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Original
Quantitative MYD88 L265P and flow cytometry levels for outcome determination in IgM gammopathies: the SAL-TO study / Dogliotti, I., Jiménez, C., Peri, V., Ferrante, M., Musto, D., Mercadante, S., Zaccaria, G.M., Ghislieri, M., Benevolo, G., Ocio, E.M., Rubio-Martínez, A., Murillo, I., Escalante, F., Aguilera, C., García-Mateo, A., García De Coca, A., Hernández-Martin, R., Davila-Valls, J., Cavallo, F., Puig, N., et al.. - In: BLOOD ADVANCES. - ISSN 2473-9529. - ELETTRONICO. - (2026). [10.1182/bloodadvances.2025018435]

Publisher:
ASH Publications

Published
DOI:10.1182/bloodadvances.2025018435

blood advances

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Quantitative MYD88 L265P and flow cytometry levels for outcome determination in IgM gammopathies: the SAL-TO study

Tracking no: ADV-2025-018435R1

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Abstract:

Waldenström macroglobulinemia (WM) is a rare indolent B-cell lymphoproliferative disorder, often preceded by a history of IgM monoclonal gammopathy of undetermined significance (IgM-MGUS). In this retrospective multicentric study, we collected real-life data from 577 IgM gammopathy patients (221 symptomatic WM, sWM, 245 asymptomatic WM, aWM, 111 IgM-MGUS) from 22 Spanish Centers, with a validation cohort of 166 patients (73 sWM, 71 aWM, 22 IgM-MGUS) from University Hospital of Torino, Italy. Median overall survival (OS) was 126.7 months for the Spanish cohort and 202.8 for the Torino cohort. Multivariate analysis identified significant age > 65 years, male gender, diagnosis of sWM and beta-2-microglobulin >3 as significant predictors for shorter OS. Additionally, age > 65 years, bone marrow (BM) biopsy infiltration, haemoglobin <11.5 g/dL and platelets <100.000/mmc were associated with shorter time to first treatment (TTFT). Pooling data from both cohorts revealed that baseline BM quantitative MYD88 L265P/MYD88 WT ratio > 0.162 (either by ddPCR or quantitative PCR) together with multiparameter flow cytometry (MFC) infiltration >4.39% had a significant impact on OS and TTFT; the combination of MYD88 and MFC levels allowed to stratify patients into high-, intermediate-, and low-risk groups, with high-risk IgM gammopathy patients showing increased disease-related death in competing risk analysis.

Conflict of interest: COI declared - see note

COI notes: S.F. is a consultant for Janssen, EUSA Pharma, Abbvie and Sandoz; is on the advisory board of Janssen, EUSA Pharma, Recordati, Incyte, Roche, Astra Zeneca, Italfarmaco and Behring; received speaker's honoraria from Janssen, EUSA Pharma, Recordati, Lilly, Beigene, Gilead and Gentili; and received research funding from Gilead and Morphosys.

Preprint server: Yes; Blood https://doi.org/10.1182/blood-2024-197940

Author contributions and disclosures: D, JC, DD, RGS and SF: Conceptualization ID, VP, MF, DM, SM, SR, GMZ: Data curation ID, JC, VP, EA: Writing - original draft DM, SM, GMZ, MG: Methodology ID, VP, GB, EO, AR, IM, EF CA, AGM, AGC, RH, JD, FC, NP, VGC, SR, MC, CC, EA, MCC, AM, NCG, FS, RGS: Investigation SF, BB, MGD, RGS: Supervision Review and editing: all Authors

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: Data are available via email by the corresponding author

Clinical trial registration information (if any):

TITLE

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KEY WORDS

Waldenström’s Macroglobulinemia, IgM-MGUS, prognostic factors, MYD88, molecular medicine, flow cytometry

KEY POINTS

Combining MYD88 L265P and flow cytometry allowed stratification into risk groups with distinct survival outcomes

RUNNING TITLE

Laboratory prognostic tools in WM and IgM-MGUS

ACKNOWLEDGMENTS FOR RESEARCH SUPPORT

The authors thank I. Isidro, T. Prieto, A. Antón, R. Maldonado and M. Hernández for their technical assistance.

ID was supported by SIE (Società Italiana di Ematologia) and AIL (Associazione Italiana contro le Leucemie, Linfomi e Mieloma).

CJ was supported by Fundación Española de Hematología y Hemoterapia (FEHHJAN23/001H).

This work has been partially supported by the Instituto de Salud Carlos III through the project PI21/00568 (co-funded by the European Union).

This work has been partially supported by grants number PI18/01866, PI21/00568.

COMPETING INTEREST

GB: speaker’s bureau and advisory board: Janssen, BMS, GSK, Novartis.

All other authors declare that they have no potential conflicts of interest.

Data Sharing

Data are available via email by the corresponding author

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Key Point

Combining MYD88 L265P and flow cytometry allowed stratification into risk groups with distinct survival outcomes

Abstract

Waldenström macroglobulinemia (WM) is a rare indolent B-cell lymphoproliferative disorder, often preceded by a history of IgM monoclonal gammopathy of undetermined significance (IgM-MGUS). In this retrospective multicentric study, we collected real-life data from 577 IgM gammopathy patients (221 symptomatic WM, sWM, 245 asymptomatic WM, aWM, 111 IgM-MGUS) from 22 Spanish Centers, with a validation cohort of 166 patients (73 sWM, 71 aWM, 22 IgM-MGUS) from University Hospital of Torino, Italy. Median overall survival (OS) was 126.7 months for the Spanish cohort and 202.8 for the Torino cohort. Multivariate analysis identified significant age > 65 years, male gender, diagnosis of sWM and beta-2-microglobulin >3 as significant predictors for shorter OS. Additionally, age > 65 years, bone marrow (BM) biopsy infiltration, haemoglobin <11.5 g/dL and platelets <100.000/mmc were associated with shorter time to first treatment (TTFT). Pooling data from both cohorts revealed that baseline BM quantitative *MYD88* L265P/*MYD88* WT ratio > 0.162 (either by ddPCR or quantitative PCR) together with multiparameter flow cytometry (MFC) infiltration >4.39% had a significant impact on OS and TTFT; the combination of *MYD88* and MFC levels allowed to stratify patients into high-, intermediate-, and low-risk groups, with high-risk IgM gammopathy patients showing increased disease-related death in competing risk analysis.

Introduction

Waldenström macroglobulinemia (WM) is a rare indolent B-cell lymphoproliferative disorder characterized by bone marrow (BM) involvement by lymphoplasmacytic cells secreting monoclonal IgM protein.[1,2]

Although a familial predisposition has been demonstrated,[3] the main risk factor for WM remains a history of IgM monoclonal gammopathy of undetermined significance (IgM-MGUS),[4] with a probability of progression to WM or to other lymphoproliferative disorders of 1.5-2% per year.[4]

Important advances in the understanding of the biology of WM have been made in the last few years. By means of whole genome sequencing (WGS), Treon et al. identified *MYD88* L265P as a highly recurrent somatic mutation in 90% of patients with WM.[5] Several studies using different techniques, such as Sanger sequencing, polymerase chain reaction (PCR) and allele-specific qPCR (ASqPCR),[6,7,8] confirmed the presence of *MYD88* L265P mutation in WM; moreover, it was also demonstrated on BM samples in 50-80% of patients with IgM-MGUS[7,8,9,10] and it can also be identified in peripheral blood (PB),[11] in particular in cell-free DNA (cfDNA) of IgM gammopathy patients.[12,13]

From a clinical standpoint, WM typically shows an indolent clinical course, with about 25% of patients being asymptomatic at initial diagnosis, despite a heterogeneous disease behaviour. Indeed, for “smoldering” WM patients there are currently no indications for the initiation of treatment until the development of significant symptoms.[14,15,16] However, these patients have a high risk of transformation from smoldering WM to active WM and should be monitored closely.[17]

Over the years, many attempts have been made to predict IgM gammopathy patients’ outcomes based on clinical assessments, although applying different criteria for WM or IgM-MGUS diagnosis and for treatment initiation.[4,18,19,20,21,22,23] For active WM patients who require treatment, the International Prognostic Scoring System for WM (IPSS-WM) was designed to assess survival after treatment initiation and OS,[24] stratifying patients into 3 risk categories with corresponding 5-year survival rates of 87%, 68% and 36%, respectively. Later on, further revisions of the IPSS-WM refined prognostication in active WM[25,26], while in the field of asymptomatic WM (aWM) patients, Bustoros et al. developed a prognostic score for disease progression, depending on BMB evaluation and basic clinical findings.[27]

Some authors have also tried to assess the impact on patients’ outcome of laboratory prognostic factors, such as multiparameter flow cytometry (MFC) features[28], cytogenetics and molecular biology characterization. Even in absence of disease-defining cytogenetic abnormalities, deletions in the chromosome 6q are present in 40-50% of cases of WM patients, and have been shown to negatively impact on patients’ prognosis,[29,30] being associated with a higher IPSS-WM score and a shorter time to treatment, PFS and OS.[31,32,33,34]

The impact of molecular factors in determining WM patients’ prognosis is still not entirely elucidated: some studies have suggested that *MYD88* L265P mutation may impact on OS and play a role in disease progression, promoting the transition from IgM-MGUS to WM or to other lymphoprolipherative diseases;[9,27,35,36] on the contrary, other studies showed no effect on OS or time to progression[14] or underlined higher progression rates to symptomatic WM (sWM) and more aggressive clinical course in *MYD88*^{WT} patients.[27,36,37,38] Moreover, *CXCR4*^{WHIM} mutations, the second most frequent genetic alteration in WM, have been proven to impact on response to therapy and PFS,[39,40] without affecting OS.[33,37] Finally, similar to many other neoplasms, *TP53* alterations confer worse outcome in terms of OS,[40,41,42] irrespectively of IPSS-WM score; of note, such alterations were not identified in patients carrying IgM-MGUS.[41,43]

Aims

The study aims to: 1) collect a large series of IgM gammopathy patients treated in real life settings; 2) determine patients' outcomes in terms of OS, time to first treatment (TTFT) and PFS; 3) identify clinical factors affecting disease progression and survival; 4) evaluate the impact of baseline molecular and flow cytometry analyses impact on survival and need for WM treatment.

Materials and methods

Patients selection

Spanish cohort.

The registry of monoclonal gammopathies based in Salamanca and in the region of Castilla y Leon (Spain) was investigated for IgM-secreting disorders (Figure 1); only patients with confirmed IgM-MGUS, aWM or sWM[1] were selected for the present study. All patients provided written informed consent in accordance with Helsinki's declaration.

Torino cohort.

Electronic health records of patients followed up or treated at the Centre of Torino (Division of Haematology 1, Department of Biotechnology and Health Sciences, University Hospital Città della Salute e della Scienza, University of Torino, Italy) were scanned to identify patients with IgM monoclonal gammopathy; patients with confirmed diagnosis of IgM-MGUS, aWM or sWM were selected by the study team, using the same criteria employed for the Spanish cohort (figure 1). All patients provided written informed consent in accordance with Helsinki's declaration.

Sample collection and laboratory analysis

BM and PB samples were extracted from patients at first diagnosis of IgM gammopathy or at disease progression according to locally established procedures, for both study cohorts; BMB was performed per clinical practice, mainly when criteria for treatment initiation or suspicion of progressive/transformed disease were met.

Genomic DNA for molecular studies from evaluated patients was extracted as previously described and analysed for the presence of the *MYD88* L265P.[44] Alternatively, *MYD88* mutational status was determined by droplet digital PCR (ddPCR) on both BM and PB samples, as previously described.[12] All patients tested in Torino were analysed by ddPCR. Of note, neither cohorts applied CD19+ cells selection prior to molecular study, while it was only performed on selected cases in Spanish cohort prior to fluorescence in situ hybridization (FISH) analysis.

Deletions of 6q were assessed in IgM-MGUS and WM by either simple interphase FISH performed on cell nuclei from whole-BM samples or CD19-selected cells using a previously published technique.[31]

Immunophenotypic evaluation was done using conventional methods, panels of monoclonal antibodies previously described[45] and following the general recommendations of the EuroFlow group for the immunophenotypic evaluation of haematological malignancies. The sensitivity of MFC assay was 0,004% for at least 500000 events.

Statistical analyses

Statistical analyses were carried out using R (v 4.3.1). Survival curves were plotted with Kaplan–Meier method and compared with log-rank test. Medians between groups for continuous variables were compared by the Kruskal-Wallis (for non-normal variables) or the one-way ANOVA test (for normal variables); the chi-squared test or Fisher's exact test for small study samples, were employed for categorical variables. OS was measured from the date of initial diagnosis to the date of death from any cause. For asymptomatic cases, TTFT was defined as the time between diagnosis and progression to sWM requiring active treatment. PFS was defined as the time between first line WM treatment and the date of progression or death from any cause. The Cox proportional hazards model was implemented for the univariate and multivariate survival analyses. In particular, for the multivariable model, an AIC-based backward stepwise algorithm (R function stats::step) was used to perform the variable selection (restricting the dataset to have no missing data) in order to determine the most relevant covariates and to analyse potential confounding factors. Once the covariates of interest were obtained from the restricted dataset, they were applied to the full dataset with respect to each endpoint.

Given the larger size of the Spanish dataset, the statistical models were constructed using these data. Subsequently, the Torino dataset was merged to evaluate whether there were differences between the two populations. An analysis to evaluate the IPSS-WM and the R-IPSS WM scores was also performed on both cohorts (Spain, Torino). Moreover, the AWM score was tested in aWM patients from the Torino series.

In addition, we worked out a cut-off for quantitative *MYD88* and MFC infiltration in BM; the cut-off points were identified using the "surv_cutpoint" function in R, on Spanish dataset, and extended to Torino analysis.

Finally, competing risk analysis was performed on OS using "cuminc" function of "tidycmprsk" package of R.

IRB Approval: Salamanca University Hospital Ethic Committee, Paseo de San Vicente, 58-182 37007 Salamanca, Spain; EC number PI91/07/2018 (date of approval: 13/08/2018), and 21/742 (date of approval: 03/11/2021). Ethic Committee of the "A.O.U. CITTA' DELLA SALUTE E DELLA SCIENZA DI TORINO - A.O. ORDINE MAURIZIANO DI TORINO - A.S.L. CITT�DI TORINO", Corso Bramante, 88/90 - 10126 Torino, Italy; EC number 476/2021 (date of approval 25/11/21).

Results

Patients' characteristics

Data from 903 patients across 22 Spanish centers were collected between 1976 and 2019 in the considered registry; 577 cases with local diagnosis of IgM-MGUS, aWM or sWM were selected, while the remaining cases were excluded due to diagnosis of other lymphoprolipherative diseases, misregistration or missing data (figure 1). Among patients followed in Torino, Italy, 166 IgM gammopathy patients with available baseline and follow up data were selected according to the same criteria; of note, these patients were diagnosed from 1988 to 2020, although the vast majority (161/166) were diagnosed after the year 2000.

Demographic and clinical data at initial diagnosis are reported in table 1 (Spanish and Torino cohort).

Table 1. *Characteristics of IgM gammopathy patients enrolled in the study – Spanish and Torino cohort. Abbreviations: MGUS, monoclonal gammopathy of undetermined significance; WM, Waldenström Macroglobulinemia; Hb, hemoglobin; B2M, beta-2-microglobulin; IgM, immunoglobulin M; LDH, lactate dehydrogenase; Ig, immunoglobulin; k, free light kappa chain; l, free light lambda chain; IPSS-WM,*

International Prognostic Score System for Waldenström Macroglobulinemia; Int, intermediate; FISH, Fluorescence in situ hybridization

When they were first evaluated, 111 Spanish patients were locally diagnosed with IgM-MGUS, 245 with aWM and 221 with sWM. IPSS-WM score for sWM patients at diagnosis was low in 30 (18.2%), intermediate in 68 (41.2%), and high in 67 (40.6%) in the Spanish cohort; low in 10 (20.0%), intermediate in 18 (36.0%), high in 22 (44.0%) in the Torino cohort (p=0.804 for the distribution in the two cohorts).

Figure 1. *Consort diagram of enrolled patients in the two cohorts. Abbreviations: MGUS, monoclonal gammopathy of undetermined significance; aWM, asymptomatic Waldenström Macroglobulinemia; WM, Waldenström Macroglobulinemia; NA, not available; FU, follow-up*

Treatments

In the Spanish retrospective series, comprising patients followed from 1976 to 2019, 78/577 (13.5%) patients had missing treatment details in first line and were excluded from analysis of treatments received. Among 499 patients with available details of treatments received, 321/499 (64.3%) (diagnosed with IgM-MGUS of aWM) were followed-up in a watch and wait (W&W) approach, while 178/499 (35.7%) WM patients received initial therapy (supplementary table 1). At last follow-up, 208/321 (64.8%) patients remained asymptomatic, while 41/321 (12.8%) had progressed to WM and required active treatment; follow-up data were missing about 72 pts.
Most employed treatments in first line included chlorambucil-based regimens (n=98/178, 55%), dexamethasone-rituximab-cyclophosphamide scheme (DRC, n=19/178, 10.7%), fludarabine-containing regimens (n=6/178, 3.4%). Overall, 51/178 patients (28.6%) received rituximab in first line (single agent or any combination).

In the Torino cohort, 5/166 patients were excluded from analysis of treatment received for missing information; 60/161 patients (37.3%) remained asymptomatic and did not receive therapy during follow up. First line treatments used at any time included DRC (n=27/73, 37%), R-Benda (n=8/73, 11%), chlorambucil-based schemes (n=6/73, 8.2%). Rituximab was administered in first line to 69.8% of treated patients.

Survival analyses

OS

In the Spanish cohort, at last follow up (FU), 305 (52.9%) patients were alive, with a median FU of 100.6 months (IQR 55.5;166.6). Median OS was 126.7 months (IQR: 58.7;193.6); OS at 5 years was 74.0% (95%CI 70.1-78.2), and at 10 years 52.5% (95%CI 47.3-58.2%) (figure 2a). Median OS for IgM-MGUS vs. aWM vs. sWM patients was 180.6 (IQR 111.3;–) vs. 143.6 (80.9;199.7) vs. 85.0 (38.8;151.6) months, (p<0.001, HR for death in SWM group: 2.481) in univariate analysis (figure 2c).

In the Torino cohort, at last FU, 132 (79.5%) patients were alive, with a median FU of 71.2 (IQR 39.6; 115.4) months. Median OS was 202.8 months (IQR 109.0;–); OS at 5 years was 85.7% (95% CI 80.0-91.8), and 71.6% (61.7-83.1) at 10 years from initial diagnosis (figure 2b). Median OS stratified for initial diagnosis was 131.0 (131.0;–) months for IgM-MGUS, not-reached (131.4;–) for aWM, 174.4 (65.9;–) months for sWM (p=0.084) (figure 2).

Figure 2. *Overall survival of Spanish cohort (A) and Torino cohort (B); overall survival stratified for initial diagnosis of MGUS, aWM or sWM (C, D)*

TTFT

TTFT was evaluated for all asymptomatic patients at baseline, including IgM-MGUS and aWM; for the Spanish cohort, median TTFT was 228.5 months (IQR 146.9;–), with 89.1% of patients not requiring treatment after 5 years of FU (95%CI 85.5-92.9). For patients followed up in Torino, median TTFT was 165.6 months (IQR 49.4;207.9), and probability to remain asymptomatic was 71.4% at 5 years (95%CI 60.9-83.7) (figure 3).

Figure 3. *Time to first treatment in asymptomatic patients belonging to Spanish cohort (A) and Torino cohort (B) (top panels); Progression free survival in patients requiring treatment at any time, belonging to Spanish cohort (A) and Torino cohort (B) (bottom panels)*

PFS

PFS was evaluated in patients receiving treatment for symptomatic WM at any time; among patients with available data, median PFS was 42.4 months (IQR 13.6;91.0) for Spanish cohort, and 38.0 months (IQR 13.9;75.6) for Torino cohort (figure 3, bottom panels). At 2 years, PFS probability was 64.6% (95%CI 58.8-70.9) and 63.3% (54.2-73.8), respectively. Importantly, there was no difference in PFS outcome between patients who received active treatment at diagnosis and patients initially undergoing watch and wait approach and then progressing to WM (p=0.34 and p=0.24 in the 2 cohorts).

Finally, 22/577 (4.8%) and 5/166 (3.1%) patients in the two examined cohorts experienced transformation from IgM gammopathy to aggressive lymphoma during FU, showing very poor survival (figure S1). No significant difference in the incidence of transformation in the two groups was highlighted (p=0.51).

Clinical prognostic factors

Clinical data at baseline, including age, sex, IgM levels, haemoglobin (Hb), platelets (Plts), ECOG PS, presence of hyperviscosity, BMB infiltration, albumin, beta2microglobulin (B2M), LDH, *MYD88* L265P mutational ratio, MFC infiltration in BM, were tested for prognostic significance in terms of OS, TTFT (for asymptomatic patients) and PFS (for patients treated at any time); significant variables for each cohort are resumed in table 3.

A multivariable model for OS, PFS, and TTFT (resumed in table 2) was built, as previously described, for the Spanish cohort. This model was then used to evaluate a merged population (Spain + Torino) in order to spot eventual differences between the two cohorts. Since a p-value >0.05 was found, we may conclude that there is no evidence of difference between these groups.
For OS prediction, the algorithm identified age > 65 years, male sex, initial diagnosis of SWM and B2M >3 as statistically significant variables. On the other hand, multivariable analysis for TTFT was built for asymptomatic patients (IgM-MGUS, aWM), producing a model with age, BMB infiltration, Hb <11.5 g/dL and Plts <100.000/mmc as significant variables. Finally, multivariable analysis for PFS in symptomatic WM patients (SWM at diagnosis + patients progressing from watch and wait approach to SWM) was performed, and age and LDH ratio resulted statistically significant.

In addition, we evaluated IPSS-WM and R-IPSS-WM scores in Spanish WM patients and found that they both retained statistical significance (p <0.001 for both scores, supplementary figure 2), confirmed also in multivariable analysis; on the other hand, AWM score could not be tested in aWM patients from the Torino series due to low number of patients with available complete data (n=29).

Table 2. *Multivariable analysis for OS, TTFT and PFS in Spanish and Torino cohort. Only variables with statistically significant effect for multivariable models are shown. Abbreviations: HR, hazard ratio; IC, interval of confidence; p, p-value; Hb, hemoglobin; BMB, bone marrow biopsy; B2M, beta-2-microglobulin; IgM, immunglobulin M; LDH, lactate dehydrogenase; IPSSWM, International Prognostic Score System for Waldenström Macroglobulinemia; MGUS, monoclonal gammopathy of undetermined significance; aWM, asymptomatic Waldenström Macroglobulinemia; sWM, symptomatic Waldenström Macroglobulinemia.*

Prognostic significance of molecular and flow cytometry factors

For patients with available BM *MYD88* L265P evaluation at baseline (n=298, 51.6% for Spanish cohort, n= 142, 85.5% for Torino cohort), we analysed outcome in terms of OS and TTFT according to both quantitative PCR methods results.

Patients from Spanish cohort and Torino cohort were pooled and divided into high vs low *MYD88* groups (cutpoint set at 0.162, defined as previously described).

In univariate analysis, MYD88^{low} group showed better OS (p=0.005, HR=0.44) and TTFT (p=0.024, HR=0.33) compared to MYD88^{high} group (table 3). In multivariable analysis, statistical significance was not confirmed (p=0.622) for OS, although there was a trend to significance for TTFT (p=0.06).

Regarding the prognostic impact of MYD88 on OS specifically in IgM-MGUS patients, data were available on 37 patients, classified as MYD88 low (n=17) or MYD88WT (n=20), while no patient scored MYD88 high; no difference in OS was observed in univariate analysis between these two subgroups (p=0.2, supplementary figure 6).

Additionally, molecular burden was compared to the percentage of marrow infiltration by BMB (available only for Torino cohort), showing a moderate positive Pearson's correlation (r=0.653, p<0.0001), as well as to the infiltration reported by BM MFC, showing a strong positive linear correlation (r = 0.73, p < 0.0001). Patients in the MYD88^{high} group generally showed higher BMB infiltration (approximately 70 to 90%), though rare outliers with low marrow infiltration (as low as 20%) were observed, indicating possible sampling errors as well as biological heterogeneity.

The pooled population was also analysed for the prognostic significance of baseline clonal B cell infiltration by MFC in available BM samples, with cut-off point set at 4.39%. In univariable analysis, MFC^{low} patients had increased OS (p=0.033, HR=0.65) and TTFT (p=0.008, HR=0.37) compared to MFC^{high} group (table 3). In multivariable analysis MFC lost statistical significance for OS (p=0.51), while it retained its importance for TTFT (p=0.025, HR=0.37). For IgM-MGUS patients, similarly, MFC burden affected TTFT (p=0.006, supplementary figure 7) but did not show impact on OS (p=0.5).

Table 3. *Univariate analysis for OS, TTFT and PFS for molecular and flow cytometry variables in Spanish and Torino cohort. Abbreviations: HR, hazard ratio; IC, interval of confidence; p, p-value*

In addition, we combined both MFC and *MYD88* baseline levels, and patients were stratified into low, intermediate and high risk (LR: MFC^{low}/*MYD88*^{low}, IR: either MFC^{low}/*MYD88*^{high} or MFC^{high}/*MYD88*^{low}; HR: MFC^{high}/*MYD88*^{high}). Based on MFC and molecular results, no IgM-MGUS patient was assigned to the HR group; 16 HR patients had sWM while 8 had aWM;

moreover, among sWM patients with high IPSS-WM risk, 7 patients were in the LR group, 18 patients in the intermediate group and 6 in the HR group.

In univariate analysis, HR group significantly differed from the others in terms of OS (n=156, p=0.005, HR=3.28, figure 4) and TTFT (n=92, p=0.003, HR=9.61); for TTFT, intermediate-risk group (p=0.015, HR=4.76) was also significantly different from reference group (low, supplementary figure 3).

Multivariable analysis confirmed these results, highlighting the strong prognostic significance of molecular and MFC evaluations combined (for OS: p=0.038, HR=2.81; for TTFT: p=0.026, HR=7.91 for intermediate vs. low risk and p=0.003, HR=24.68 for high vs. low risk).

Finally, competing risk analysis was performed; among 545 evaluable cases, at 5 years from diagnosis 12.0% (95%CI:9.6%, 16%) deaths were not related to WM, while 7.1% (95%CI: 5.0%, 9.6%) were considered related (supplementary figure 4); among patients in the *MYD88*/MFC HR group, there was a statistically significant increase in disease-related, but not unrelated deaths at 5 years (p=0.002, 2.2%, 95%CI 0.17%-10% vs 19%, 95%CI 5.6%-38% for LR vs HR respectively, supplementary figure 5).

Figure 4. *Outcome in terms of OS according to BM MYD88^{L65P} levels and to BM MFC levels*

Discussion

Previous real life studies reporting on survival outcomes of WM patients, for instance the Rory Morrison Registry in the UK and the Swedish lymphoma registry, showed a five-year OS ranging from 60 to 90%, both with a median follow-up of around 6 years;[20,46] in our series, including also asymptomatic WM and IgM-MGUS, 5-years OS was comparable (85% for the Torino cohort, 74% for the Spanish cohort), after a median FU of 71 vs 100 months respectively. Of note, the slightly superior OS outcome of the Torino cohort might be explained by the more recent IgM gammopathy diagnosis and the more extensive adoption of rituximab-containing regimens in first and subsequent treatment lines.

However, in published registry studies, molecular evaluations and MFC data were limited or not available, so prognostic evaluation relied solely on clinical factors. Indeed, prognostication for symptomatic WM was historically established based on the IPSS-WM score and its revised version,[24,25] stratifying patients based on age and baseline characteristics.

In our study, both IPSS-WM and R-IPSS-WM were confirmed able to correctly identify sWM patients' risk, while for aWM, the score proposed by Bustoros et al was not reproducible, probably due to the low number of patients with data about BMB percentage of invasion available.[27] Moreover, analysing the whole IgM gammopathy series (IgM-MGUS, aWM and sWM taken together), clinical factors were confirmed important both for OS (age, sex, B2M and diagnosis) and TTFT (age, Hb, Plts and BMB involvement). Indeed, IPSS-WM was initially built at a time (prior to 2002) when nucleoside analogues represented around one third of first line therapies, while rituximab was only administered to 4% of patients; nevertheless, it has been still confirmed a feasible and powerful tool. However, in the rapidly changing field of WM, where both understanding of its biology and treatment were revolutionized by the discovery of *MYD88* L265P mutation, the need to incorporate translational knowledge to prognostic assessment is still unmet. Indeed, while *MYD88* L265P evaluation is already considered as part of standard initial evaluation in suspected WM, particularly helpful in the differential diagnosis with other lymphoproliferative neoplasms, its role in prognostication is still debated.

Actually, previous findings have highlighted that *MYD88*^{WT} WM patients have inferior survival outcomes,[36] and show slower and less profound responses with Bruton's tyrosine kinase inhibitor (BTKi), especially with ibrutinib;[40] on the other hand, a large cohort study by Abeykoon et al. did not show inferior OS or time to next treatment in *MYD88*^{WT} vs *MYD88* L265P WM patients, while there was an increased occurrence of transformation to diffuse large B-cell lymphoma (DLBCL).[14]

For IgM-MGUS patients, a study by Varettoni et al. showed lower progression rates to overt WM in *MYD88*^{WT} compared to *MYD88* L265P cases.[9] In the same study, *MYD88* L265P allele burden by ASqPCR on BM CD19+ selected mononuclear cells showed a significant correlation with progression from MGUS to WM, albeit with a mild effect; these findings were later confirmed by a recent study by Moreno et al. using ddPCR in IgM-MGUS and aWM.[47] Of note, for IgM-MGUS patients, reproducibility of prognostic effect of molecular factors might be affected by low disease burden; the use of highly sensitive techniques is therefore key to correctly estimate prognosis. Indeed, past studies have shown heterogeneous results due to different PCR techniques, the variable application of CD19+ sorting and the adoption of different diagnostic criteria[48].

To date, however, there were no published data regarding the effect of high versus low *MYD88* L265P baseline levels on OS and TTFT in WM patients. In our study, highly sensitive techniques for *MYD88* quantitative evaluation, either by ddPCR or ASqPCR, together with MFC analysis, allowed for a precise definition of disease burden at the time of IgM gammopathy diagnosis. Interestingly, the identified MFC cut-off points (4.39% clonal B cell infiltration in BM) to separate high vs low disease burden was actually quite low, close to the median results in aWM subgroup (3.55%). On the contrary, *MYD88* L265P cut-off point (ratio MUT/WT=0.162 (AF 13,4%)) was higher than median *MYD88* L265P identified in sWM group (ratio MUT/WT=0.04 (AF 3.85%)), thus identifying patients with very high molecular disease burden. We also observed a strong correlation of *MYD88* L265P results with BM MFC infiltration, higher than the one between *MYD88* L265P and the percentage of infiltration in BMB by conventional histopathology observation.

From a technical standpoint, molecular analysis was not homogeneous between the two cohorts: all Torino samples were analyzed using ddPCR, which offers higher sensitivity, whereas the vast majority (>95%) of Spanish samples were assessed by ASqPCR, reflecting clinical practice at the time of initial diagnosis. Although both techniques correctly identified molecular high-risk patients, this discrepancy represents a limitation of our retrospective, real-world study and highlights the absence of standardized molecular approaches in this disease. This lack of harmonization underscores the need for future efforts aimed at method standardization to ensure comparability and consistency across studies and clinical settings.

By multivariable analysis, the combination of MFC and molecular variables identified a high-risk group consisting in around 15% of the evaluable patients. Interestingly, a minority of aWM cases (around 9%), but no IgM-MGUS patients, was classified as HR in terms of the *MYD88*/MFC score: thus, it is conceivable that this kind of evaluation could help represent the risk of progression and disease-related death even in asymptomatic patients. Indeed, a significant fraction (around 22%) of the patients who scored high at IPSS-WM was re-classified as LR by the molecular/MFC score.

Conclusions

To our knowledge, this is the first time quantitative *MYD88* L265P evaluation and MFC degree of infiltration in BM were proven prognostic of patients' survival and need for treatment in a large IgM gammopathy series; moreover, molecular and flow cytometry results have never been combined before to generate translational data. Validation of these results in prospective studies might add greatly to prognostic evaluation of IgM gammopathy, particularly to improve definition of high-risk WM patients.

Author Contribution Statement

D, JC, DD, RGS and SF: Conceptualization
ID, VP, MF, DM, SM, SR, GMZ: Data curation
ID, JC, VP, EA: Writing - original draft
DM, SM, GMZ, MG: Methodology
ID, VP, GB, EO, AR, IM, EF CA, AGM, AGC, RH, JD, FC, NP, VGC, SR, MC, CC, EA, MCC, AM, NCG, FS, RGS: Investigation
SF, BB, MGD, RGS: Supervision
Review and editing: all Authors

Conflict of Interest Statement

S.F. is a consultant for Janssen, EUSA Pharma, Abbvie and Sandoz; is on the advisory board of Janssen, EUSA Pharma, Recordati, Incyte, Roche, Astra Zeneca, Italfarmaco and Behring; received speaker's honoraria from Janssen, EUSA Pharma, Recordati, Lilly, Beigene, Gilead and Gentili; and received research funding from Gilead and Morphosys.

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Tables

Spanish cohort

	IgM MGUS (n=111)	asymptomatic WM (n=245)	symptomatic WM (n=221)	p-value
Age (Median [IQR])	69.5 [29.0, 87.0]	72.0 [34.0, 93.0]	71.0 [30.0, 94.0]	0.070
Sex				
Female	46 (41.4%)	88 (35.9%)	67 (30.3%)	0.120
Male	65 (58.6%)	157 (64.1%)	154 (69.7%)	
Hb (Median [IQR])	13.9 [8.1, 17.4]	13.2 [6.0, 20.0]	10.4 [3.6, 18.0]	<0.001
ECOG				
0	68 (61.3%)	136 (55.5%)	46 (20.8%)	<0.001
1	19 (17.1%)	55 (22.4%)	74 (33.5%)	
2	3 (2.7%)	12 (4.9%)	48 (21.7%)	
3	0 (0%)	3 (1.2%)	10 (4.5%)	
4	1 (0.9%)	2 (0.8%)	2 (0.9%)	
Missing	20 (18.0%)	37 (15.1%)	41 (18.6%)	
B symptoms				
No	92 (82.9%)	203 (82.9%)	117 (52.9%)	<0.001
Yes	1 (0.9%)	7 (2.9%)	73 (33.0%)	
Missing	18 (16.2%)	35 (14.3%)	31 (14.0%)	
Platelets (Median [IQR])	227 [73, 1970]	248 [30, 644]	210 [2, 684]	<0.001
B2M ((Median [IQR])	2.00 [0.5, 6.4]	2.46 [0.15, 18.0]	3.26 [0.4, 18.6]	<0.001
Missing	21 (18.9%)	55 (22.4%)	45 (20.4%)	
Albumin (Median [IQR])	4.10 [2.9, 5.0]	3.90 [2.3, 5.2]	3.60 [1.6, 4.9]	<0.001
Missing	20 (18.0%)	38 (15.5%)	32 (14.5%)	
IgM (Median [IQR])	781 [87, 6130]	1460 [225, 9220]	3300 [19.0, 13000]	<0.001
Missing	9 (8.1%)	23 (9.4%)	22 (10.0%)	
LDH ratio (Median [IQR])	0.73 [0.27, 1.8]	0.65 [0.28, 1.76]	0.65 [0.25, 4.0]	0.043
Missing	18 (16.2%)	35 (14.3%)	35 (15.8%)	
Light Ig type				
K	69 (62.2%)	160 (65.3%)	157 (71.0%)	0.039
L	37 (33.3%)	62 (25.3%)	38 (17.2%)	
Negative	0 (0%)	2 (0.8%)	1 (0.5%)	
Missing	5 (4.5%)	21 (8.6%)	25 (11.3%)	
IPSS-WM				
High	0 (0%)	13 (5.3%)	67 (30.3%)	<0.001
Int	45 (40.5%)	126 (51.4%)	68 (30.8%)	
Low	30 (27.0%)	45 (18.4%)	30 (13.6%)	
Missing	36 (32.4%)	61 (24.9%)	56 (25.3%)	
FISH				
Abnormal	4 (3.6%)	20 (8.1%)	34 (15.5%)	0.001
Normal	26 (23.4%)	93 (38.0%)	65 (29.5%)	
Not evaluable	81 (73%)	132 (53.9%)	121 (54.1%)	
***MYD88* L265P mutation**				
Negative	20 (18.0%)	25 (10.2%)	26 (11.8%)	<0.001
Positive	18 (16.2%)	111 (45.3%)	98 (44.3%)	
Not evaluable	73 (65.8%)	109 (44.5%)	97 (43.9%)	
***MYD88* L265P mutational ratio** (Median [IQR])	0.02 [0.001, 0.0868]	0.0165 [0.00131, 1.76]	0.0362 [0.00158, 1.36]	0.004

Torino cohort

	IgM MGUS (n=22)	asymptomatic WM (n=71)	symptomatic WM (n=73)	p-value
Age (Median [IQR])	73.0 [48.0, 91.0]	67.0 [24.0, 84.0]	70.0 [43.0, 95.0]	0.091
Sex				
Female	8 (36.4%)	33 (46.5%)	24 (32.9%)	0.237
Male	14 (63.6%)	38 (53.5%)	49 (67.1%)	

Hb (Median [IQR])	10.4 [3.60, 18.0]	14.0 [8.30, 16.0]	13.0 [0, 18.0]	<0.001
ECOG				
0	16 (72.7%)	59 (83.1%)	30 (41.1%)	<0.001
1	4 (18.2%)	8 (11.3%)	15 (20.5%)	
2	1 (4.5%)	1 (1.4%)	10 (13.7%)	
3	0 (0%)	0 (0%)	7 (9.6%)	
4	0 (0%)	0 (0%)	1 (1.4%)	
Missing	1 (4.5%)	3 (4.2%)	10 (13.7%)	
BMB% (Median [IQR])	0 [0, 0]	30.0 [10.0, 90.0]	70.0 [10.0, 95.0]	<0.001
Missing	15 (68.2%)	8 (11.3%)	10 (13.7%)	
B symptoms				
No	20 (90.9%)	69 (97.2%)	53 (72.6%)	0.002
Yes	1 (4.5%)	0 (0%)	10 (13.7%)	
	1 (4.5%)	2 (2.8%)	10 (13.7%)	
Platelets (Median [IQR])	209 [114, 315]	260 [71.0, 632]	203 [29.0, 543]	0.013
B2m ((Median [IQR])	2.52 [1.4, 6.6]	1.95 [1.1, 13.2]	3.10 [1.5, 12.6]	0.002
Missing	10 (45.5%)	23 (32.4%)	42 (57.5%)	
Albumin (Median [IQR])	3.80 [3.0, 5.1]	4.00 [3.0, 5.3]	3.75 [2.5, 4.8]	0.012
Missing	11 (50.0%)	34 (47.9%)	35 (47.9%)	
IgM (Median [IQR])	781 [195, 5360]	1370 [210, 5480]	2960 [205, 9620]	<0.001
Missing	0 (0%)	5 (7.0%)	11 (15.1%)	
LDH (Median [IQR])	0.8 [0.3, 1.8]	0.8 [0.3, 1.4]	0.7 [0.4, 2.4]	0.554
	8 (36.4%)	25 (35.2%)	22 (30.1%)	
Light Ig type				
K	15 (68.2%)	50 (70.4%)	52 (71.2%)	0.982
L	4 (18.2%)	15 (21.1%)	15 (20.5%)	
Missing	3 (13.6%)	6 (8.5%)	6 (8.2%)	
IPSS WM				
High	0 (0%)	4 (5.6%)	22 (30.1%)	<0.001
Int	15 (68.2%)	30 (42.3%)	18 (24.7%)	
Low	6 (27.3%)	23 (32.4%)	10 (13.7%)	
Missing	1 (4.5%)	14 (19.7%)	23 (31.5%)	
FISH				
Abnormal	0 (0%)	2 (2.8%)	2 (2.9%)	0.193
Normal	0 (0%)	2 (2.8%)	0 (0%)	
Not evaluable	22 (100%)	67 (94.4%)	68 (97.1%)	
***MYD88* L265P mutation**				
Negative	3 (13.6%)	5 (7.0%)	10 (13.7%)	0.376
Positive	11 (50.0%)	50 (70.4%)	53 (72.6%)	
Missing	8 (36.4%)	16 (22.5%)	10 (13.7%)	
***MYD88* L265P mutational ratio (**Median [IQR])	0.00497 [0.000551, 0.0371]	0.0216 [0.000520, 0.283]	0.0278 [0.000400, 0.713]	0.003

Table 1. *Characteristics of IgM gammopathy patients enrolled in the study – Spanish and Torino cohort. Abbreviations: MGUS, monoclonal gammopathy of undetermined significance; WM, Waldenström Macroglobulinemia; Hb, hemoglobin; B2M, beta-2-microglobulin; IgM, immunoglobulin M; LDH, lactate dehydrogenase; Ig, immunoglobulin; k, free light kappa chain; l, free light lambda chain; IPSS-WM, International Prognostic Score System for Waldenström Macroglobulinemia; Int, intermediate; FISH, Fluorescence in situ hybridization*

Spanish cohort									
	OS			**PFS**			**TTFT**		
	HR	**95% IC**	**p**	**HR**	**95% IC**	**p**	**HR**	**95%IC**	**p**
Age									
≤65	-	-		-	-		-	-	
65-75	2.221	1.514-3.260	**<0.001**	1.389	0.931-2.072	0.107	1.002	0.483-2.083	0.996
≥76	4.136	2.812-6.085	**<0.001**	2.561	1.675-3.918	**<0.001**	0.238	0.075-0.757	**0.015**
Sex									
Female	-	-					-	-	

Male	1.651	1.192-2.287	**0.003**				2.115	0.971-4.606	0.059
Hb									
≤11,5							-	-	
>11,5							0.353	0.136-0.918	**0.033**
Diagnosis code									
MGUS	-	-							
aWM	1.172	0.722-1.902	0.521						
sWM	2.086	1.277-3.407	**0.003**						
BMB categ									
Not evaluable				-	-		-	-	
Negative				0.937	0.423-2.074	0.873	1.498	0.523-4.291	0.452
Positive				1.332	0.950-1.869	0.096	2.902	1.337-6.298	**0.007**
Platelets									
≤100				-	-	-	-	-	
>100				1.567	0.950-1.869	0.091	0.096	0.027-0.345	**<0.001**
B2m									
≤3	-	-		-	-				
>3	1.617	1.200-2.180	**0.002**	1.262	0.905-1.761	0.171			
Albumin									
<3,5							-	-	-
≥3,5							0.449	0,200-1.010	0,053
LDH									
≤1				-	-				
>1				0.533	0.326-0.872	**0.012**			
Hyperviscosity									
No	-	-							
Yes	1.407	0.943-2.098	0.094						
				Torino cohort					
	OS			**PFS**			**TTFT**		
	HR	**95% IC**	**p**	**HR**	**95% IC**	**p**	**HR**	**95%IC**	**p**
Age									
≤65	-	-		-	-		-	-	
65-75	2.221	1.514-3.260	**<0.001**	1.389	0.931-2.072	0.107	1.002	0.483-2.083	0.996
≥76	4.136	2.812-6.085	**<0.001**	2.561	1.675-3.918	**<0.001**	0.238	0.075-0.757	**0.015**
Sex									
Female	-	-					-	-	
Male	1.651	1.192-2.287	**0.003**				2.115	0.971-4.606	0.059
Hb									
≤11,5							-	-	
>11,5							0.353	0.136-0.918	**0.033**
Diagnosis code									
MGUS	-	-							
aWM	1.172	0.722-1.902	0.521						
sWM	2.086	1.277-3.407	**0.003**						
BMB categ									
Not evaluable				-	-		-	-	
Negative				0.937	0.423-2.074	0.873	1.498	0.523-4.291	0.452
Positive				1.332	0.950-1.869	0.096	2.902	1.337-6.298	**0.007**
Platelets									
≤100				-	-	-	-	-	
>100				1.567	0.950-1.869	0.091	0.096	0.027-0.345	**<0.001**
B2m									
≤3	-	-		-	-				
>3	1.617	1.200-2.180	**0.002**	1.262	0.905-1.761	0.171			
Albumin									
<3,5							-	-	-
≥3,5							0.449	0.200-1.010	0.053
LDH									
≤1				-	-				
>1				0.533	0.326-0.872	**0.012**			
Hyperviscosity									
No	-	-							
Yes	1.407	0.943-2.098	0.094						

Table 2. *Multivariable analysis for OS, TTFT and PFS in Spanish and Torino cohort. Only variables with statistically significant effect for multivariable models are shown. Abbreviations: HR, hazard ratio; IC, interval of confidence; p, p-value; Hb, hemoglobin; BMB, bone marrow biopsy; B2M, beta-2-microglobulin; IgM, immunglobulin M; LDH, lactate dehydrogenase; IPSSWM, International Prognostic Score System for Waldenström Macroglobulinemia; MGUS, monoclonal gammopathy of undetermined significance; aWM, asymptomatic Waldenström Macroglobulinemia; sWM, symptomatic Waldenström Macroglobulinemia.*

Spanish cohort									
	OS			**PFS**			**TTFT**		
	HR	**95% IC**	**p**	**HR**	**95% IC**	**p**	**HR**	**95% IC**	**p**
***MYD88* ratio – cutoff >0,1624318**									
Neg	-		-	-		-	-		-
Low	0.995		0.982	0.789		0.333	0.864		0.754
High	1.823		0.061	1.013		0.969	3.478		**0.049**
Flow ratio - cutoff >4,39%									
Neg	-		-	-		-	-		-
Low	0.912		0.792	**<0.001**		0.995	3.215		0.268
High	1.421		0.279	**<0.001**		0.995	7.196		0.056
Torino cohort									
	OS			**PFS**			**TTFT**		
	HR	**95% IC**	**p**	**HR**	**95% IC**	**p**	**HR**	**95% IC**	**p**
***MYD88* ratio – cutoff >0,1624318**									
Neg	-		-	-		-	-		-
Low	1.153		0.822	0.811		0.575	0.724		0.670
High	3.063		0.16	1.430		0.434	2.874		0.395
Flow ratio - cutoff >4,39%									
Neg	-		-	-		-	-		-
Low	**<0.001**		0.998	**<0.001**		0.997	0.35		0.396
High	**<0.001**		0.998	**<0.001**		0.997	1.48		0.621

Table 3. *Univariate analysis for OS, TTFT and PFS for molecular and flow cytometry variables in Spanish and Torino cohort. Abbreviations: HR, hazard ratio; IC, interval of confidence; p, p-value*

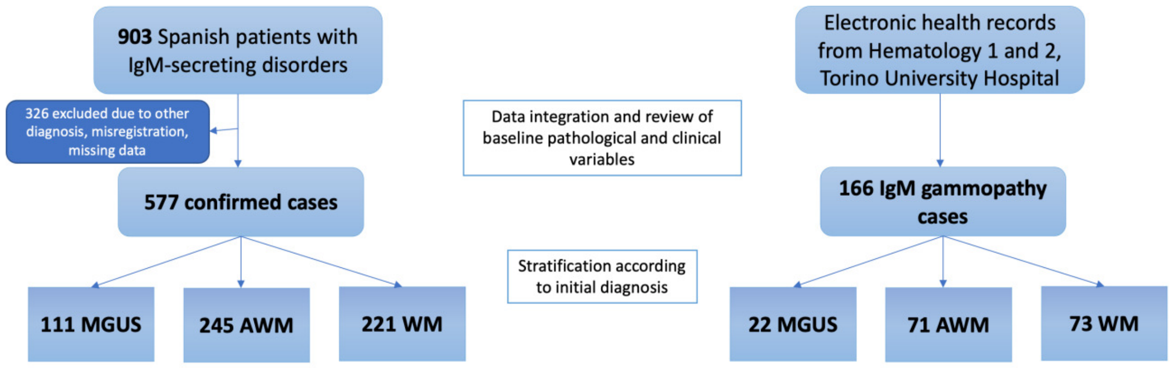


Figure 1. *Consort diagram of enrolled patients in the two cohorts. Abbreviations: MGUS, monoclonal gammopathy of undetermined significance; aWM, asymptomatic Waldenström Macroglobulinemia; WM, Waldenström Macroglobulinemia; NA, not available; FU, follow-up*

Figure 2

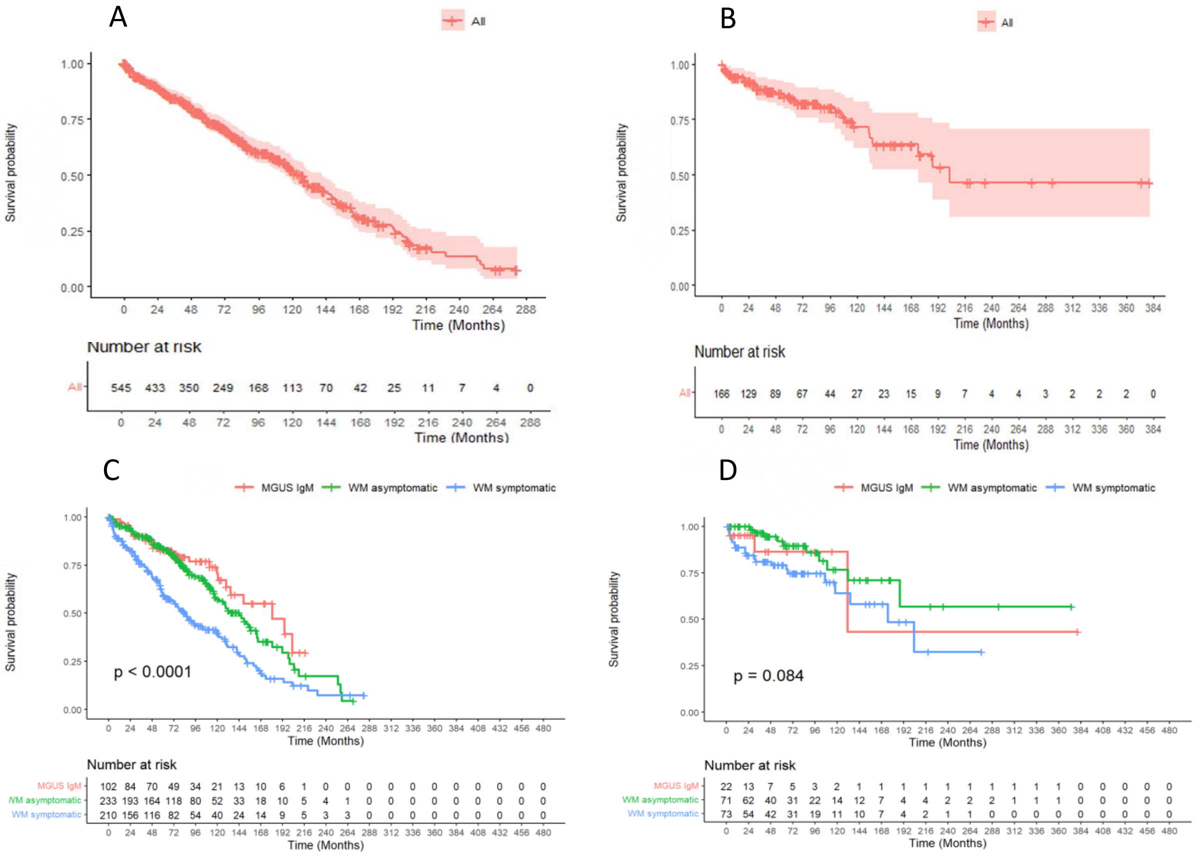


Figure 2. *Overall survival of Spanish cohort (A) and Torino cohort (B); overall survival stratified for initial diagnosis of MGUS, aWM or sWM (C, D)*

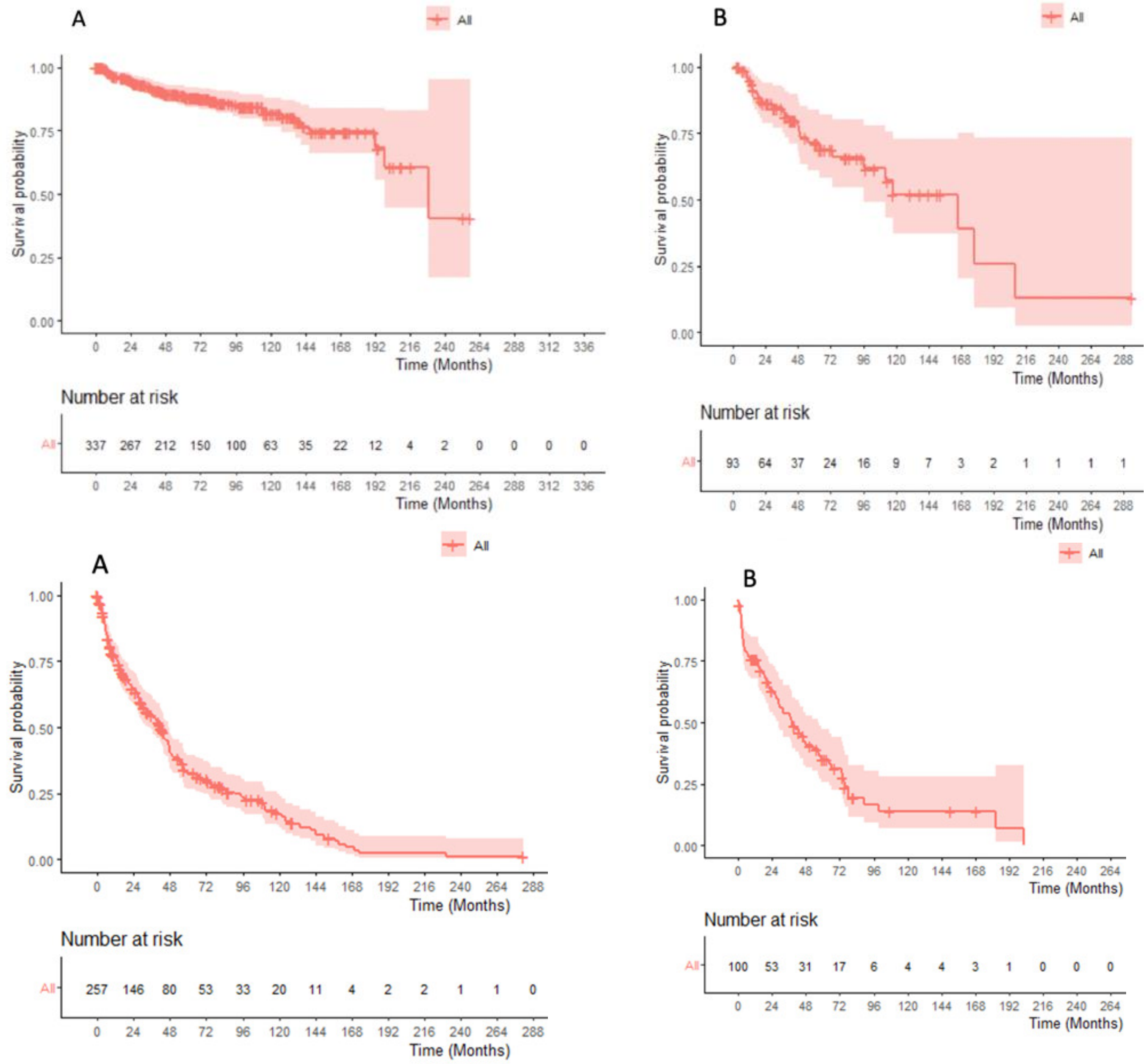
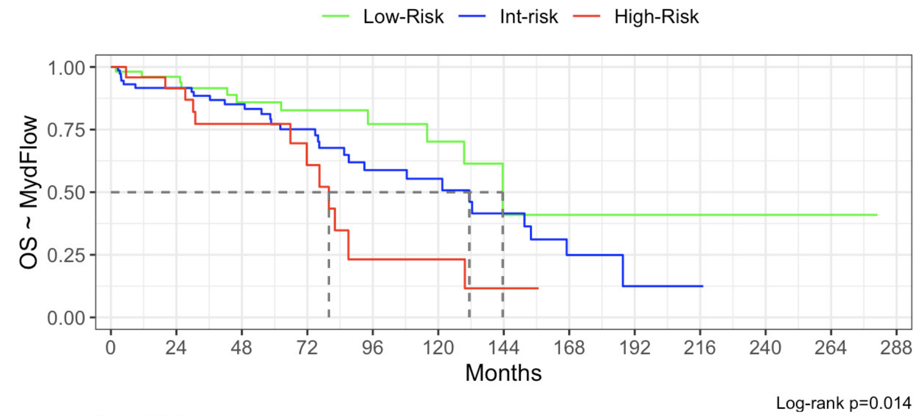


Figure 3. *Time to first treatment in asymptomatic patients belonging to Spanish cohort (A) and Torino cohort (B) (top panels); Progression free survival in patients requiring treatment at any time, belonging to Spanish cohort (A) and Torino cohort (B) (bottom panels)*

Figure 4



Low-Risk

At Risk	55	43	28	20	14	9	2	1	1	1	1	1	0
Events	0	2	6	7	8	9	11	11	11	11	11	11	11
Censored	0	10	21	28	33	37	42	43	43	43	43	43	44

Int-risk

At Risk	77	60	46	32	19	12	9	4	1	1	0	0	0
Events	0	6	10	15	21	22	25	28	29	29	29	29	29
Censored	0	11	21	30	37	43	43	45	47	47	48	48	48

High-Risk

At Risk	24	20	10	7	2	2	1	0	0	0	0	0	0
Events	0	2	5	7	11	11	12	12	12	12	12	12	12
Censored	0	2	9	10	11	11	11	12	12	12	12	12	12

Figure 4. *Outcome in terms of OS according to BM MYD88^{L65P} levels and to BM MFC levels*