Multiparameter flow cytometry quantification of bone marrow plasma cells at diagnosis provides more prognostic information than morphological assessment in myeloma patients

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ABSTRACT

Quantification of bone marrow plasma cells in multiple myeloma patients using conventional morphology is of limited prognostic value, while the merit of multiparameter flow cytometry immunophenotyping is still considered unproven. Here we compare the bone marrow plasma cell counts obtained by morphology and multiparameter flow cytometry and explore the potential prognostic impact of both techniques in 765 newly diagnosed, uniformly treated multiple myeloma patients. Although multiparameter flow cytometry generally yields lower plasma cell counts (median percentage of 11% *vs*. 40%, respectively; $p<0.001$), there is a significant positive correlation between the two techniques ($R_2=0.46$, $p<0.001$). Regarding prognosis, multivariate analysis selected the bone marrow plasma cell counts obtained by multiparameter flow cytometry as an independent prognostic factor for overall survival ($p=0.007$), supporting the incorporation of multiparameter flow cytometry immunophenotyping into the routine diagnostic evaluation of multiple myeloma patients and validating the clinical utility of bone marrow plasma cell counting by multiparameter flow cytometry approaches. *(clinicaltrials.gov identifier: NCT00560053).*

Key words: multiple myeloma, immunophenotyping, conventional morphology, multiparameter flow cytometry quantification.

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Introduction

Quantification of bone marrow (BM) plasma cells (PCs) by conventional morphology (CM) is a mandatory test for the diagnosis and response assessment in multiple myeloma (MM).[1] However, the degree of BM infiltration by CM may vary significantly not only among but also within patients. This has been attributed to the heterogeneous pattern of BMPC infiltration in MM[2-4] and could help to explain its inconsistency as a prognostic factor.[5-9] One alternative would be to use multiparameter flow cytometry (MFC) immunophenotyping to quantify BMPC, although this is currently still limited to research studies and the differential diagnosis of unusual cases.[4] The current study aimed to compare the BMPC counts obtained by CM and MFC and to assess the prognostic value of both techniques in a large series of uniformly treated MM patients.

Design and Methods

A total of 765 newly diagnosed MM patients were included in this study. Written consent was obtained from all patients. Baseline demographics and disease characteristics are shown in Table 1. At the time of concluding this study, 495 patients (65%) had relapsed/progressed and 306 (40%) had died. The median follow-up was 51 months. All 765 patients were uniformly treated according to the GEM2000 protocol (six alternating cycles of VBCMP/VBAD followed by high-dose therapy supported by autologous stem-cell transplantation (ASCT)). Median progression-free survival (PFS) and overall survival (OS) were 40 and 73 months, respectively.

BM *first-pull* aspirate samples were used for morphological assessment, with May-Grümwald-Giemsa staining. Morphology PC counts were obtained from a 200-cell differential count, using conventional bright-field microscopy.

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Table 1. Patient demographics and baseline characteristics.

	n=765
Male/female (%)	421 (55%) / 354 (45%)
Age, years; median (range)	60 (32–74)
Myeloma subtype, (%)	
IgG	405 (53%)
IgA	207 (27%)
Bence-Jones protein	138 (18%)
Non-secretory	11 (1.5%)
Other	4 (0.5%)
Durie-Salmon disease stage I / II / III, (%)	54 (7%)/275 (36%)/436 (57%)
International Staging System disease stage I/II/III, (%)	268 (35%)/314 (41%)/183 (24%)
β2-microglobulin, mmol/L; median (range)	27 (0–441)
Hemoglobin, g/L; median (range)	105 (10–168)
Creatinine, μmol/L; median (range)	84 (23–1014)
Calcium, mmol/L; median (range)	2 (0.5–5)
C-reactive protein, mg/dL; median (range)	1 (0–97.2)
Albumin, g/dL; median (range)	36 (3–70)
% of plasma cells in S-phase; median (range)	1.6 (0–15.3)
Cytogenetics	n=255
IgH translocations, (%)	
t(4;14)	24 (9%)
t(11;14)	32 (13%)
t(14;16)	8 (3%)
Others	24 (9%)
Del(13q), (%)	101 (40%)
Del(17p), (%)	20 (8%)
High-risk (any t(4;14), t(14;16), and/or del(17p)), (%)	47 (18%)

Immunophenotypic studies were performed on erythrocyte-lysed BM aspirate samples, according to previously described methods,[10-12] using a four-color direct immunofluorescence technique (FITC/PE/PerCP-Cy5.5/APC: CD38/CD56/CD19/CD45, CD138/CD28/CD33/CD38 and CD20/CD117/CD138/CD38) aimed at identifying, quantifying and characterizing PC. MFC data were acquired in a FACSCalibur™ flow cytometer (BD Biosciences [BDB], San Jose, CA, USA) using the CellQUEST software (BDB), with a double-step acquisition procedure for selected cases. In the first step, $2x10^4$ cells from the whole BM cellularity were measured. If the number of PCs acquired was $<3x10^3$, a second acquisition step was performed using a broad *live-gate* drawn to select and measure a total of $3x10^3$ $CD38^{hi}$ events showing low-intermediate SSC events. The Paint-A-Gate PRO program (BDB) was used for data analysis, following recommendations of the European Myeloma Network.[4] Plasma cell enumeration was performed using the CD38/CD138/SSC and the CD38/SSC strategies, both of which gave similar results. The overall number of nucleated BM cells was used as the denominator to calculate the overall percentage of BMPCs. All cases included in the study showed erythroid nucleated cells evaluated in the BM aspirate by MFC. The sample may therefore be considered representative of the BM.

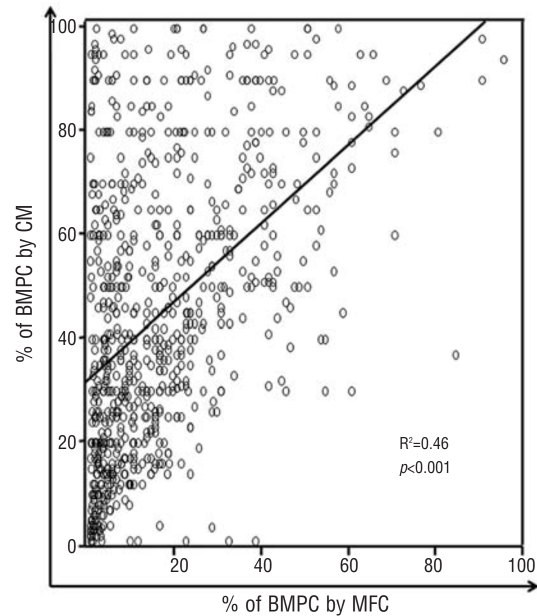

Figure 1. Correlation of morphological and multiparameter flow cytometry bone marrow plasma cell enumeration at diagnosis of multiple myeloma patients (n = 765).

Statistical analyses included one-tailed Student's independent sample *t*-tests and Pearson correlations, calculated with SPSS (version 15.0; SPSS Inc., Chicago, IL, USA), as previously described.[10,13-15] Survival curves were plotted according to the Kaplan-Meier method, differences being assessed by the log-rank test. The Cox regression proportional hazard model (stepwise regression) was used in a multivariate analysis of PFS and OS, retaining those variables with a statistically significant predictive value ($p<0.05$) in the predictive model.

Results and Discussion

Differences in bone marrow plasma cell counts between conventional morphology and multiparameter flow cytometry

As expected, the median percentage of BMPC measured by CM (40%; range: 5-100%) was significantly higher ($p<0.001$) than that obtained by MFC (11%; range: 0.5-95%), confirming previous results in smaller series of patients.[2,4,16] Greater infiltration by CM was detected in the majority of patients (93%, n=709), with equal PC levels in 3% of cases (n=27). The PC count obtained by MFC was greater than that with CM in only 29 patients (4%). Despite these differences, the PC counts with the two techniques were significantly correlated ($R^2=0.46$; regression coefficient=0.28, $p<0.001$; intercept=3.79; Figure 1). The fewer PCs detected by MFC could be explained by the different quality of BM samples used for the two techniques, whereby there was greater peripheral blood contamination in the MFC

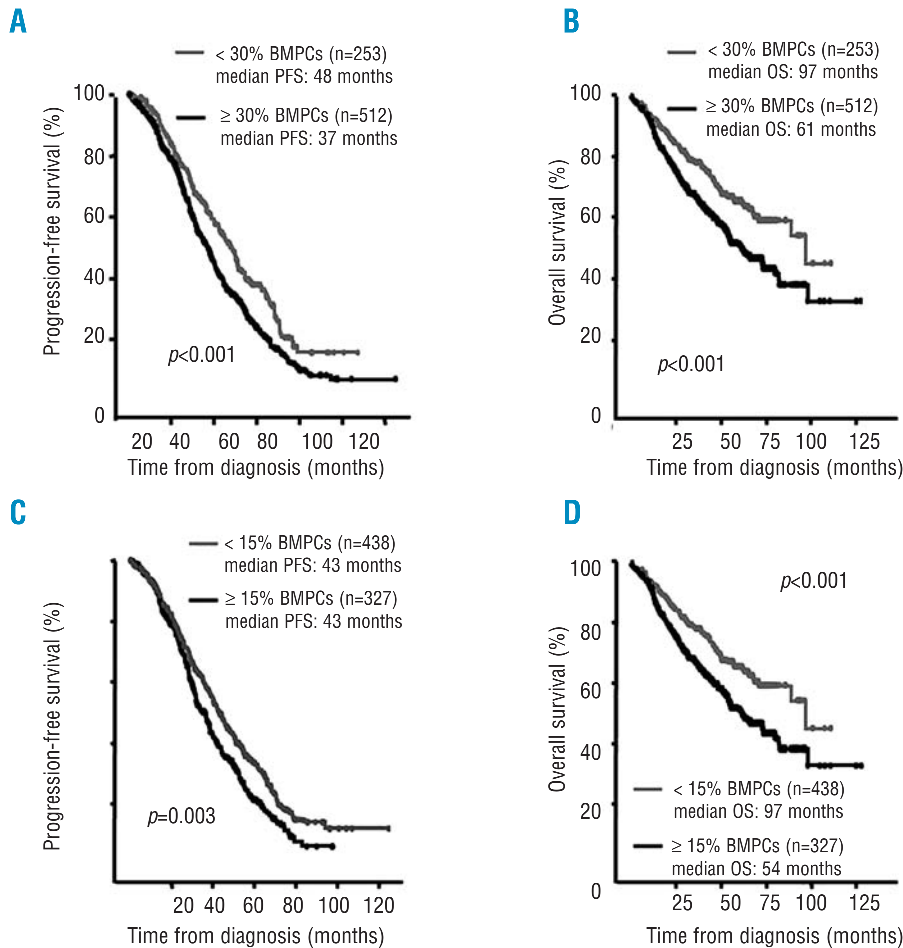

Figure 2. Progression-free survival (PFS; panels A and C) and overall survival (OS; panels B and D) of myeloma patients (n = 765) according to the percentage of bone marrow plasma cells (BMPCs) detected at diagnosis by conventional morphology (cut-off value of ≥ 30% BMPCs; panels A and B) and multiparameter flow cytometry (cut-off value of ≥ 15% BMPCs; panels C and D).

samples, and by the existence of small PC clusters (microaggregates) in the BM.[17] In this sense, Rawstron *et al*.[4] found a significantly lower percentage of PCs detected by MFC than by CM performed on first-pull BM aspirates, but similar values when the CM was performed on cytocentrifuged samples.[4] Also, samples evaluated by CM contain cells associated with lipid-enriched spicules, whereas MFC is performed on the BM fluid, which is depleted in the lipid-adhesive PC.[2] In fact, morphological quantification of BMPC may focus on those microaggregates where PCs are abundant, rather than from a randomly chosen field.

Prognostic impact of bone marrow plasma cell aspirate counts on progression-free survival and overall survival

PC counts assessed by CM and MFC distinguish between groups of patients with different prognosis, with optimal cut-off values of 30% and 15% BMPC for CM and MFC, respectively. Thus, patients with less than 30% BMPC at diagnosis, detected by CM, had significantly longer PFS (median of 48 *vs.* 37 months; $p<0.001$; Figure 2A) and OS (median of 97 *vs.* 61 months; $p<0.001$; Figure 2B) than patients with or more than 30% BMPC: PFS and OS rates at 5 years of 40% *vs.* 25% ($p<0.001$) and 65% *vs.* 50% ($p<0.001$), respectively. Regarding MFC, patients with less than 15% BMPC had significantly longer PFS (median 43 *vs.* 36 months; $p=0.003$; Figure 2C) and OS (median 97 *vs.* 54 months; $p<0.001$; Figure 2D) than cases with or more than 15% BMPC: PFS and OS at 5 years of 37% *vs.* 21% ($p=0.003$), and 68% *vs.* 53% ($p<0.001$), respectively. Other baseline variables of significance in the univariate analyses of survival were: age (>60 years; $p\leq0.02$), low hemoglobin (≤100 g/L; $p<0.001$), high serum levels of calcium (>10mg/dL; $p\leq0.004$) and creatinine (>2 mg/dL; $p<0.001$), advanced disease (ISS Stage III; $p<0.001$), high percentage of PC in S-phase (>0.5%; $p\leq0.01$) and high-risk cytogenetics (t(4;14), t(14;16), and/or del (17p); $p<0.001$). In the multivariate analysis, only high-risk cytogenetics was selected as an independent prognostic factor for both PFS and OS (Hazard ratio, HR: 2.7; $p<0.001$ and HR: 2.6; $p<0.001$, respectively), while for OS, patient age (HR: 1.6; $p=0.03$) and the percentage of PC detected by MFC at diagnosis (HR: 2.3; $p=0.006$) were also selected. Our results confirm previous reports in which the BMPC infiltration measured by CM did not maintain its independent prognostic value, irrespective of the cut-off value applied.[5,6,8,9]

We further investigated if different phenotypic characteristics could be detected according to the percentage of BMPC detected by MFC at diagnosis. We found that within the group with less than 15% BMPC there was a greater incidence of cases with the good prognosis

phenotypic pattern (CD28-negative, CD117-positive)[10] than for those with or more than 15% BMPC group (25% and 15%, respectively; p=0.004). Finally, we also investigated whether MFC remission (minimal residual disease negative) rates assessed at day 100 after ASCT was different in these two cohorts of patients. We found that cases with less than 15% PC by MFC at diagnosis more frequently reached immunophenotypical remission (46% of these cases became MRD-negative after autologous transplantation) compared with cases with more than 15% of BMPC at diagnosis (only 30% of these cases became MRD-negative) (p=0.02).

In summary, our results show in a very large series of uniformly treated myeloma patients that MFC immunophenotyping is a valid method for evaluating the PC burden in the BM of symptomatic MM patients at diagnosis, with a significant correlation with CM. More importantly, it is of independent prognostic value for predicting patient survival. Taken together, these results support the incorporation of MFC immunophenotyping into the routine evaluation of all MM patients at diagnosis.

Authorship and Disclosures

BP and MBV equally contributed to this work. JFSM, JJL and AO conceived the idea, and together with MBV designed the study protocol; BP, MBV, GM, JJP, and MAM analyzed the flow cytometry data; MVM, JJL, JB, together with all Pethema/GEM members contributed with provision of study material or patients; BP, MBV, and GM collected and assembled data; BP, MBV, and JFSM analyzed and interpreted data; BP, and MBV performed statistical analysis; and BP, MBV, and JFSM wrote the manuscript. All authors reviewed and approved the manuscript.

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