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Tumorigenesis and Neoplastic Progression

Multiparameter Flow Cytometry Evaluation of Plasma Cell DNA Content and Proliferation in 595 Transplant-Eligible Patients with Myeloma Included in the Spanish GEM2000 and GEM2005<65y Trials

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The incorporation of high-dose therapy/autologous stem cell transplantation (HDT/ASCT) and novel agents has significantly improved survival in patients with multiple myeloma (MM), but whether this improvement also benefits patients harboring poor prognostic features, such as nonhyperdiploid MM (NH-MM) and a high proliferation index, remains largely unknown. We analyzed the DNA content and proliferation index of bone marrow plasma cells (PCs) by multiparameter flow cytometry in 595 newly diagnosed transplant-eligible patients with MM included in two consecutive PETHEMA/GEM trials: GEM2000 [VBMCP/VBAD (vincristine, carmustine, melphalan, cyclophosphamide, prednisone/vincristine, bischloroethylnitrosourea, adriamycin, and dexamethasone) followed by HDT/ASCT; *n* = 319] and GEM2005<65y (randomized induction with VBMCP/VBAD/bortezomib or thalidomide/dexamethasone or bortezomib/thalidomide/dexamethasone followed by HDT/ASCT; *n* = 276). Of the 595 patients, 295 were classified as NH-MM (49.6%) and 336 (56.5%) as high-proliferative MM (≥1% PCs in S-phase). Detection of NH-MM DNA content and ≥1% PCs in S-phase were of independent prognostic value for overall survival. Treatment with bortezomib-based regimens abrogated the inferior overall survival of patients with ≥1% PCs in S-phase but not of patients with NH-MM. Finally, a comparative analysis of PC proliferation index at diagnosis versus disease progression showed a two-

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fold increase at relapse in 44 of 52 patients (85%) analyzed at both time points. NH-MM and a high proliferation index assessed by multiparameter flow cytometry remain as independent prognostic factors in MM, but the latter may be overcome by incorporating novel agents in the HDT/ASCT setting. ***(Am J Pathol 2012, 181: 1870–1878; http://dx.doi.org/S0002-9440(12)00587-1)***

The incorporation of high-dose therapy/autologous stem cell transplantation (HDT/ASCT) and novel therapeutic agents[1–4] along with intensive scientific research that unraveled key genetic features[5–9] have markedly changed the landscape of multiple myeloma (MM). Overall, MM is mainly divided into two major categories: hyperdiploid (H-MM; usually harboring multiple numerical chromosomal alterations) and nonhyperdiploid (NH-MM; typically enriched for IgH translocations),[7] the latter being associated with inferior survival.[10–13] However, in-depth analysis through microarray gene expression profiling (GEP) has shed significant light on the molecular heterogeneity of MM plasma cells (PCs), with up to 10 patient subgroups being identified.[14,15] Among these, the proliferation, MMSET, and MAF/MAFB subgroups encompass patients with high-risk disease and a dismal outcome.[15,16] Since the MMSET molecular subgroup is intrinsically associated with t(4;14) and this abnormality can be systematically evaluated by fluorescence *in situ* hybridization (FISH) analysis, important data are emerging suggesting that the poor outcome of patients harboring t(4;14) may be partially overcome by some regimens.[17,18] In turn, information on the remaining high-risk subgroups is limited.

Assessment of the proliferation of the tumor cells through the PC labeling index (PCLI) is an important prognostic factor in MM,[19] but its routine use has been hampered by the labor-intensive fluorescent microscope slide–based method required for its application.[20] Multiparameter flow cytometry (MFC) immunophenotyping is a widely available tool in routine laboratories. It provides not only rapid measurement of PC proliferation but also simultaneous assessment of PC total DNA content.[21,22] Importantly, we recently observed in elderly patients with MM receiving novel agents during induction and maintenance that the poor prognosis of NH-MM was not abrogated,[23] but whether survival of transplant-eligible patients with NH-MM and high PC proliferation is improved by the incorporation of novel agents up front remains largely unknown.[24,25]

In the present study, we applied MFC to analyze the DNA content and proliferation index of myelomatous PCs from a series of 595 newly diagnosed transplant-eligible patients with MM. These results show that the PC DNA content and proliferation status are independent prognostic factors in MM and that novel agents–based regimens cannot overcome the poor prognosis of NH-MM but rather prolong survival of patients with MM and high proliferation indices. In addition, we investigated which of the commonly assessed cytogenetic abnormalities drives PC proliferation. Finally, we assessed whether there is a difference in individual patients in the proliferative rate of PCs between diagnosis and disease progression.

Materials and Methods

Patients

The study included 595 patients with MM diagnosed according to the International Myeloma Working Group criteria.[26] Patients were included in two consecutive PETHEMA (Programa para el Estudio de la Terapéutica en Hemopatías Malignas)/GEM (Grupo Español de MM) trials: GEM2000 [VBMCP/VBAD (vincristine, carmustine, melphalan, cyclophosphamide, prednisone/vincristine, bischloroethylnitrosourea, adriamycin, and dexamethasone) followed by HDT/ASCT and 2 years of maintenance with interferon plus prednisone; $n = 319$] and GEM2005<65y [randomized induction with the same chemotherapy plus bortezomib in the last two cycles or thalidomide/dexamethasone (TD) or bortezomib/thalidomide/dexamethasone (VTD) followed by HDT/ASCT, and 3 years of maintenance with interferon-a2b or thalidomide or thalidomide/bortezomib; $n = 276$]. Patients included in the GEM2000 protocol with >65 years, levels of serum calcium >14 mg/dL and/or serum creatinine >2 mg/dL were excluded from the analysis to avoid confounding survival bias. Median follow-up was 38 months (range, 1 to 123 months). All the samples were collected after informed consent was given, and the study was approved by the clinical research ethical committee.

MFC Immunophenotypic DNA Studies

Simultaneous staining for DNA (with propidium iodide) and PC surface antigens (CD38 and CD138) was performed as described elsewhere.[27] A minimum of 2×10^6 nucleated bone marrow (BM) cells per case were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using FACSDiva software version 6.0 (BD Biosciences). Total PC DNA content was calculated by dividing the median fluorescence channel of the G_0/G_1 peak of CD38/CD138^{hi} myelomatous PCs by the median fluorescence channel of G_0/G_1 residual normal BM cells present in the sample. Patients were considered to be hyperdiploid (H-MM) when the PC DNA content ranged from 1.06 to 1.74; nonhyperdiploid (NH-MM) cases included patients with a PC DNA content <0.95 (hypodiploid), >1.74 (tetraploid/near-tetraploid), and ranging from 0.95 to 1.05 (diploid).[23] The distribution of myelomatous PCs in the G_0/G_1, S-phase, and G_2/M cell-cycle phases was assessed according to well-established methods[28] after specifically selecting CD38/CD138^{hi} PCs and excluding debris and cell doublets. The PC proliferation index was calculated as the percentage of PCs in S-phase in the whole BM PC compartment.

FISH Analysis

FISH was performed at baseline in immunomagnetically enriched PCs from 325 patients. Patients harboring

t(4;14), t(14;16), and/or del(17p13) were classified as having high-risk disease and all other cases as standard risk, according to the International Myeloma Working Group guidelines.[7]

Statistical Analysis

The Mann-Whitney *U*-test was used to estimate the statistical significance of differences between groups. Progression-free survival (PFS) and overall survival (OS) curves were plotted using the Kaplan-Meier method, and the log-rank test was used to estimate the statistical significance of differences observed between curves. A univariate analysis was conducted to assess the impact of various baseline prognostic factors on PFS and OS (Table 1). The Cox proportional hazards regression model (stepwise regression) was used in a multivariate analysis of PFS and OS, retaining those variables with a significant predictive value ($P \leq 0.05$) in the predictive model. For all the statistical analyses, SPSS software version 15.0 (SPSS Inc., Chicago, IL) was used.

Results

PC DNA Content and Its Relationship with Disease Characteristics and Patient Survival

Of the 595 patients included in the present study, 295 were classified as having NH-MM (49.6%) and the remaining 300 as having H-MM (50.4%). As expected, patients with NH-MM showed an increased frequency of t(4;14) (20% versus 9%, $P = 0.008$), t(11;14) (28% versus 5%; $P < 0.001$), t(14;16) (5% versus 1%; $P = 0.055$), and del(13q14) (50% versus 33%, $P = 0.002$) but not del(17p13) (5% versus 7%, $P = 0.37$). Moreover, NH-MM showed more frequently advanced disease (International Staging System stage III, 21% versus 13%; $P = 0.03$) and higher BM PC infiltration by MFC (16% versus 9%; $P = 0.008$).

As expected, patients with NH-MM had significantly inferior median PFS (34 versus 44 months; $P = 0.004$; Figure 1A) and OS (67 versus 84 months; $P = 0.005$; Figure 1B) than those with H-MM. We detected the presence of two different clones of myelomatous PCs (with different DNA content) in 34 of the 595 patients (5.7%) by

Table 1. Baseline Disease Features with a Significant Effect on PFS and/or OS (Univariate and Multivariate Analyses)

	Univariate analysis				Multivariate analysis			
	PFS		OS		PFS		OS	
Feature	Median (months)	*P* value	Median (months)	*P* value	HR	*P* value	HR	*P* value
ISS disease stage					1.2	0.34	1.9	0.02
I	48	<0.001	NR	<0.001				
II	35		64					
III	25		46					
Age (years)					—	—	0.6	0.084
<60	41	0.1	86	0.002				
≥60	34		62					
Hemoglobin (g/L)					1.1	0.71	1.4	0.18
>100	45	<0.001	93	<0.001				
≤100	28		57					
Serum β_2-microglobulin (mg/L)					1.0	0.86	1.6	0.13
≤3.5	43	0.006	84	0.001				
>3.5	28		61					
LDH (IU/L)					2.0	0.004	2.2	0.009
Normal	43	<0.001	80	<0.001				
Increased	24		46					
BM PCs (%)					1.5	0.07	—	—
<30	48	0.004	79	0.15				
≥30	34		75					
BM PCs by MFC (%)					1.6	0.02	1.4	0.14
<15	47	<0.001	79	0.004				
≥15	31		61					
Normal PCs/BM PCs by MFC (%)					6.5	0.009	4.1	0.17
>5	53	<0.001	78	0.04				
≤5	38		76					
% PCs in S-phase					1.9	<0.001	2.0	0.003
<1	43	<0.001	93	0.001				
≥1	34		66					
DNA ploidy status by MFC					1.2	0.21	1.7	0.02
Hyperdiploid	44	0.004	84	0.005				
Nonhyperdiploid	34		67					
Interphase FISH cytogenetics					1.6	0.01	2.1	0.003
Standard risk	44	<0.001	80	<0.001				
High risk*	23		41					

*High-risk cytogenetics includes any t(4;14), t(14;16), and del(17p); standard-risk cytogenetics includes all other cases.
HR, hazard ratio; ISS, International Staging System; NR, not reported; —, not tested.

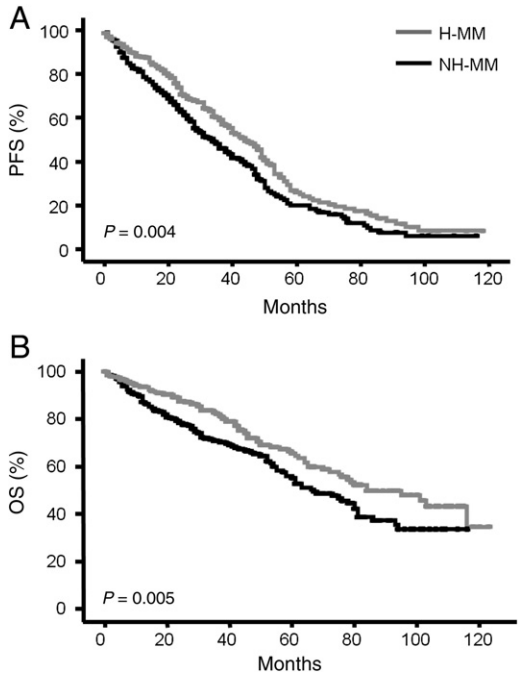

Figure 1. PFS (**A**) and OS (**B**) of patients with symptomatic MM treated with HDT/ASCT grouped according to the presence of H-MM (n = 300) and NH-MM (n = 295) PC DNA content assessed by MFC at diagnosis. The median PFS was 44 months in the H-MM group and 34 months in the NH-MM group. The median OS was 84 months in the H-MM group and 67 months in the NH-MM group.

MFC (Figure 2), and although the number is small, this subgroup had a dismal outcome compared with patients in whom only one PC clone was detected (median PFS and OS of 26 and 52 months, respectively; $P \leq 0.005$). After this analysis in the overall MM population, we specifically looked at whether the outcome of NH-MM versus H-MM differed according to the trial: GEM2000 and GEM2005<65y. As occurred in the overall series, patients with NH-MM included in the GEM2000 trial had a significantly inferior PFS (34 versus 42 months, $P = 0.04$; Figure 3A) and a trend toward decreased OS (63 versus 79 months, $P = 0.11$; Figure 3B). Likewise, significant differences emerged for PFS (40 months versus not reached; $P = 0.03$; Figure 3C) and OS (both medians not reached; $P = 0.004$; Figure 3D) for patients included in the GEM2005<65y trial according to the NH-MM versus H-MM classification.

PC Proliferation Index and Its Relationship with Disease Characteristics and Patient Survival

The median percentage of PCs in S-phase in the whole series was 1.14% (range, 0% to 13%). When we looked for a specific association between the percentage of PCs in S-phase and other baseline parameters, no significant correlations were found (data not shown), except for an inferior proliferation index in patients with NH-MM versus those with H-MM (1.00% versus 1.23%; $P = 0.008$). We then investigated the potential association between specific cytogenetic alterations and PC proliferation. Patients harboring t(11;14) showed a significantly decreased percentage of PCs in S-phase (0.7% versus 1.2%, $P < 0.001$), but no significant differences were recorded for t(4;14), t(14;16), del(13q14), and del(17p13). Accordingly, the presence or absence of high-risk cytogenetics was not associated with different PC proliferation, irrespective of whether we looked in all patients (1.12% versus 1.16%, respectively; $P = 0.33$) or specifically in patients with NH-MM versus H-MM (see Supplemental Table S1 at *http://ajp.amjpathol.org*).

In accordance with the median proliferation index of the whole series (1.14%), patients were stratified using a cutoff point of ≥1% versus <1% S-phase PCs, which translated into a significantly different PFS (median of 34 versus 43 months; $P < 0.001$) and OS (median of 66 versus 93 months; $P = 0.001$). Moreover, we confirmed that the higher the percentage of S-phase PCs, the worse the associated survival. In particular, a proliferation index ≥3% identified a subgroup of patients (92 of 595, 15.5%) with high-risk disease (median PFS and OS of 22 and 45

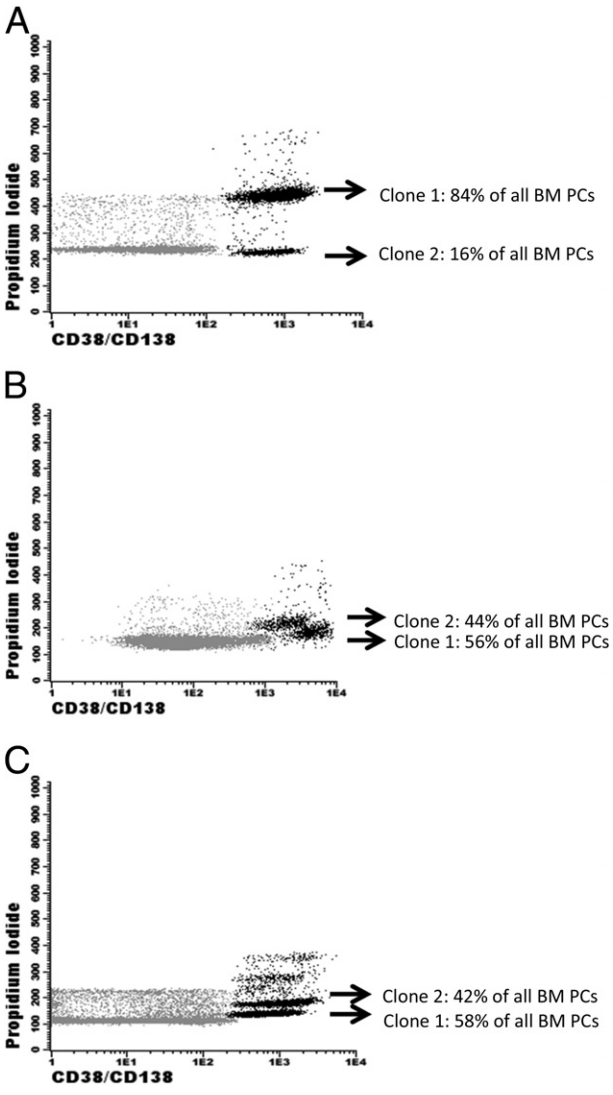

Figure 2. Bivariate dot plot histograms illustrating the detection of two different PC clones (with different DNA content) by MFC in the BM of three newly diagnosed patients with myeloma. **A** represents a case with diploid and tetraploid PC clones, whereas on **B** and **C,** two hyperdiploid PC clones coexist.

Figure 3. PFS and OS of patients with symptomatic MM treated with HDT/ASCT grouped according to the presence of H-MM and NH-MM PC DNA content assessed by MFC at diagnosis. **A** and **B:** PFS and OS of patients with MM included in the GEM2000 protocol ($n = 319$). **C** and **D:** PFS and OS of patients with MM included in the GEM2005<65y trial ($n = 276$).

months, respectively; $P < 0.001$; Figure 4). In the group of patients with ≥1% S-phase PCs ($n = 336$), the complete response rate was slightly higher than that in the remaining patients (45% versus 36%, $P = 0.053$); however, the duration of the complete response tended to be shorter (PFS at 3 years: 65% versus 80%; $P = 0.057$). Thereafter, we explored whether the poor prognosis of patients with a high proliferation index (≥1% PCs in S-phase) could be abrogated in the most recent GEM2005<65y trial. The results show that while for patients included in the GEM2000 trial patient stratification into ≥1% versus <1% S-phase PCs predicted for different PFS (33 versus 43 months; $P = 0.003$; Figure 5A) and OS (61 versus 93 months; $P < 0.001$; Figure 5B), patients with ≥1% S-phase PCs receiving novel agents up front still showed only slightly inferior PFS (40 months versus not reached; $P = 0.09$; Figure 5C) and similar OS (both medians not reached; $P = 0.99$; Figure 5D) compared with patients with <1% PCs in S-phase. Accordingly, it seems that treatment with novel agents overcomes the adverse prognosis of a high proliferation index (≥1% S-phase PCs); however, this applied only to patients treated with either VTD or VBMCP/VBAD/bortezomib (median PFS of 40 and 44 months, respectively) but not to patients who received TD (PFS of 23 months; $P = 0.03$).

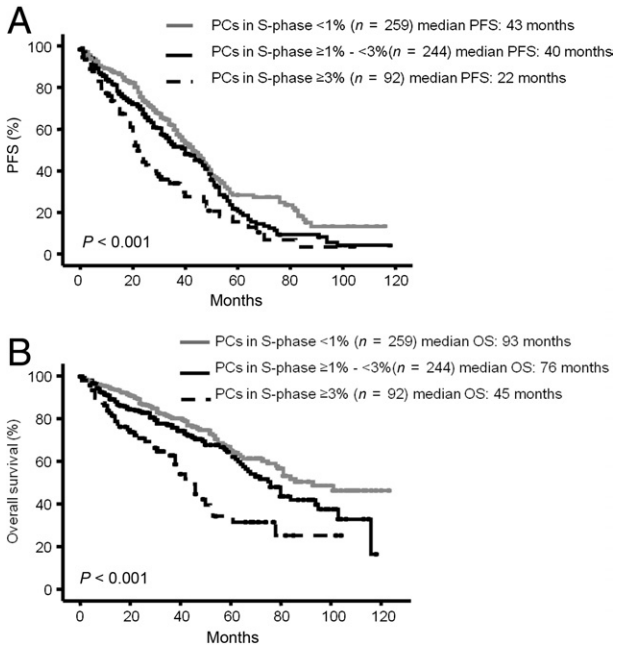

Figure 4. PFS (**A**) and OS (**B**) of patients with symptomatic MM treated with HDT/ASCT grouped according to the presence of <1% ($n = 259$), ≥1% to <3% ($n = 244$), and ≥3% ($n = 92$) S-phase PCs as assessed by MFC at diagnosis.

Prognostic Factors for PFS and Overall Survival by Univariate and Multivariate Analyses

On univariate analysis, eight factors in addition to the PC DNA content and proliferation index by MFC were identified as having a significant adverse effect on PFS (Table 1): International Staging System disease stage, baseline anemia, serum β_2-microglobulin, lactate dehydrogenase, BM PC infiltration by morphology and by MFC, persis-

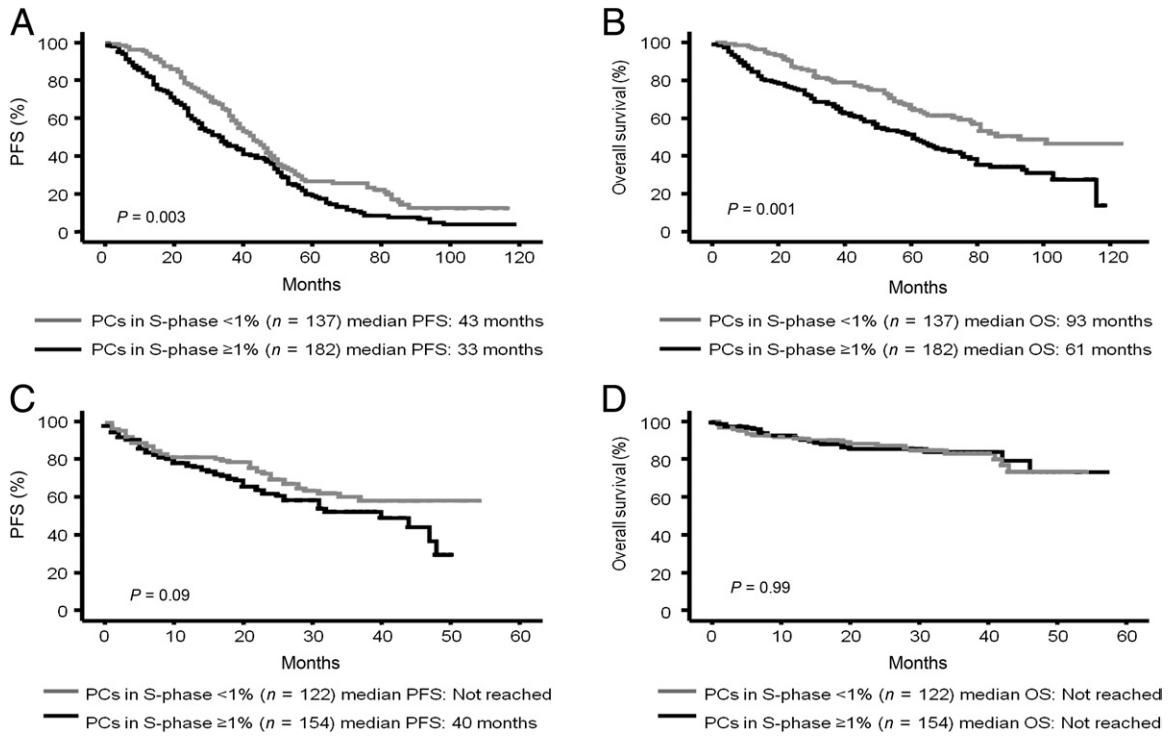

Figure 5. PFS and OS of patients with symptomatic MM submitted to HDT/ASCT grouped according to the presence of <1% and ≥1% S-phase PCs as assessed by MFC at diagnosis. **A** and **B:** PFS and OS of patients with MM included in the GEM2000 protocol (n = 319). **C** and **D:** PFS and OS of patients with MM included in the GEM2005<65y trial (n = 276).

tence of normal PC levels (>5%) at baseline by MFC, and high-risk cytogenetics. For OS, all the same factors except BM PC infiltration by morphology retained their prognostic value, in addition to patient age.

In the multivariate analysis (Table 1), the presence of high-risk cytogenetics, increased lactate dehydrogenase level, ≥1% S-phase PCs, >15% PCs by MFC, and >5% normal PCs in the BM PC compartment remained as independent prognostic factors for PFS; in turn, for OS, the presence of high-risk cytogenetics, increased lactate dehydrogenase level, ≥1% S-phase PCs, International Staging System disease stage, and NH-MM PC DNA content were selected (Table 1). It should be noted that the prognostic significance of PC proliferation according to ≥3% S-phase PCs was not superior to high-risk cytogenetics in the multivariate analysis (data not shown).

Proliferation Index at Diagnosis Versus Disease Progression

To address the final question on whether there is a difference in the proliferation of PCs between diagnosis and disease progression, we compared the proliferative rate of PCs from 52 patients with paired BM samples at diagnosis and at relapse (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). Of note, 44 of the 52 patients (84.6%) showed an increased percentage of S-phase PCs at relapse, with a median twofold difference between the proliferative rate at diagnosis versus that at relapse (0.97% versus 2.29%, respectively; $P < 0.001$).

Discussion

Introduction of HDT/ASCT in the 1990s and the discovery and incorporation of novel agents in the past decade represent a major cornerstone in the treatment of MM.[1–3,29,30] In fact, this contribution opened the debate about whether patients defined in the era of conventional agents as being at high risk (identified by PCLI,[19,31] FISH cytogenetics,[5,8,9] GEP,[16] or immunophenotyping[32]) may now have a better outcome in the era of the novel agents.[33] Accordingly, a growing body of data is emerging regarding the impact of novel agents on survival of high-risk patients carrying cytogenetic abnormalities, such as t(4;14) or del(17p13),[23,34] with promising results for some specific subtypes of high-risk MM.[18,35,36] However, this type of information on other poor prognostic features remains limited.[24,31] Herein, we report on the prognostic relevance of the PC DNA content and proliferation index assessed by MFC in a large series of transplant-eligible patients, with approximately half of them receiving novel agents up front.

In line with results of previous studies based on conventional cytogenetics,[13] copy number variation,[11] and GEP,[15,16] we found that patients with H-MM have increased survival compared with patients with NH-MM. Moreover, in the present series, NH-MM DNA content emerged as an independent prognostic factor for OS but not for PFS, which is also in line with previous observations.[10,23] Although the molecular mechanisms responsible for these findings remain largely unknown, it should be considered that based on recent observations, pa-

tients with a hyperdiploid GEP signature have superior postrelapse survival.[12] Moreover, NH-MM DNA content was independent of high-risk cytogenetics, stressing that the high-risk factors associated with patients with NH-MM might not be fully captured by FISH cytogenetics. In the present study, we identified a small fraction (5.7%) of patients with two different PC clones with different PC DNA content. High-density genomic tools are now shedding some light on the cytogenetic heterogeneity of clonal PCs in MM,[37,38] which can be found in up to 20% of the patients via RT-PCR analysis.[39] Herein, we show that patients with clonal heterogeneity potentially relating to increased chromosomal instability can be easily identified by MFC and have a dismal outcome (median PFS and OS of 26 and 52 months, respectively). Specific analysis of the cytogenetic profile of these patients showed no significant differences compared with those with only one PC clone according to the DNA content (data not shown). Altogether, these results require further investigation and validation.

The assessment of PCLI has repeatedly been shown to be a powerful prognostic tool in patients with newly diagnosed myeloma,[40–43] but analysis of PC proliferation by fluorescent microscope slide–based methods is labor intensive and time-consuming and, therefore, difficult to incorporate into routine clinical practice.[20] In the past, MFC has proved to be a valid methodological alternative.[44] In the present study, we confirm early results[28,45–47] in which the proliferation index of PCs measured by MFC emerged as a powerful prognostic factor in transplant and nontransplant settings. Herein, patients with a proliferation index greater than the median (>1% of S-phase PCs) had inferior PFS and OS; these results were further confirmed in the multivariate analysis. Moreover, it was also possible to identify a subgroup of patients (≈15%) showing extremely increased proliferation (≥3% of S-phase PCs) associated with a high-risk signature (median OS of <4 years), in accordance with data derived from high-throughput GEP analysis.[15,16,25] In one of such studies, a higher proliferation index as assessed by GEP was significantly associated with del(13q14).[25] The present results do not confirm this, and neither do other associations with cytogenetic abnormalities, such as t(4;14), t(14;16), and del(17p11). In turn, patients with NH-MM and/or t(11;14) showed a significantly lower percentage of S-phase PCs.

One of the major goals of this study was to assess the impact of novel agents–based HDT/ASCT regimens on the survival of patients with NH-MM and a high proliferation index. To answer this question, we compared the outcome of patients stratified according to the GEM2000 and GEM2005<65y trials. Overall, the present results show that patients with NH-MM had inferior PFS and OS than did patients with H-MM, particularly in the GEM2005<65y trial, which may suggest that the survival benefit of incorporating novel agents in the HDT/ASCT setting was mainly favoring patients with H-MM. Regarding the prognostic value of the proliferation index, we observed that the poor outcome detected for patients with ≥1% S-phase PCs included in the GEM2000 trial partially disappeared in the GEM2005<65y trial since their OS was identical to that of patients with <1% S-phase PCs. These findings suggest that HDT/ASCT schemes based on the novel agents may overcome the poor prognosis of patients with a high PC proliferation index. Accordingly, the Mayo Stratification of Myeloma and Risk-Adapted Therapy criteria now consider increased proliferation as an intermediate-risk feature in the era of novel agents.

Finally, an interesting comparison between the PCLI measured at diagnosis and after treatment has been recently reported[20] in which a reduction in the PCLI after initial therapy predicted improved survival. This led us to investigate the potential role of the proliferation index of PCs in disease progression through the sequential analysis of paired diagnostic and relapse samples from a series of 52 patients; in most of the studied cases (85%), the percentage of S-phase PCs doubles at relapse. These results confirm and expand on previous observations derived by GEP showing an increment of patients with the proliferation signature at relapse,[12] suggesting that the investigation of novel agents targeting a high-proliferative PC could be of value, particularly in relapsing patients with a high proliferation index.

In summary, the present results show that the PC DNA content and proliferation index by MFC immunophenotyping remain as independent prognostic factors in MM, and that the incorporation of novel agents in the HDT/ASCT setting may improve the survival of patients with a high proliferation index but not that of patients with NH-MM. Moreover, we found that patients show an increased proliferation index at relapse; therefore, the precise mechanisms leading to PC proliferation deserve further investigation.

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