

ORIGINAL ARTICLE

Bone-marrow immunophenotypic analysis allows the identification of high risk of progression and immune condition-related monoclonal gammopathy of undetermined significance

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Abstract

Background. Monoclonal gammopathy of undetermined significance (MGUS) progresses to plasma cell dyscrasia at a rate of 1% per year. A high prevalence of MGUS has been noted in series of patients with immune disorders or chronic infections.

Methods. Retrospective cohort and a cross-sectional study to analyze the prognostic value of aberrant (CD38⁺⁺CD138⁺CD19⁻CD45^{weak}) to normal phenotype (CD38⁺⁺CD138⁺CD19⁺CD45⁺) bone-marrow plasma cells ratio (A/N ratio) for the development of a plasma cell dyscrasia and the association with the presence of a chronic immune disorder.

Results. A total of 322 patients were included with a median follow-up of 46 months. Analysis for progression revealed an increased A/N ratio as the main independent prognostic variable. A significant association between a reduced A/N ratio and the diagnosis of a chronic immune condition was found. Using receiver-operating characteristic analysis we created an A/N ratio range from 4 to 0.20. Values of 4 or higher define a group at high risk of progression (OR 10.7). A/N values of 0.20 or lower are associated with immune disorders or chronic infections (OR 20.9).

Conclusions. Extreme values of the A/N ratio seem to be related with two different conditions: high risk of progression, and immune condition-related MGUS.

Key words: Flow cytometry, immune system diseases, monoclonal gammopathy of undetermined significance, multiple myeloma

Introduction

Monoclonal gammopathy of undetermined significance (MGUS) is a plasma cell disorder found in approximately 3% of the general population 50 years of age or older (1). MGUS is associated with progression to multiple myeloma (MM) or related malignancy at a rate of 1% per year (2). In addition, there is no decline in the risk of progression over time, making lifelong follow-up necessary in anyone diagnosed with MGUS (2). Moreover, two recent studies have shown that multiple myeloma is nearly always preceded by MGUS, encouraging

investigators to find better predictors for MM development in order to implement strategies to prevent or delay progression (3,4).

The association between monoclonal gammopathies and autoimmune disorders or chronic infections has been reported by several authors. Brown et al. found significantly elevated risk of MGUS associated with broad categories of autoimmune, infectious, and inflammatory disorders (5). In this regard, an increased risk of MGUS was found among cohorts of patients with hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infections (6,7). Basic research has demonstrated

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Key messages

- We herein evaluate the relationship between aberrant and normal phenotype bone-marrow plasma cells detected by flow cytometry in a large series of patients with newly diagnosed monoclonal gammopathy of undetermined significance (MGUS), with the aim of assessing its association with either immune or chronic infectious disorders or the development of multiple myeloma (MM).
- Extreme values of the aberrant-to-normal phenotype bone-marrow plasma cells ratio seem to be related with two different conditions: high-risk MGUS, more likely to progress to MM, and immune condition-related MGUS.

that the bone-marrow is the deposit organ for autoantibodies-producing long-lived plasma cells (8–10), and reactive plasmacytosis has been reported in peripheral blood of patients with infections (11).

Various studies have demonstrated that two populations of plasma cells (aberrant and normal) can be identified both in MM and MGUS patients based on their immunophenotypic profile (12,13). The prognostic value related to the predominance of the phenotypically aberrant bone-marrow plasma cells (BMPC) in MGUS was preliminarily reported in an abstract by Rawstron et al. and has been recently assessed by ourselves and others (14–16).

We herein evaluate the relationship between aberrant and normal phenotype bone-marrow plasma cells detected by flow cytometry in a large series of patients with newly diagnosed MGUS, with the aim of assessing its association with either immune or chronic infectious disorders or the development of MM.

Methods

Patient, clinical, and laboratory data

Patients were included in this study if they had a diagnosis of immunoglobulin (Ig-) G or IgA MGUS and a bone-marrow aspirate allowing a flow cytometry analysis at diagnosis. The criteria used for the diagnosis of MGUS were: a monoclonal protein in the serum of 30 g/L or less, absence or less than 1 g/24 h Bence-Jones proteinuria, no lytic bone lesions, anemia, hypercalcemia, or renal failure secondary to the plasma cell dyscrasia, and less than 10% plasma cells in the bone-marrow evaluation. Polyclonal immunoglobulin (Ig) reduction was defined as the

Abbreviations

APC	allophycocyanin
BJ	Bence-Jones
BMPC	bone-marrow plasma cells
CD	cytoplasmic domain
ESR	erythrocyte sedimentation rate
FITC	fluorescein isothiocyanate
HCV	hepatitis C virus
HIV	human immunodeficiency virus
Ig	immunoglobulin
IQR	interquartile range
MC	monoclonal component
MGUS	monoclonal gammopathy of undetermined significance
MM	multiple myeloma
A/N ratio	aberrant-to-normal phenotype bone-marrow plasma cells ratio
PC	plasma cell
PE	phycoerythrin
PerCP-Cy5.5	peridin/chlorophyll protein
Q	quartiles
ROC	receiver-operating characteristic

presence of at least one of the uninvolved immunoglobulin below normal levels. Serum monoclonal component (MC) was defined by means of immunoelectrophoresis on agarose gel. Any serum thought to have a band was subjected to immunofixation. Serum monoclonal component was quantified by electrophoresis on agarose gel and serum polyclonal Ig levels were measured by nephelometry. Urinary paraprotein was quantified using electrophoresis of unconcentrated urine performed on cellulose acetate and stained with colloidal gold. BMPC percentages on light microscopy were the mean values of a 400-cell count. Plasma cells were considered atypical if they fulfilled the morphological criteria described elsewhere (17).

In each patient, the following variables were examined: age, sex, hemoglobin, serum creatinine, serum calcium, type and size of the serum MC, isotype of the MC Ig, presence of urine MC, quantification of serum Ig levels, erythrocyte sedimentation rate (ESR), BMPC percentage and presence of atypical plasma cells on light microscopy, and aberrant and normal phenotype BMPC percentages. β_2 -Microglobulin at base-line was also considered; however, as it was only studied in a subset of patients (68%), it was not included in multivariate analysis.

Data were obtained by review of the medical records and the computerized laboratory database of each cohort patient. We considered the following autoimmune disease diagnosis: polymyositis,

dermatomyositis, rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis, amyotrophic lateral sclerosis, autoimmune hemolytic anemia, celiac disease, chronic rheumatic heart disease, discoid lupus erythematosus, immune thrombocytopenic purpura, myasthenia gravis, pernicious anemia, primary biliary cirrhosis, Graves's disease, autoimmune thyroiditis, Sjögren's syndrome, ankylosing spondylitis, Crohn's disease, psoriasis, sarcoidosis, ulcerative colitis, Wegener's granulomatosis, and microscopic polyarteritis. We defined chronic infection as a history of an infection of more than six months. Diagnoses of autoimmune disorders and chronic infections were established by specialists from various fields: rheumatology, internal medicine, and gastroenterology. Progression to multiple myeloma was established according to the criteria defined by the International Myeloma Working Group (18).

We also studied 31 patients who underwent a flow cytometry analysis of BMPC due to suspicion of MM prior to having the results of the immunoelectrophoresis and immunofixation, both tests finally being negative. We used these non-MGUS patients (not included in the 322 MGUS patients cohort) to phenotypically characterize the BMPC compartment in the absence of a serum monoclonal component.

Informed consent was obtained from all the patients in accordance with the declaration of Helsinki.

Flow cytometry analysis

Bone-marrow samples were obtained and processed as described (19) within 6 hours following bone-marrow aspiration. Mononuclear cells were stained with fluorescence-labeled CD138-FITC (fluorescein isothiocyanate), CD38-PE (phycoerythrin), CD45-PerCP-Cy5.5 (peridin/chlorophyll protein), and CD19-APC (allophycocyanin) antibodies (the first of them from Immunoquality, Groningen, NL and the rest from BD Biosciences, San Jose, CA). A negative control tube, only stained for CD38-APC, was always included in the panel in order to establish a cut-off for positivity based on the level of the natural autofluorescence of plasma cells (PCs). Data were acquired by means of a Becton Dickinson fluorescence-activated cell-sorting FACScalibur with CellQuest Pro software (BD Biosciences). For data analysis, the Paint-a-Gate Plus software (Becton Dickinson) was used. As a minimum, 100,000 total cells were acquired in each test. Normal and aberrant PCs were identified and quantified using a sequential gating strategy (Figure 1). Briefly, a primary gate included $CD38^{++}CD138^{+}$ events, selecting PCs. Normal plasma cells are $CD19^{+}CD45^{+}$, whereas

aberrant PCs are $CD19^{-}CD45^{weak}$. A minimum of 100 events satisfying the gating strategy were required for the characterization of the BMPC compartment.

Statistical methods

Categorical data were presented as frequencies and percentages, and continuous data were presented as median (25th, 75th percentiles). Receiver-operating characteristic (ROC) curves analyses were generated to test the predictive discrimination of different continuous variables to identify association with immune or chronic infection or progression during follow-up. The cut point with the best sensitivity and specificity was chosen. If the area under the ROC curve was lower than 0.65, standard cut-off points were considered to dichotomize continuous variables (Table I).

The cumulative probability of transformation into MM or a related disorder was analyzed using the method described by Kaplan and Meier and compared between groups by the log-rank test (20). The effect of variables on progression was calculated using a Cox proportional hazards regression model. A risk score system was then constructed by assigning weighted points to each independent and significant predictor proportional to the β regression coefficient values (rounded to the nearest integer). Based on their risk score, patients were categorized into three different risk groups using the terciles as cut-off values.

We also conducted a cross-sectional study to identify variables at base-line associated with immune or chronic infectious disorders. In this regard, a series of univariate and multivariate analyses was fitted using a binary logistic regression strategy.

Only those values with $P < 0.150$ in a univariate analysis were incorporated in multivariate models. All P -values < 0.05 were accepted as statistically significant. Statistical analysis was performed using SPSS 15.0 for Windows (SPSS, Inc., Chicago, Illinois).

Results

Between March 1997 and April 2008, flow cytometry analysis on bone-marrow samples was performed on 322 patients with newly diagnosed MGUS. Median patient age was 71 years (interquartile range (IQR) 63–78 years) with a slightly male predominance (51%). Median follow-up was 46 months (IQR 23–58 months). Additional patient characteristics are shown in Table II.

During the period of observation, in 23 (7.1%) patients a transformation was registered into: MM ($n = 22$), and primary amyloidosis ($n = 1$). A total of

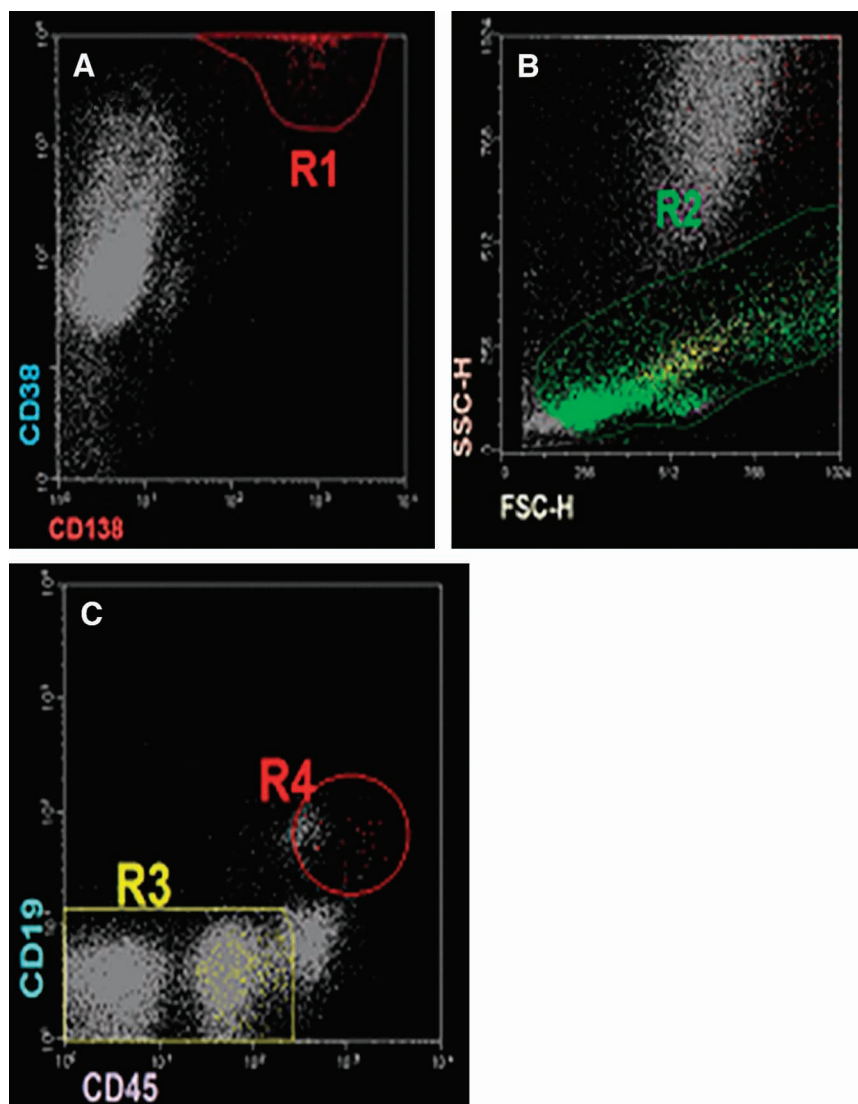


Figure 1. Sequential gating strategy for identification and characterization of BMPCs. A minimum of 100,000 events were analyzed for each patient. The gating strategy is demonstrated in a patient with IgG MGUS. An initial region R1 was set around cells expressing a high level of CD38 and positivity for CD138 (A). A second region R2 was set on the light scatter of the cells satisfying R1 to exclude debris or apoptotic PCs (B). Then two PC subpopulations were identified on the CD45 versus CD19: R3 was set around PCs expressing a high level of CD45 and positivity for CD19, while R4 was set around PCs with a low or intermediate CD45 and negative CD19 (C). BMPC = bone marrow plasma cells; Ig = immunoglobulin; MGUS = monoclonal gammopathy of undetermined significance; R = region; PC = plasma cell.

Table I. Continuous variables cut-off values derived from ROC analysis to maximize sensitivity and specificity.

Variable and cut-off value for progression to plasma cell dyscrasia	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
A/N ratio ≥ 4	0.87 (0.77–0.96)	0.79 (0.70–0.81)	0.92 (0.89–0.95)
MC size ≥ 16 g/L	0.73 (0.63–0.85)	0.69 (0.58–0.76)	0.74 (0.71–0.78)
BMPC on light microscopy $\geq 5\%$	0.71 (0.59–0.82)	0.69 (0.64–0.73)	0.62 (0.59–0.65)
Variable and cut-off value for immune condition association			
A/N ratio ≤ 0.20	0.82 (0.74–0.90)	0.77 (0.73–0.81)	0.87 (0.84–0.92)
Age ≤ 65 years	0.65 (0.55–0.75)	0.58 (0.54–0.62)	0.68 (0.64–0.82)

ROC = receiver-operating characteristic; AUC = area under the curve; CI = confidence interval; A/N ratio = aberrant-to-normal phenotype bone-marrow plasma cells ratio; MC = monoclonal component; BMPC = bone marrow plasma cells.

Table II. Patient characteristics at base-line.

	Total MGUS population (<i>n</i> = 322)	MGUS progressed to MM (<i>n</i> = 23)	MGUS associated to immune conditions (<i>n</i> = 42)	Non-MGUS group (<i>n</i> = 31)
Age, years				
Median	71	71	64	72
Q1–Q3	63–78	63–75	49–76	67–88
Males/Females	163/159	13/10	18/24	15/9
Serum MC, g/L				—
Median	13.9	19.6	16.2	
Q1–Q3	9.8–16.8	15.1–25	11–22	
MC type, %				—
IgG	73.6	65.2	61.9	
IgA	24.8	34.8	35.7	
Double component	1.6	0	2.4	
Isotype of the MC Ig, %				
κ	59	57	59	—
λ	41	43	41	—
BMPCs, %				
Median	2	4	2.5	1.5
Q1–Q3	2–4	2–6	1–5	1–4
Patients with BMPC morphologic atypias, %	33.2	78	19	22
Patients with BJ proteinuria, %	10.6	39	2.4	0
β ₂ -Microglobulin, mg/L				
Median	2.5	3.4	2.8	2.9
Q1–Q3	2–3.6	2–5.4	2.1–4	2–3
ESR, mm/h				
Median	21.5	32	49	19
Q1–Q3	12–37	15–43	28–90	11–35
Creatinine mg/dL				
Median	0.98	1.2	0.88	0.8
Q1–Q3	0.8–1.2	0.9–1.4	0.7–1.1	0.7–1
Hemoglobin g/dL				
Median	12.4	12.7	12	12.5
Q1–Q3	11–13.8	11–13.4	10.7–13	11–13
Serum calcium mg/dL				
Median	9.1	9.6	9	9.5
Q1–Q3	8.6–9.5	9–10	8.4–9.4	9–10
Non-involved Igs				
Reduced, %	14.4	37.8	2.5	—
Increased, %	6.5	0	40.4	
Months of follow up				
Median	46	51	40.5	41
Q1–Q3	23–58	38–65	18–69	16–70
Aberrant phenotype BMPC, %				
Median	0.20	1.14	0.08	0.01
Q1–Q3	0.10–0.57	0.65–2.57	0.05–0.19	0–0.02
Normal phenotype BMPC, %				
Median	0.30	0.15	0.97	0.22
Q1–Q3	0.16–0.58	0.11–0.27	0.55–1.47	0.11–0.35
A/N ratio				
Median	0.65	8.6	0.13	0.01
Q1–Q3	0.24–2	4–13	0.06–0.21	0–0.04

SI conversion factors: To convert creatinine to micromoles per liter, multiply by 88.4; hemoglobin to grams per liter, multiply by 10; serum calcium to millimoles per liter, multiply by 0.25.

MGUS = monoclonal gammopathy of undetermined significance; MC = monoclonal component; Ig = immunoglobulin; BMPC = bone – marrow plasma cells; BJ = Bence – Jones; ESR = erythrocyte sedimentation rate; A/N ratio = aberrant to normal phenotype bone-marrow plasma cells ratio; Q = quartiles.

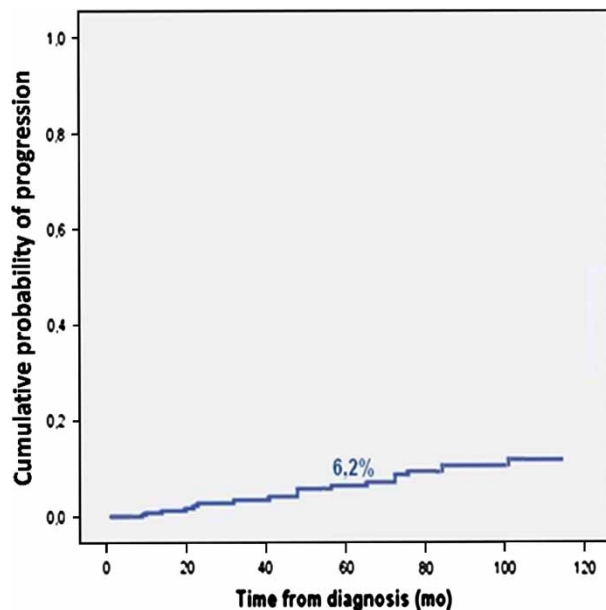


Figure 2. Risk of progression to myeloma or related disorder in 322 patients with monoclonal gammopathy of undetermined significance.

24 (7.4%) patients had a diagnosis of autoimmune disorder, and 18 (5.6%) patients of a chronic infection. Other relevant diagnoses reached through the follow-up were: 8 solid tumors (lung cancer ($n=4$), breast ($n=3$), germ cell ($n=1$)), 3 lymphomas (non-Hodgkin's lymphoma ($n=2$) and Hodgkin's lymphoma ($n=1$)), and myelodysplastic syndrome ($n=3$).

Phenotypically aberrant and normal BMPC were detected in all bone-marrow samples assessed. The median aberrant percentage was 0.20% (IQR 0.10%–0.57%), whereas the median normal percentage was 0.30% (IQR 0.16%–0.58%). The median aberrant-to-normal phenotype bone-marrow plasma cell (A/N) ratio was 0.65 (IQR 0.24–2). Altogether 264 (81.9%) patients were alive at the end of this study.

High risk of progression MGUS

The main clinical and laboratory parameters at diagnosis of the 23 patients that evolved to MM are summarized in Table II. The progression occurred at a median duration of 51 months (IQR 38–65 months). The cumulative probability of progression was 6.2% at 5 years and 1.2% per year (Figure 1). The median normal phenotype BMPC percentage was 0.15% (IQR 0.11%–0.27%), whereas the median aberrant phenotype BMPC percentage was 1.14% (IQR 0.65%–2.57%). The median A/N ratio was 8.6 (IQR 3–12).

At univariate analysis, the variables associated with an increased probability of evolution were:

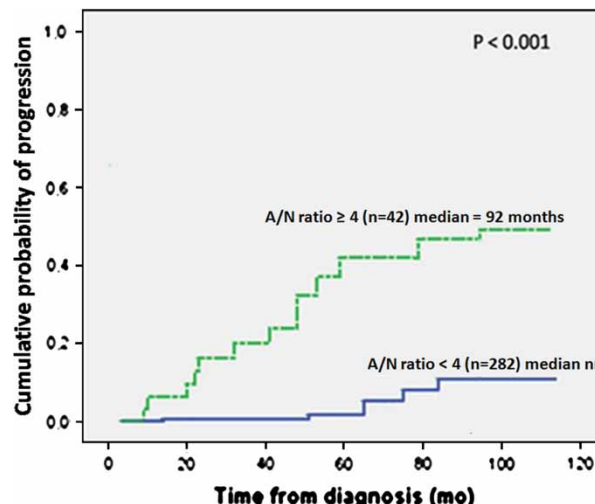


Figure 3. Time to progression in MGUS according to the A/N ratio at diagnosis. Patients classification into two groups based on the best ROC A/N ratio cut point. MGUS = monoclonal gammopathy of undetermined significance; A/N ratio = aberrant-to-normal phenotype bone-marrow plasma cells ratio; ROC = receiver-operating characteristic.

detectable Bence-Jones proteinuria, a BMPC percentage on light microscopy of 5 or higher, the reduction of one or two serum polyclonal Igs, serum MC level of 16 g/L or higher, and an A/N ratio equal to or higher than 4. The presence of plasma cell atypias and a serum β_2 -microglobulin equal to or higher than 3 mg/L were not significant, although a trend was noted (Table III).

At multivariate analysis, only an A/N ratio of 4 or higher, an MC size equal to or greater than 16 g/L, and the reduction of uninvolved Igs were independently associated with MGUS malignant transformation (Table III, Figure 5). A total of 282 patients had an A/N ratio below this cut-off value, and 42 had values equal to or above the cut-off. Patients with an A/N ratio ≥ 4 had a cumulative probability of transformation of 35% at 5 years, compared with 3% for those with an A/N ratio < 4 (Figure 3).

We performed a new Cox regression analysis replacing our A/N ratio by the ratio proposed by Perez Persona et al. (16): percentage of abnormal phenotype plasma cells within the BMPC compartment (aPC/BMPC) greater than or equal to 95%. It showed itself to be an independent prognostic factor in multivariate analysis (OR 6.9 (2.2–21.6, 95% CI)). The area under the ROC curve of the aPC/BMPC $\geq 95\%$ was 0.75 (0.62–0.88, 95% CI), its sensitivity was 0.58 (0.53–0.63, 95% CI), and its specificity was 0.96 (0.93–0.98, 95% CI).

Based on the previous results, we defined a prognostic scoring system including A/N ratio ≥ 4 , the reduction of uninvolved Igs, and the M component size ≥ 16 g/L. The weight assigned to each

Table III. Univariate and multivariate Cox proportional hazards regression model of factors that might be associated with the development of a plasma cell dyscrasia ^a.

Variable	Univariate			Multivariate		
	<i>P</i>	OR	(95% CI)	<i>P</i>	OR	(95% CI)
A/N phenotype BMPCs ratio ≥ 4	≤ 0.001	31.6	(11.7–85.4)	≤ 0.001	10.7	(4.2–39)
Reduction of one or two ‘uninvolved’ Igs	≤ 0.001	11.3	(4.8–26.6)	0.002	5.3	(1.8–15.5)
Size MC ≥ 16 g/L	≤ 0.001	6.3	(2.6–15.4)	0.008	4.8	(1.5–13.8)
BMPCs $\geq 5\%$	0.01	4.2	(1.8–9.7)	NS		
Detectable BJ proteinuria	0.02	2.8	(1.1–7.8)	NS		
β_2 -Microglobulin ≥ 3 mg/L	0.08	3.7	(1.4–9.2)	NT		
Presence of PCs atypias	0.09	1.9	(0.9–4.5)	NS		
Isotype of the MC Ig, %						
κ	NS			NT		
λ	NS			NT		
Non-IgG MC	NS			NT		
Age ≥ 65 years	NS			NT		
Sex, male	NS			NT		
ESR ≥ 45 mm/h	NS			NT		
Calcium ≥ 9.5 mg/dL	NS			NT		
Serum creatinine ≥ 1.5 mg/dL	NS			NT		
Hemoglobin ≤ 10 g/dL	NS			NT		

^aOnly values with $P < 0.150$ in a univariate analysis were incorporated in multivariate models.

NT = not tested; NS = not significant; A/N = aberrant to normal; BMPC = bone marrow plasma cells; Ig = immunoglobulin; MC = monoclonal component; BJ = Bence Jones; PC = plasma cell; κ = kappa; λ = lambda; ESR = erythrocyte sedimentation rate.

variable was based on the regression coefficients from the proportional hazards models (A/N ratio: 2 points; reduction of uninvolved Igs: 1 point; M component size ≥ 16 g/L: 1 point). Risk groups were as follows: low risk (score ≤ 1), intermediate (score = 2), and high (score 3–4). The scores stratified patients into three groups with different progression-free survival ($P < 0.001$) (Figure 4). Patients included in low, intermediate, and

high-risk groups had a cumulative probability of 5-year progression of 1%, 16%, and 58%, respectively.

Immune condition-related MGUS

The main clinical and laboratory parameters at baseline of patients that had a diagnosis of an immune disorder or a chronic infection are summarized in Table II. Immune disorders included rheumatoid arthritis ($n = 11$), systemic lupus erythematosus ($n = 4$), ulcerative colitis ($n = 2$), Graves-Basedow ($n = 2$), Crohn’s disease ($n = 2$), myasthenia gravis ($n = 1$), sarcoidosis ($n = 1$), and microscopic polyarteritis ($n = 1$). Chronic infections included chronic HCV hepatitis ($n = 10$), HIV infection ($n = 5$), and chronic HBV hepatitis ($n = 3$).

A total of 35 patients were diagnosed with a chronic immune response condition before the monoclonal component was found and the BMPC immunophenotypic evaluation performed. Median time from immune condition diagnosis to MGUS diagnosis was 5 months (IQR 1–11 months). Interestingly, in eight patients the diagnosis of immune disorder or chronic infection was established after the immunophenotypic assessment; among those patients, diagnosis was made within one ($n = 5$), two ($n = 2$), and three ($n = 1$) months from A/N ratio determination, respectively.

The median normal phenotype BMPC percentage was 0.97% (IQR 0.55%–1.47%), whereas the

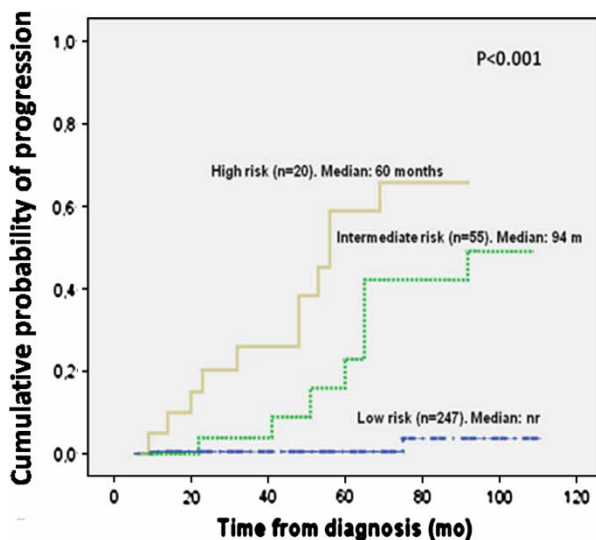


Figure 4. Time to progression in cohort classified into our prognostic scoring system.

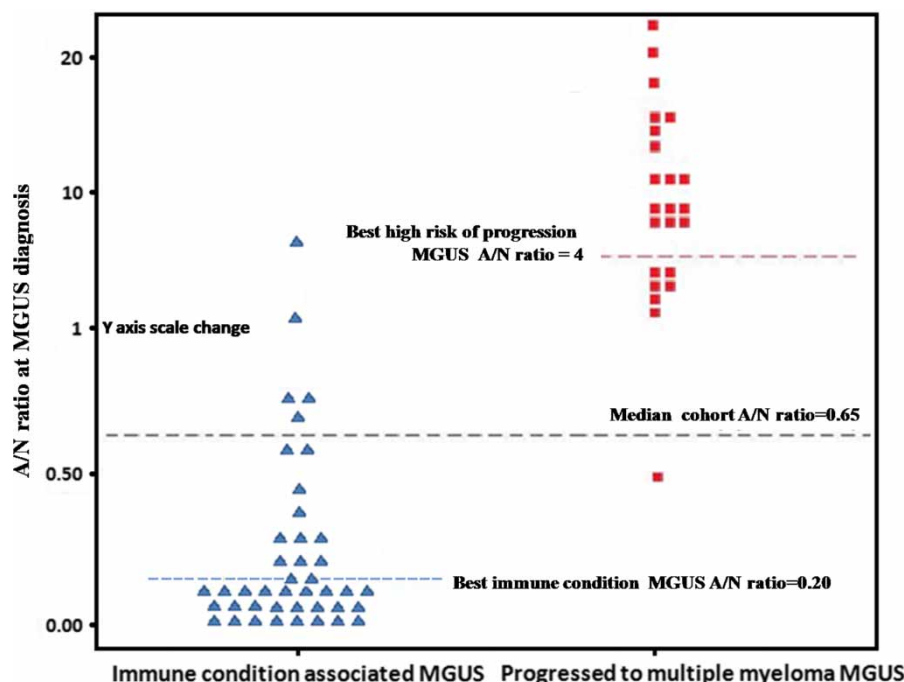


Figure 5. Cases of immune condition MGUS and progressed to MM MGUS grouped by similar A/N ratio. Immune condition MGUS cases grouped by decimals. Progressed to MM MGUS cases grouped by units. MGUS=monoclonal gammopathy of undetermined significance; A/N ratio=aberrant-to-normal phenotype bone-marrow plasma cells ratio; MM=multiple myeloma.

median aberrant phenotype BMPC percentage was 0.08% (IQR 0.05%–0.19%). The median A/N ratio was 0.13 (IQR 0.06–0.21). No significant differences were found between autoimmune disorder MGUS and chronic infections MGUS regarding clinical, laboratory, or flow cytometry data (data not shown).

At univariate analysis, the variables associated with a diagnosis of immune disorder or chronic infection were: age below 65, the increase of one or two serum polyclonal Igs, a serum MC level of 16 g/L or higher, an ESR level of 45 mm/h or higher, and an A/N ratio equal to or lower than 0.20. Non-IgG monoclonal protein, a BMPC percentage on light microscopy of 5 or higher, and a serum β_2 -microglobulin level of 3 mg/L or higher were not significant, although a trend was noted (Table IV).

At multivariate analysis, an A/N ratio of 0.20 or lower, the increase of uninvolved Igs, and an age equal to or below 65 were independently associated with a diagnosis of immune disorder or chronic infection (Table IV, Figure 5).

Non-MGUS bone-marrow plasma cell enumeration

During a median follow-up of 41 months, none of the 31 non-MGUS patients had a diagnosis of MM, autoimmune disease, or chronic infection. Two patients were diagnosed with myelodysplastic syndrome and one patient with Hodgkin's lymphoma. The median normal phenotype BMPC

percentage was 0.22% (IQR 0.11%–0.35%), whereas aberrant phenotype BMPC was undetectable or in the limit of test sensitivity (median 0.01%; IQR 0%–0.02%). Additional non-MGUS cases characteristics are shown in Table II.

Discussion

In a sample of 322 newly diagnosed IgG and IgA MGUS patients, Cox regression analysis showed that the finding of an elevated aberrant-to-normal phenotype BMPC ratio, by means of flow cytometry, was the best predictor of progression to MM, among another 13 clinical and laboratory variables. In addition, a low aberrant-to-normal phenotype BMPC ratio at base-line was the factor that showed the strongest association with the presence of a chronic immune response condition.

The development of multiparametric immunophenotyping throughout the 1990s permitted an accurate identification and characterization of aberrant and normal PCs. This has enabled basic investigators to isolate pure myeloma cells for essential studies in the knowledge of MM kinetics and microenvironment, and has allowed clinicians to either avoid a false diagnosis of MM, evaluate minimal residual disease, or identify prognostic factors (21,22). A common problem to all flow cytometry laboratories is that the sum of plasma

Table IV. Univariate and multivariate logistic regression model of factors that might be associated with the presence of a chronic immune response disorder^a.

Variable	Univariate			Multivariate		
	<i>P</i>	OR	(95% CI)	<i>P</i>	OR	(95% CI)
A/N phenotype BMPCs ratio ≤ 0.20	≤ 0.001	21.7	(9.8–47.8)	≤ 0.001	20.9	(8.5–51.1)
Increase of one or two ‘uninvolved’ Igs	≤ 0.001	13.6	(5.2–35.9)	0.02	5.1	(1.3–19.4)
Age ≤ 65 years	0.001	3.1	(1.6–5.9)	0.005	3.5	(1.4–8.6)
Size MC ≥ 16 g/L	0.003	2.5	(1.3–5.2)	0.06	2.2	(0.9–5.7)
ESR ≥ 45 mm/h	0.009	2.4	(1.2–4.6)	NS		
BMPCs $\geq 5\%$	0.08	1.9	(0.9–4)	NS		
Non-IgG MC	0.07	1.8	(0.9–3.7)	NS		
β_2 -Microglobulin ≥ 3.0 mg/L	0.14	2.2	(0.8–4.2)	NT		
Presence of PCs atypias	NS			NT		
Isotype of the MC Ig, %						
κ	NS			NT		
λ	NS			NT		
Detectable BJ proteinuria	NS			NT		
Sex, female	NS			NT		
Serum calcium ≥ 9.5 mg/dL	NS			NT		
Serum creatinine ≥ 1.5 mg/dL	NS			NT		

^aOnly values with $P < 0.150$ in a univariate analysis were incorporated in multivariate models.

NT = not tested; NS = not significant; A/N = aberrant to normal; BMPC = bone marrow plasma cells; Ig = immunoglobulin; MC = monoclonal component; BJ = Bence Jones; PC = plasma cell; κ = kappa; λ = lambda; ESR = erythrocyte sedimentation rate.

cells evaluated by flow cytometry is considerably lower than what is found in the bone-marrow smears or biopsies. In our cohort the median BMPC percentage on light microscopy was 2 (IQR 2%–4%) and the median BMPC percentage by means of flow cytometry was 0.60 (IQR 0.30%–0.95%). As stated by Rawstron et al. (22), the major cause of this discrepancy, in addition to counting errors and expression of certain adhesion molecules, is that the marrow samples taken for morphological review are often the highest quality ‘first-pull’ aspirate samples while those used for flow cytometry analysis are often secondary aspirate samples with a higher degree of peripheral blood contamination.

In our MGUS cohort we observed a 5-year progression to MM rate of 6%. This value is in accordance with previously published studies. Several authors identified simple hematologic parameters of value as predictors of progression (23–26). Kyle et al. reported the results of a large population-based study of MGUS, in which the size and type of M protein were predictive of progression (2). In addition, a bone-marrow plasma cell percentage of 6% to 9%, an abnormal serum free light chain ratio, and the presence of circulating plasma cells have all been associated with a higher risk of progression (27–29).

Our results show that an elevated aberrant-to-normal phenotype BMPC ratio, determined by multiparametric flow cytometry, is significantly correlated with progression to MM. Interestingly, the value of this parameter turns out to be independent

of the size of the M component or the uninvolved Igs reduction, two well known prognostic factors. Unlike larger studies (27,28), a monoclonal protein other than IgG did not predict a higher risk of progression in our cohort. It is possible that the exclusion of IgM MGUS and progression to Waldenström macroglobulinemia in our design could explain part of this discrepancy.

The ROC curve analysis identified an A/N ratio of 4 or higher as the best predictive cut point with a 10.7-fold increased risk of progression to MM. In addition, our results suggest that the aberrant phenotype population seems to be exclusive of patients with a monoclonal protein in serum as shown by our data from non-MGUS cases. We also tested the aPC/BMPC ratio proposed by Perez-Persona et al. (16) and validated its prognostic value in our cohort. Their cut-off point seems to be more specific but less sensitive than ours in our patients. However, as they included the positivity of CD56 to characterize aberrant plasma cells, both cut-off points cannot be compared accurately, and the optimal cut-off for prognostic classification should not be decided yet. As stated above, an abnormal serum free light chain ratio has proved to be an independent risk factor for progression in MGUS (27). Since two recent studies have shown that almost all multiple myeloma cases are preceded by MGUS (3,4), the need for accurate prognostic factors is increasingly important, and it is of much interest to investigate both phenotypic BMPC and

free light chain ratios, which seem to be the more powerful predictors by now, to determine if each test can provide independent prognostic information.

We also developed an accurate and easy-to-use prognostic scoring system to stratify patients with MGUS into low, intermediate, and high-risk groups, with a cumulative probability of 5-year progression of 1%, 16%, and 58%, respectively. Multiple myeloma is currently an incurable disease, therefore the development of preventive strategies in this setting may be an attractive approach. In this regard, on-going trials are testing the use of new active agents in the treatment of smoldering multiple myeloma, and promising results have been advanced (30–32). The assessment of our results could eventually justify testing similar strategies in high-risk MGUS patients, as defined by our scoring system: a fraction of patients that progress rapidly, with a rate of annual progression of 11.6%. Contrarily, it is of particular interest that the predominance of polyclonal BMPC (A/N ratio < 1), a small M component size (< 16 g/L), and the absence of uninvolved Igs reduction define a group of patients in which no progression was observed. This group of patients accounts for half (49.7%; $n = 160$) of our cohort and may benefit from a reassuring patient counseling, in which frequent monitoring may not be needed.

A secondary finding demonstrates that a low A/N ratio also seems to have some value in the diagnosis of a group of patients with immune and chronic infection disorders. An A/N ratio of 0.20 or lower, determined by ROC analysis, is strongly associated with the presence of an immune or chronic infection disorder. In our hands, the A/N ratio enables not only excluding the presence of a concomitant MM or a high risk of progression MGUS but also suggests the diagnosis of an immune or infectious disorder as shown by the fact that eight of our patients received the diagnosis after the A/N ratio determination. In our view, the finding of a low A/N ratio in a patient not diagnosed with a chronic immune response disorder should encourage the clinician to also consider testing for autoantibodies, acute phase reactants, liver function, and viral serologies in order to exclude the presence of an autoimmune or chronic infection disorder.

Manz et al. (8) showed that a large fraction of the anti-self-antibodies-producing plasma cells localizes in the bone-marrow. In this regard, an interesting finding of the present study was that in autoimmune disorder-related MGUS, the median size of the normal phenotype BMPC compartment (0.97%) exceeded 5-fold the normal phenotype BMPC compartment of the rest of our cohort (0.23%).

During the 1980s and early 1990s several studies investigated the hypothesis that repeated or chronic stimulation of the immune system may lead to MM, but results were not entirely consistent (33–35). It is worth noting that in our series, 40 out of 42 of immune condition-related MGUS showed a predominance of normal phenotype BMPC, and that interestingly no progression to MM was observed in this particular patient subset. Whether immune condition-related MGUS is included in the non-evolving MGUS concept, proposed by Rosiñol et al. (36) and characterized by a stable MC level and a longer time to progression until the onset of a symptomatic disease, is beyond the scope of this study due to the reduced number of events and the relatively short follow-up. Recently, Brown et al. (5) found a significant relation of MM development with broad categories of autoimmune, infectious, and inflammatory disorders in a large cohort of male US veterans. Most of the conditions that showed an increased risk of developing a MM revealed a significantly higher risk in the latency interval of 5 or more years. It may support the idea that those patients do not evolve rapidly and that, maybe, further oncogenic events are necessary in immune condition-related MGUS to favor the myelomatous plasma cell clone predominance.

Our study has several limitations. First, this is not a screening trial, and patients were included if they had a bone-marrow aspiration at diagnosis that allowed us to phenotypically characterize the BMPC compartment. The indication of a bone-marrow aspiration in newly diagnosed MGUS patients, although subjective, may be supported in patients with some risk factors previously reported, therefore the number of events may be overestimated. We attempted to minimize concerns about this effect by adjusting for known risk markers of MM progression. In spite of an eventual compromise of the accuracy of our results due to selection bias, our cohort characteristics at base-line are similar to those found in large population-based studies (2), and the associations found are strong enough to suggest that our results may be extrapolated to most patients with MGUS. Second, we created a prognostic scoring system based on the information retrospectively obtained from our cohort. The clinical use of this score is limited by the relatively short follow-up and the sample size, and it will need validation through the study of an independent cohort. Third, we found no differences regarding clinical and laboratory data among patients bearing autoimmune diseases or chronic infections; however, due to the low number of patients in each group our results cannot be

conclusive in this respect. Fourth, a recent consensus on multiparametric flow cytometry in multiple myeloma and related disorders stated that the test antigens for classifying aberrant plasma cells should be CD19 and CD56 (21). In spite of the lack of CD56 in our strategy, we were able to discriminate between aberrant and normal plasma cells in all of our cohort samples. In ten patients, that discrimination was based on the aberrant expression of just one of the two antigens used for that purpose (CD19 and CD45). Finally, missing β_2 -microglobulin values accounted for 32% of patients. In order to retain the maximum sample size and control for any bias associated with missing values, we decided not to include it in multivariate analysis, precluding us from evaluating its effect as a possible confounding factor.

In summary, according to our results, extreme values of the A/N ratio seem to be related with two different conditions: high-risk MGUS, more likely to progress to MM, and immune condition-related MGUS. Our findings further support the routine use of phenotypic characterization of bone-marrow plasma cells in patients with MGUS at diagnosis.

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