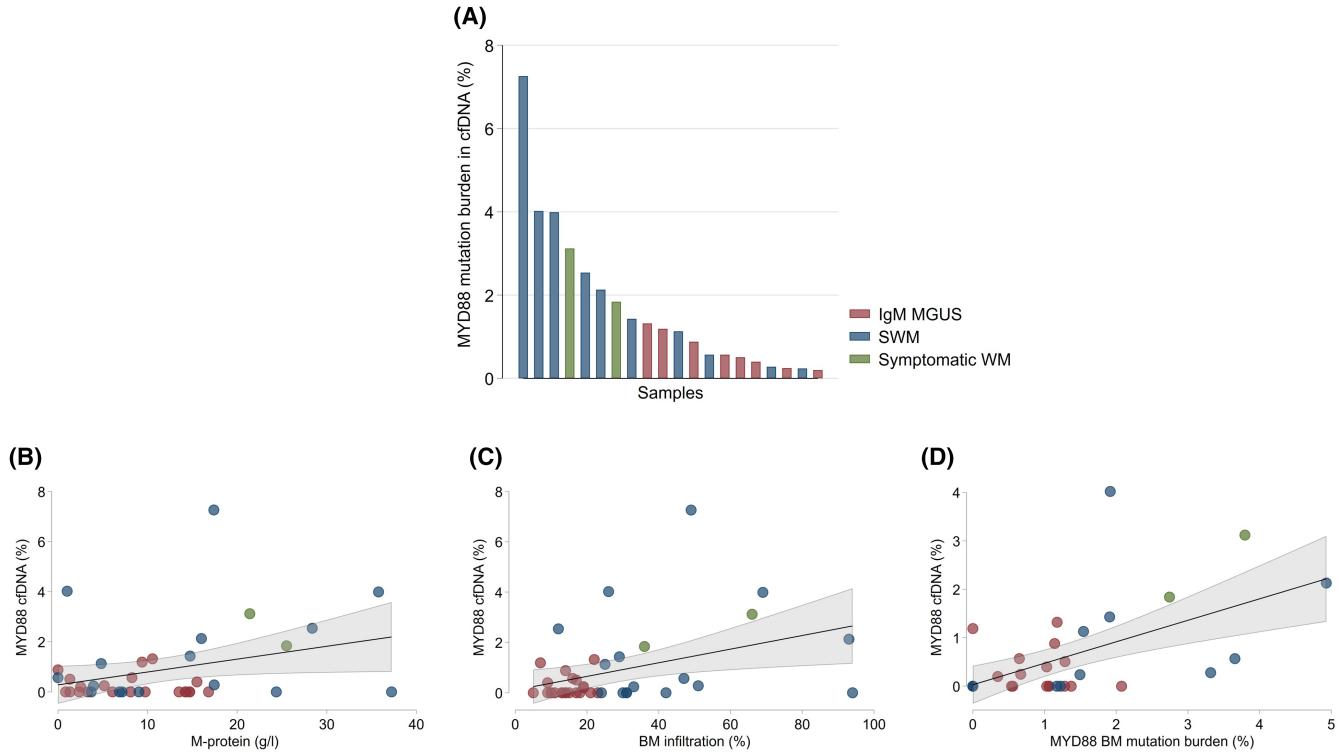

FIGURE 4 Analysis of *MYD88* L265P cell-free DNA (cfDNA) by ddPCR in plasma from IgM MGUS ($n = 21$), SWM ($n = 16$), and symptomatic WM ($n = 2$). (A) Distribution of *MYD88* L265P burden in cfDNA in samples of patients with IgM MGUS, SWM and symptomatic WM. (B) Correlation plot of *MYD88* L265P cfDNA (%) and M-protein size ($n = 39$; $r = 0.3$; $p = 0.047$). (C) Correlation plot of *MYD88* L265P cfDNA (%) and the BM infiltration by morphology ($n = 39$; $r = 0.4$; $p = 0.015$). (D) Correlation plot of *MYD88* L265P cfDNA (%) and the *MYD88* BM mutation burden (values transformed to fit in the scatterplot) by ddPCR ($n = 35$; $r = 0.5$; $p = 0.001$). BM, bone marrow; ddPCR, droplet digital polymerase chain reaction; MGUS, monoclonal gammopathy of undetermined significance; SWM, smouldering Waldenström macroglobulinaemia; WM, Waldenström macroglobulinaemia.

patients (7 MGUS and 16 SWM patients). Death without progression accounted for up to 37 (22%) patients. All patients progressed due to disease complications related to tumour burden: significant peripheral blood cytopenia (anaemia and/or thrombocytopenia) and symptomatic lymphadenopathy and/or splenomegaly. There were no patients lost to follow-up. At five and 10 years, the cumulative incidence of progression of patients with IgM MGUS to symptomatic WM was 5% (95% CI 2–12) and 7% (95% CI 3–19) respectively. In the case of SWM, the cumulative incidence of progression was 22% (95% CI 12–36) at five years, and 30% (95% CI 17–47) at 10 years. Median

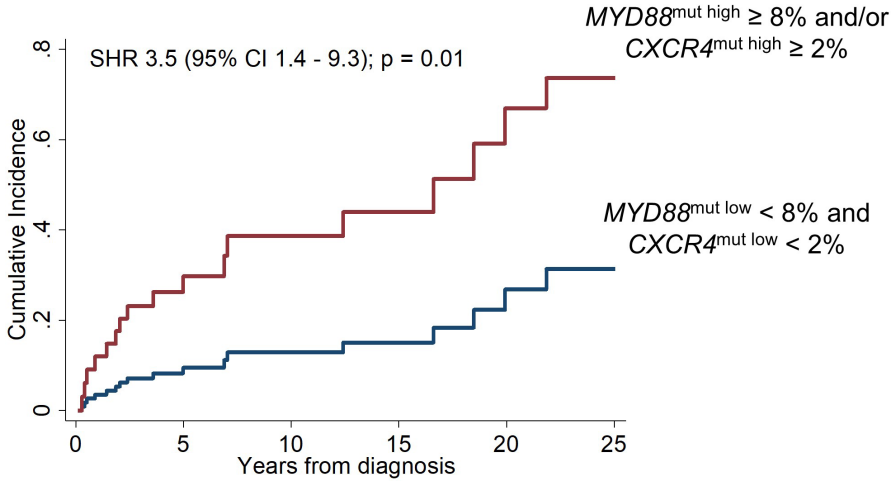

FIGURE 5 Risk of progression to symptomatic Waldenström macroglobulinaemia. Cut-off points were calculated using an X-tile approach. Shown is the cumulative incidence of progression for both IgM monoclonal gammopathy of undetermined significance and smouldering Waldenström macroglobulinaemia considering *MYD88* L265P and *CXCR4* S338* C1013G tumour burden when assessed by droplet digital polymerase chain reaction in the bone marrow.

overall survival of all patients was 13 years (95% CI 11–20) (Figure S4).

To assess the impact of BM ddPCR-detected *MYD88* and *CXCR4* mutations on the risk of progression, we fitted a competing-risk framework as described previously. Using the Fine and Grey regression model, we first analysed the mutation burden of each of the mutations as continuous variables. In the univariate analysis, the subhazard ratios (SHR) of the *MYD88* mutation burden were 1.2 (95% CI 1.04–1.36; $p = 0.012$) and 1.04 (95% CI 1.01–1.08; $p = 0.040$) in IgM MGUS and SWM respectively. The SHRs of *CXCR4* C1013G were 1.8 (95% CI 1.33–2.41; $p < 0.001$) and 1.02 (95% CI 0.9–1.04; $p = 0.065$) in IgM MGUS and SWM respectively (Table S4).

We then established cut-off points for the *MYD88* and *CXCR4* mutation burden to identify risk categories of progression to symptomatic WM. For the whole series, patients who had a *MYD88* mutation burden higher than 8% [SHR 4.8, 95% confidence interval (CI) 2–11.2; $p < 0.001$] or a *CXCR4* mutation burden higher than 2% (SHR 4.2, 95% CI 1.7–10.7; $p = 0.003$) had a cumulative incidence of progression of 30% and 25% at five years respectively. In our series, 19 patients who progressed were previously tested for *MYD88* and *CXCR4* mutations. Two out of six IgM MGUS patients and nine out of 13 SWM patients having a *MYD88* or *CXCR4* high mutation burden (>8% and >2% respectively) progressed (SHR 3.5, 95% CI 1.4–9.3; $p = 0.01$) (Figure 5). Based only on the *MYD88* L265P mutation, we identified a small group of patients with high risk at each stage. Thus, IgM MGUS patients with a *MYD88* mutation burden higher than 4% (SHR 7.8, 95% CI 1–32; $p = 0.005$) had a cumulative incidence of progression of 20% at five years. In the case of SWM, patients with a *MYD88* mutation burden higher than 25% (SHR 3.4, 95% CI 1–8; $p = 0.012$) had a cumulative incidence of progression of 45% at five years (Figure S5). Regarding patients who were *MYD88* L265P wt using ddPCR, only three out of 51 (6%) patients progressed to symptomatic disease. On the other hand, 21 out of 117 (18%) *MYD88*-mutated patients by ddPCR progressed. So, *MYD88* L265P wt did not impact the progression to symptomatic disease (SHR 0.4, 95% CI 0.1–1.2; $p = 0.112$).

DISCUSSION

Our study highlighted the usefulness of ddPCR to detect *MYD88* and *CXCR4* mutations in the clinical setting of asymptomatic IgM monoclonal gammopathies. We showed the feasibility of *MYD88* L265P detection and quantification using ddPCR in unsorted BM samples with low tumour burden. In matched samples, we found that ddPCR was able to detect more *MYD88*-mutated cases than conventional AS-PCR, especially in IgM MGUS. Moreover, ddPCR identified the mutation even in samples for which flow cytometry could not detect B-cell clonality. This in fact could be explained by either the sensitivity of multiparameter flow cytometry or, as recently reported, the presence of the *MYD88* mutation in precursor lymphocytes.[7,8] Although our manuscript is not able to answer this question, we can infer from our data that the *MYD88* mutation is again a very early event. Further studies comparing next-generation flow cytometry to detect B-cell clonality along with ddPCR could help to solve this issue.

Using *MYD88* mutation burden as a continuous variable, we demonstrated that the mutation distribution was higher with successive disease stages. Moreover, *MYD88* mutation burden by ddPCR positively correlated with well-known biomarkers, such as involvement of the BM either evaluated by flow cytometry or morphology; therefore, it could be considered a specific biomarker that accurately reflects disease burden. In addition, inclusion of this novel biomarker along

with the classical laboratory features in early disease stages of WM might increase the predictive power of risk models.

Previous studies have found that nonsense *CXCR4* mutations were also recurrently prevalent in WM.[3] Among all variants, nonsense *CXCR4* S338* mutations were the most prevalent. Up to 50% are transversions C>G and C>A at nucleotide position 1013.[11,13,27,28] These variants have been associated with hyperviscosity and worst outcome regarding progression-free survival in WM patients.[3,11,27,29–31] However, most of these studies have been done in purified tumour BM samples. Due to the high sensitivity of ddPCR, we could detect the two most recurrent *CXCR4* mutations (C1013G and C1013A) in unsorted BM samples. To our knowledge, this is the first report of testing *CXCR4* mutations by ddPCR in IgM MGUS and SWM. We detected the *CXCR4* C1013G mutation in 35% of IgM MGUS cases and in 43% of SWM carrying also the *MYD88* mutation. The variant *CXCR4* C1013A was identified in fewer cases, all of them with low mutation burden. Most of the samples that harboured *CXCR4* mutations had a mutation burden lower than that of *MYD88*. The only three SWM cases that harboured higher *CXCR4* mutation burden did not differ in any other clinical or laboratory characteristic from the whole series, including *MYD88* mutation burden itself, which was quite similar. Moreover, together with two recent single-cell studies suggesting that *CXCR4* mutations behave as second clonal hits,[7,8] these three cases could be better explained by technical sample-processing issues. In addition, *CXCR4* C1013G positively correlated with the BM involvement, as was reported previously in $CD19^+$ selected BM cells in WM.[28] Here, we showed that ddPCR can reliably detect *CXCR4* mutations in unsorted BM cells, which, considering cell sorting is not feasible in most laboratories, makes it more easily transferable to the clinic.

Considering that *MYD88* testing is based on single BM samples for each patient and that BM disease distribution can be somehow heterogeneous and patchy, it could affect mutation detection. Therefore, we also analysed cfDNA in a small set of cases, following the promising results observed in WM.[21,32–34] We found that ddPCR was able to detect and quantify *MYD88* mutation in cfDNA in IgM MGUS and SWM, allowing us to infer correlation with other biomarkers. Regarding *CXCR4* mutations in cfDNA, previous reports have been mostly focused on WM samples[21,33]; while one study using a different sequencing approach reported that two out of nine IgM MGUS patients harboured *CXCR4* mutations.[32] In our study, it has been more difficult to show solid cfDNA data in asymptomatic IgM patients. This may be explained by the fact that *CXCR4* mutations are subclonal and that we have only assessed the two most common mutations. We consider that performing both ddPCR as well as deep next-generation sequencing in cfDNA could allow us to draw more conclusions on *CXCR4* mutation detection in samples with very low tumour burden. Taken together, we have demonstrated that cfDNA is a promising source of material for biomarker detection in IgM MGUS and SWM and thus can overcome diagnostic challenges such as performing BM biopsy especially in an ageing population.

Given the previous results, we analysed the impact of *MYD88* and *CXCR4* mutations on risk of progression to symptomatic WM. We established cut-off points for IgM MGUS and SWM, both individually and combined, in a competing-risk framework.[35] We found that the cumulative incidence of progression was higher with greater *MYD88* and *CXCR4* BM mutation burden, either analysed as continuous or categorical variables. Larger studies could demonstrate the independent impact of the mutation burden on the risk of progression compared to other well-known biomarkers related to tumour burden. Nevertheless, our data suggested that both the presence and the mutation BM tumour burden confer a greater risk of progression. Previous studies have shown nonconcordant results regarding the clinical impact of *MYD88* and *CXCR4* mutations in IgM MGUS and SWM.[10,14,15,18] The low number of patients tested, the technical issues regarding sample preparation in IgM MGUS and SWM, along with the heterogeneous histological features of wt *MYD88* cases,[36,37] might explain the variability of previous findings. Of note, we only observed disease progression to symptomatic WM and no other lymphoproliferative disorder or amyloid light-chain (AL) amyloidosis. Another important driver of disease progression is the presence of del(6q). Although we did not have data to draw conclusions about the interaction between *MYD88*, *CXCR4* and del(6q) in our series, we consider it a potential field of future research, partially solved recently by single-cell technology.[8] Regarding cfDNA mutation burden, we were unable to predict the risk of progression given that plasma samples were only collected in the last five years, and most of these patients have not yet progressed during the follow-up. Longer follow-up will help to elucidate if cfDNA could also predict the risk of progression.

Overall, our study establishes that *MYD88* and *CXCR4* mutations can be analysed by ddPCR with high sensitivity, making them excellent disease biomarkers for asymptomatic IgM monoclonal gammopathies in the clinic. The main advantages of ddPCR are that it can be applied in almost any academic centre, due to its easy setup, and that it avoids the need of sample sorting, making it an excellent candidate to replace standard AS-PCR for *MYD88* mutation analysis. We also showed that *MYD88* testing in cfDNA is a promising tool that might overcome diagnostic challenges. In addition, we propose the first genomic risk classification of asymptomatic IgM monoclonal gammopathies using novel techniques.

AUTHOR CONTRIBUTIONS

David F. Moreno, Mónica López-Guerra, Dolors Colomer, and Carlos Fernández de Larrea designed the research project, analysed and interpreted data, and wrote the manuscript. David F. Moreno, Carlos Fernández de Larrea, Laura Rosiñol, Joan Bladé, and María Teresa Cibeira recruited and followed the patients, and collected the clinical data from the patients' registry. Sara Paz, David F. Moreno, Mari-Pau Mena, Miguel Osuna, Alfredo Rivas-Delgado, and Oriol Cardús conducted the experiments. Aina Oliver-Caldés, Juan G. Correa, David F. Moreno, Luis Gerardo Rodríguez-Lobato,

Oriol Cardús, Anthony M. Battram, and Carlos Fernández de Larrea analysed the data. All authors reviewed and approved the final manuscript.

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CONFLICT OF INTERESTS

Joan Bladé: honoraria for lectures from Janssen, Celgene, Amgen, Takeda, and Oncopeptides. Laura Rosiñol: consulting fees from Amgen, Celgene, Sanofi, Janssen, and Takeda. Carlos Fernández de Larrea: advisory boards from Amgen, Janssen, and BMS; research grants from Janssen, BMS, Takeda, and Amgen; honoraria for lectures: BMS, Takeda, Sanofi, Amgen, Janssen, GSK, and Beigene. María Teresa Cibeira: honoraria from Amgen and Janssen. Luis Gerardo Rodríguez-Lobato: honoraria from Janssen and travel grants from Janssen and Amgen. David F. Moreno and Aina Oliver-Caldés: travel grants from Janssen. Mónica López-Guerra, Juan G. Correa, Dolors Colomer, Anthony M. Battram, Sara Paz, Mari-Pau Mena, Oriol Cardús, Raquel Jiménez-Segura, Natalia Tovar, Miguel Osuna, and Alfredo Rivas-Delgado have nothing to disclose.

The results from this study were presented as an oral presentation at the 63rd American Society of Haematology Annual Meeting (December, 2021).

DATA AVAILABILITY STATEMENT

Data are available on request to the corresponding authors, Dolors Colomer (dcolomer@clinic.cat) and Carlos Fernández de Larrea (cfernan1@clinic.cat).

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SUPPORTING INFORMATION

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