

# Immunophenotypic Aberrations, DNA Content, and Cell Cycle Analysis of Plasma Cells in Patients with Myeloma and Monoclonal Gammopathies

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**ABSTRACT:** We describe the immunophenotypic and gross DNA defects in 55 patients with myeloma and 50 patients with monoclonal gammopathy and review the literature on this subject (MedLine, 1994–2000). Our data confirmed previous reports indicating that in myeloma nearly all marrow plasma cells are abnormal ( $98.7 \pm 8.1\%$ ). In monoclonal gammopathy the fraction of abnormal plasma cells was  $35.0 \pm 32.8\%$ . In both myeloma and monoclonal gammopathy, the most frequent aberrant phenotypic features consisted of absence of expression of CD19, strong expression of CD56, and decreased intensity of expression of CD38; aberrant expression of CD10, CD20, CD22, or CD28 was observed in less than one-third of myeloma cases. The vast majority of cases had two or more phenotypic aberrations. In the DNA studies, 7% of myeloma cases were biclonal and 93% of cases were monoclonal. In those studies with only one plasma cell mitotic cycle, 37% had normal DNA content and 63% were aneuploid (hyperploid, 61%; hypoploid, 2%). The mean percentages of plasma cells in S- and G2M phases were  $4.9 \pm 8.5$  and  $4.4 \pm 6.9\%$ , respectively. Thirty-eight percent of cases had more than 3% of plasma cells in S phase. In monoclonal gammopathy, the DNA index of abnormal plasma cells ranged from 0.89 to 1.30 and the percentage of diploid (31%) and aneuploid (69%) cases was not different from the results found in myeloma. The differences in percentage of abnormal plasma cells in S- ( $7.4 \pm 8.6\%$ ) and G2M-phases ( $2.4 \pm 1.7\%$ ) in patients with monoclonal gammopathy were not statistically significant. © 2000 Academic Press

**Key Words:** plasma cells; DNA; immunophenotype; myeloma, monoclonal gammopathy.

## INTRODUCTION

Plasma cell dyscrasias comprise a number of diseases including myeloma and essential monoclonal gammopathy (1–5). Essential monoclonal gammopathy or monoclonal gammopathy of unknown significance, here designated “MG,” is a term used to designate patients with a monoclonal protein without other criteria for myeloma, macroglobulinemia, amyloidosis or other lymphoproliferative disorder and without other conditions that may be associated with a secondary mono-

clonal gammopathy (6–17). Follow-up studies revealed that a large fraction of patients with MG evolve into myeloma or another lymphoplasma-cytic disorder. The actuarial probability of malignant transformation is estimated to be around 5–10, 15–20, 25–35, and 30–40% at 5, 10 and 20, and 25 years (11–17). There is no a reliable single variable to predict this transformation.

Prognostic factors in myeloma are related to the tumor mass ( $\beta$ 2-microglobulin, reactive C protein and lactic dehydrogenase) and to the characteristics of the myelomatous plasma cells (plas-

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mablastic morphology), effects on normal hematopoiesis (cytopenias), renal function (creatinine), the performance status, and response to treatment (18–32). Some of these factors were identified more than two decades ago and were used in classical staging schemas (18–24). Other variables, including  $\beta$ 2-microglobulin, reactive C protein, interleukin 6 (IL 6), IL 6 receptor and aminopterin were described more recently (25–32). Plasma cell labeling-index (LI) and Ki-67 proliferation index were also described as variables useful in discriminating between myeloma and MG and to distinguish myeloma cases with poor prognosis from stable myeloma patients requiring no immediate therapy (33–41). Difficulties in implementing these assays for the clinical laboratory have limited their usefulness. DNA analysis and immunophenotypic studies are now more available. Phenotypic and DNA abnormalities proved to be useful in predicting the clinical aggressiveness and response to therapy (42, 43) as well as in distinguishing between MG and myeloma (44, 45). Other variables that may improve accuracy in predicting both progression of MG and prognosis of myeloma patients are cytogenetic abnormalities (46–52), activation of oncogenes or inactivation of tumor suppressor genes (53–57), imbalance between apoptosis-suppressor and -inducer proteins (58, 59) and multidrug resistance gene expression (41, 60, 61).

We describe the major phenotypic and DNA aberrations found in a series of 55 patients with untreated myeloma and 50 patients with MG and evaluate the value of plasma cell phenotyping in establishing the differential diagnosis between these entities. We also review the literature on this subject and discuss the biology of plasma cell disorders.

## MATERIALS AND METHODS

### *Patients and Samples*

Data presented are derived from 55 patients with untreated myeloma and 50 patients with MG studied at the Hospital Geral de Santo António, Porto, Portugal (57 males, 48 females; median age of 61 years, range 29–95 years). Marrow samples were collected into EDTA-K3 and cell concentra-

tion was determined and adjusted with phosphate buffered saline to give  $5\text{--}15 \times 10^6$  cells/ml. The mean percentage of plasma cells was  $2.2 \pm 1.9\%$  (0.2–6.0) and  $27 \pm 29\%$  (2–77%) of all nucleated marrow cells for MG and myeloma, respectively (mean  $\pm$  standard deviation, minimum–maximum). Patients with myeloma had <10, 10–20, and >20% plasma cells, in 12, 12, and 31 cases, respectively.

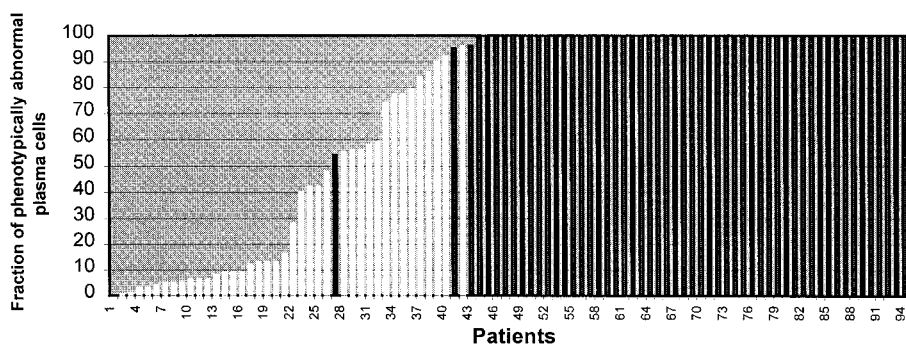
### *Immunophenotypic Studies*

Immunophenotyping was performed in all 55 myeloma patients and in a subset of 40 MG patients, using a direct immunofluorescence technique. Briefly, 100  $\mu$ l of whole blood containing between 0.5 and  $2 \times 10^6$  nucleated cells was incubated for 15 min at room temperature in the darkness with saturating amounts of the monoclonal antibodies indicated below. Lysis of red blood cells and fixation of the leukocytes was then performed, processing samples in the Q-Prep workstation using Immunoprep (Beckman Coulter, Miami, FL).

The following three-color panel (FITC/PE/PE-Cy5) was used in both MG and myeloma: CD138/CD56/CD38 and CD10/CD19/CD38. In myeloma two additional stainings were performed in order to better characterize plasma cells: CD20/CD22/CD38 and CD45/CD28/CD38. The source of antibodies were as follows: CD19, CD20, CD22, and CD38 (Beckman Coulter); CD28 and CD56 (Becton–Dickinson, San José, CA); CD138 (ImmunoQuality Products); and CD45 (Dako A/S, Glostrup, Denmark).

### *DNA Studies*

DNA studies were performed in 55 patients with myeloma and in a subset of 27 patients with MG using a two step- washing method developed in our laboratory. This method is based on the use of both FITC-conjugated primary (antigen-specific) and secondary (anti-immunoglobulin) antibodies, a strategy that increases the FITC-signal of CD38-positive cells without an appreciable increase in nonspecific staining. This procedure obviated difficulties usually imposed by



**FIG. 1.** Fraction of abnormal plasma cells in patients with MG (white columns) and myeloma (black columns).

the decrease of expression of cell surface antigens when cells are simultaneously stained for DNA (data not shown). As a first step cells were incubated with 10  $\mu$ l of FITC-conjugated mouse anti-human CD38 (Cytognos, Salamanca, Spain) for 15 min at room temperature, protected from light, and washed twice in phosphate-buffered saline. As a second step 20  $\mu$ l of FITC-conjugated rabbit anti-mouse immunoglobulins (Dako) was added and cells were incubated under the same conditions. Once this incubation period was completed, cells were washed once in buffered saline and then processed with DNA-Prep either automatically (DNA-Prep workstation) or manually, according to the manufacturer's instructions (Beckman Coulter). Briefly, 100  $\mu$ l of DNA Prep LPR (reagent to lyse red cells and permeabilize cell membranes) was added and vortexed for 8 s; after that, 2 ml of DNA-Prep stain (propidium iodide-PI solution with RNase) were added and vortexed for 10 s. Finally, the samples were incubated at 4°C in the darkness for 15 min before acquisition.

#### *Data Acquisition and Analysis*

Data acquisition was carried out in a EPICS-XL-MCL flow cytometer (Beckman Coulter) equipped with a 15-mW air-cooled 488-nm argon laser, using the XL2 software program (Beckman Coulter). Instrument alignment and standardization were performed daily and electronic compensation was used to remove spectral overlap. A normal blood sample was processed in parallel every day and the red fluorescence high voltage was adjusted to place the mean channel of the

G0/G1 peak of normal blood lymphocytes at the channel  $200 \pm 10$ . Information on a minimum of  $1.5 \times 10^3$  plasma cells was acquired and stored as list mode data for each staining.

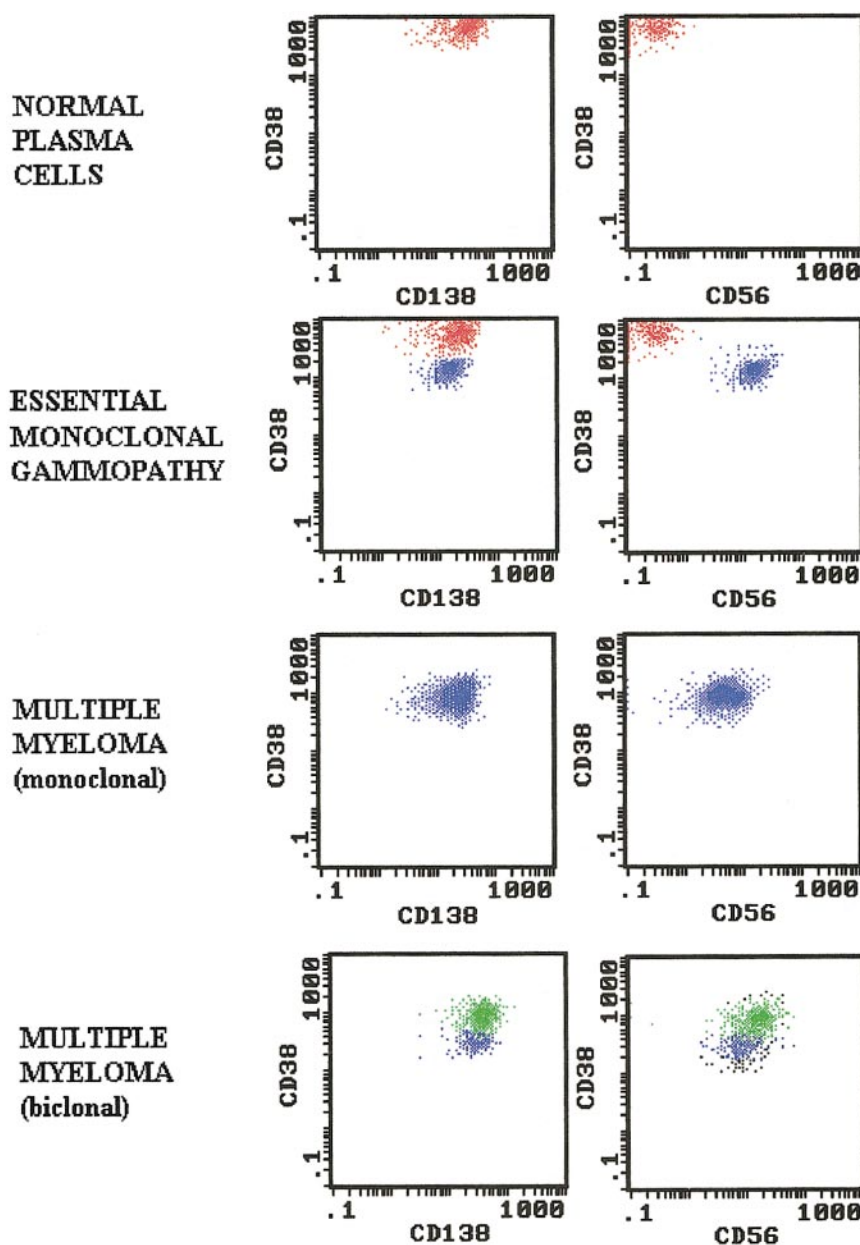
Immunophenotypic analysis was performed using the XL2 software (Beckman Coulter). Briefly, plasma cells were first identified based on the expression of CD138 antigen and high levels of expression of CD38; then, normal and abnormal plasma cells were identified and characterized for the expression of each antigen and the fraction of each plasma cell population was calculated. Criteria used for distinguished normal and abnormal plasma cell populations were based on the expression of CD38, CD19, and CD56 and were previously described in detail elsewhere (44, 45).

For DNA analysis, cell doublets were excluded and CD38-brightly positive plasma cells and CD38-dim normal residual marrow cells were gated separately in the SSC/CD38 histogram; the DNA content and cell cycle distribution of each cell population were analyzed individually using the Multicycle software (Phoenix Flow Systems, San Diego, CA). The DNA index was calculated as the ratio of the modal channel of G0/G1 peak of the CD38 brightly positive plasma cells and that of the CD38-dim residual normal marrow cells. Plasma cells were considered aneuploid when the DNA index was out of the range 0.95–1.05.

## RESULTS

### *Immunophenotype*

As illustrated in Fig. 1, when all patients with plasma cell dyscrasias were considered together,



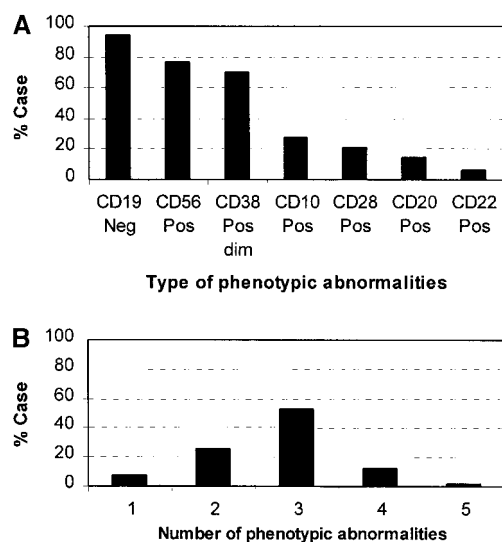
**FIG. 2.** Illustrative dot plots showing normal plasma cells (red dots) and abnormal plasma cells (blue dots) in marrow from normal individuals, myeloma and MG patients.

the fraction of BM plasma cells that had an abnormal immunophenotype was highly variable ranging from 0 to 100%.

In patients with MG, a large fraction of marrow plasma cells had a normal immunophenotype. Indeed, the fraction of abnormal plasma cells was only  $35.0 \pm 32.8$  (0.0–96.0) and was greater than 95% in only 1 case. The typical phenotypic features observed in MG are shown in Fig. 2. Absence of expression of CD19 (100%) and strong expression of CD56 (90%) together

with a decreased expression of CD38, were the most important phenotypic features in distinguishing the abnormal plasma cell population. Abnormal plasma cells usually had a slightly higher size and granularity, as evaluated by forward-scatter and side-scatter, respectively (data not shown). In the only case in which abnormal plasma cells were not detected in the BM, B-cells were monoclonal, although there were neither clinical nor other laboratory criteria for the diagnosis of a B-cell lymphoproliferative disorder.





**FIG. 3.** Type (A) and number (B) of phenotypic abnormalities found in myelomatous plasma cells.

In contrast to that observed in MG, abnormal plasma cells comprised  $98.7 \pm 8.1\%$  (40–100%) of total marrow plasma cell in myeloma patients, only one case having less than 95% of abnormal plasma cells. Illustrative examples of monoclonal and biclonal myeloma cases are shown in Fig. 2. As in MG, the most frequent phenotypic aberrations were the absence of expression of CD19 (94%), the expression of CD56 (77%) and decreased expression of CD38 (70%) (Fig. 3A). Other phenotypic abnormalities occurred less frequently and included: expression of CD10 (28%); expression of CD28 (21%); expression of CD20 (15%) and expression of CD22 (6%). As shown in Fig. 3B, the vast majority of cases (93%) had two or more aberrant immunophenotypic features. In two cases two different abnormal plasma cell populations were identified.

#### *DNA Content and Cell Cycle Distribution*

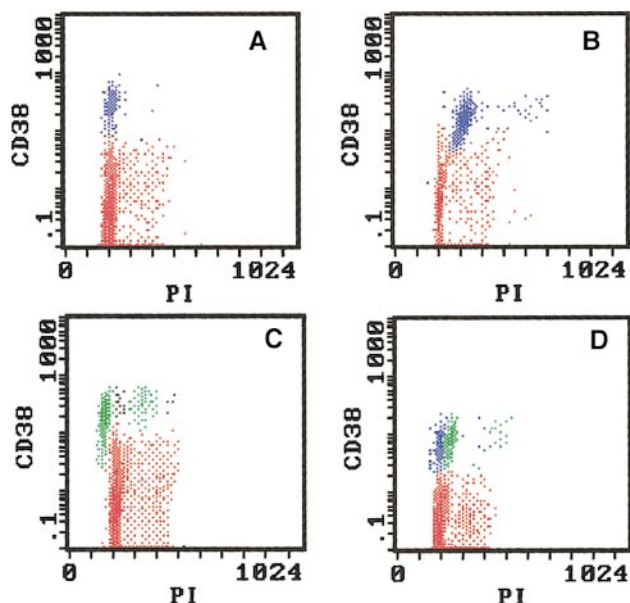
From a total of 27 cases of MG in which DNA analysis was performed, 18 cases had immunophenotypic studies whereas the remaining nine cases did not. Information on DNA content and cell cycle distribution of abnormal plasma cells was conclusive in only 16 of 27 cases analyzed. In the remaining 11 cases it was not possible to distinguish an abnormal plasma cell cycle from the normal one. These cases were: seven cases in

which the fraction of phenotypically abnormal plasma cells was very low (less than 10% of plasma cells), in which only a diploid cell cycle was observed, and four cases in which immunophenotypic studies were not available and only one diploid cell cycle was observed. In 27 MG cases studied the DNA index ranged from 0.89–1.30 ( $1.07 \pm 0.10$ ) and the cell cycle distribution was as follows: G0/G1 phases  $91.2 \pm 8.4\%$  (68.2–100.0), S phase  $6.1 \pm 7.0\%$  (0.0–25.2) and G2/M phases  $2.7 \pm 2.8$  (0.0–13.2). If only the cell cycles that corresponded were considered, abnormal plasma cells the following values were obtained: G0/G1 phases  $90.2 \pm 9.6\%$  (68.3–97.8%), S phase  $7.4 \pm 8.6$  (0.0–25.2) and G2/M phases  $2.4 \pm 1.7$  (0.0–6.5). In this case DNA index ranged from 0.89 to 1.30 ( $1.09 \pm 0.13$ ) five cases had a normal DNA content and 11 were aneuploid (10 hyperploid and 1 hypoploid). In cases classified as aneuploid, two plasma cells cycles were simultaneously identified: one, aneuploid, corresponding to the abnormal plasma cells and the other, diploid, corresponding to residual normal plasma cells.

Normal residual marrow cells had the following cell cycle distribution: G0/G1 phases  $88.3 \pm 5.0\%$  (77.7–98.8%), S phase  $9.4 \pm 4.2\%$  (1.1–18.4%) and G2/M phases  $1.7 \pm 0.9\%$  (0.0–3.9%).

In patients with myeloma, the DNA index of the abnormal plasma cell population ranged from 0.78 to 1.54%. Four cases (7%) were biclonal (two of them corresponding to those being also classified as biclonal based on immunophenotyping) and 51 cases (93%) monoclonal. Of those cases having only one plasma cell cycle, 19 (37%) had an normal DNA content, whereas the remaining were aneuploid (hyperploid, 31 cases (61%); hypoploid, 1 case (2%). Illustrative PI/CD38 dot plots of normal marrow plasma cells and diploid, hyperploid, hypoploid and biclonal myelomatous plasma cells are shown in Fig. 4. Cell cycle distribution was as follows: G0/G1 phases  $90.2 \pm 13.9\%$  (12.1–98.8%), S phase  $4.9 \pm 8.5\%$  (0.0–46.5%) and G2/M phases  $4.4 \pm 6.9\%$  (0.0–50.0%). Thirty eight percent of cases had a percentage of plasma cells in S phase higher than 3%.

Normal residual marrow cells had cell cycle distribution similar to that found in MG: G0/G1



**FIG. 4.** Illustrative CD38/PI dot plots showing the DNA content and cell cycle distribution of plasma cells (blue or green dots) and normal residual marrow cells (red dots) in diploid (A), hyperploid (B), hypoploid (C), and biclonal (D) plasma cell disorders.

phases  $90.4 \pm 5.3\%$  (77.4–99.2%), S phase  $7.4 \pm 4.7\%$  (0.1–19.9%) and G2/M phases  $1.9 \pm 1.3\%$  (0.0–5.5%).

## DISCUSSION AND REVIEW OF THE LITERATURE

### *Immunophenotypic and DNA Abnormalities of Malignant Plasma Cells*

Many studies have focused on the immunophenotypic aberrations of myeloma although comparative studies with normal marrow plasma cells are less frequent (62–67). Expression of CD138 and very strong reactivity for CD38 are the best markers for identifying myelomatous as well as normal plasma cells (68–71). Aberrant immunophenotypic features are frequent in myelomatous plasma cells and the frequency of each abnormality has been similar to that found in this series. Normal and myelomatous plasma cells usually do not express CD45 although some cases, especially those with a more immature morphology, may be CD45-positive (63, 67, 69, 72–74). Myelomatous plasma cells usually do not

express CD19, whereas normal plasma cells do (66, 75, 76). Malignant plasma cells may express other B-cell associated markers that are usually negative in normal plasma cells, such as CD20, CD21 and CD22 (63, 67, 69, 72–75). In contrast to normal, myelomatous plasma cells are usually strongly positive for CD56, although they may not express other natural killer associated markers, including CD16 and/or CD57 (75, 77). Myelomatous plasma cells do not express CD34 (78, 79), whereas CD117 is expressed on cells in nearly one third of myeloma cases (80). A considerable fraction of myelomatous plasma cells express CD10 (81, 82), CD28 (83, 84), and CD40 (83, 85), and in a minor fraction of patients myelomatous plasma cells also express markers that have been associated to the myelomonocytic, erythroid, and megakaryocytic lineages, such as CD13, CD14, CD15, and CD33 (72, 86–88).

Until recently, flow cytometric DNA quantification and cell cycle analysis were limited by the fact that most studies were based on single DNA staining, limiting its application to samples with a high proportion of plasma cells. Multiparametric DNA analysis using double staining with propidium iodide and anti-CD38 overcame this problem by allowing DNA analysis of plasma cells, specifically (102, 103). Using a different technical approach for DNA studies in plasma cells, we obtained results similar to those reported previously; the majority of myeloma cases have an abnormal DNA content and hyperploid cases predominate over hypoploid (104–111). The percentage of abnormal plasma cells in S phase is not higher in myeloma than in MG and in both cases the percent of cells in S phase is lower in plasma cells than in normal hematopoietic cells (45, 102).

### *Immunophenotypic and DNA Abnormalities, Disease Expression, and Prognosis*

Adhesion molecules may have an important role in determining the localization of malignant plasma cells in the marrow extracellular matrix and in extramedullary spread (89–96). Imbalance of molecules implicated in plasma cell proliferation—such as CD40 and CD117 (85, 97, 98)—and of proteins that regulate apoptosis—including

p26/bcl2 and CD95/Fas (56, 57, 59)—may also be crucial for plasma cell survival and may be implicated in the pathogenesis of malignant gammopathy by prolonging the survival of plasma cells and increasing the chance of acquiring additional gene defects. Some of the antigens aberrantly expressed on myelomatous plasma cells may have prognostic implications and expression of CD10, CD20, CD28, CD45, myelomonocytic markers, and surface immunoglobulins correlates with aggressive biological and clinical characteristics, as did the absence of expression of CD56 and VLA-5/CD49e (72–74, 77, 81, 83, 84, 89, 99–101). Disease progression may be accompanied by immunophenotypic changes, such as an increase of expression of CD28 and CD86 (84). Expression of proteins associated with multidrug resistance phenotype may determine nonresponsiveness to therapy (41, 61).

Abnormalities in plasma cell DNA content and cell cycle distribution may also have important prognostic implications in myeloma (104–112). Survival is significantly longer in hyperploid compared with diploid myeloma (45, 109–111). Hypoploid or bichlonal myeloma apparently have a worst prognosis and higher resistance to chemotherapy, when compared to those having a diploid or a hyperploid DNA content in plasma cells (107, 108, 111), although some recent data questioned this finding (112). The percentage of S phase plasma cells is an independent prognostic factor in myeloma cases; a high number of S phase plasma cells (higher than 2 or 3%, depending on the study) predicts a poor prognosis (113, 114).

### *Progression of MG to Myeloma*

Studies of the phenotypic differences between MG and myeloma plasma cells (44, 45, 76) established that virtually all marrow plasma cells from patients with myeloma have an abnormal phenotype, characterized by a slightly lower reactivity for CD38, strong expression of CD56 and absence of expression of CD19, in contrast to normal plasma cells, which are CD38-strongly positive/CD56-negative/CD19-positive. Patients with MG have two clearly defined plasma cells

populations, one identical to normal plasma cells and the other having an immunophenotype similar to myelomatous plasma cells (44, 45). Our results support these observations indicating that the fraction of residual normal plasma cells is a powerful variable to use for the discrimination of MG and myeloma patients. The fact that MG and myeloma form a spectrum of plasma cells disorders in which the monoclonal plasma cell population progressively occupy the entire plasma cell pool in marrow might suggest that MG patients having a higher proportion of abnormal plasma cells are those with a higher probability of developing myeloma in a shorter period of time. This surmise is consistent with transformation to myeloma as a multi-step process (115–118). The state of the B-cell differentiation in which the first oncogenic event lead to a monoclonal expansion occurs probably in a mature B-cell that has been in contact with antigen (119). However, as in the case of MG, monoclonal plasma cell expansion occurs without clinical manifestations and additional mutational events are required in order to develop clinical features of malignancy. The fact that MG patients acquire chromosomal changes gradually within several related plasma cell subclones (120) and that some chromosomal abnormalities, such as monosomy 13, are associated with transition of MG to myeloma also supports this hypothesis (46, 47). Oncogenes also play a role in the conversion of MG into myeloma as well in the proliferation and survival of malignant plasma cells (53–57). For instance, loss or inactivation of Rb1 and p53 tumor suppressor (54) and mutational activation of N- and K-ras oncogenes (55, 57) are frequent findings in myeloma but not in MG, suggesting that these molecular events occur late during the process of oncogenesis. Cell adhesion molecules that mediate interactions between plasma cells and marrow stroma cells and stimulate production of cytokines (121–123) may also be important in this process. IL 6 has a crucial role in maintaining plasma cell proliferation (124) whereas IL1 $\beta$  leads to osteoclast activation and osteolysis and can increase the expression of adhesion molecules and induce IL 6 secretion by either autocrine (plasma cells) or paracrine (stromal cells) mechanisms (125). An-



giogenic cytokines such as vascular endothelial growth factor and basic fibroblast growth factor expressed by myeloma cells may also be involved (126). Recent data also suggest that a virus (Kaposi-associated virus–HHV-8) may be associated with development of myeloma (127). Long-term follow-up of MG patients will determine if a high fraction of abnormal plasma cells should be considered as an index of an imminent transformation into myeloma.

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