Cytometry Part B (Clinical Cytometry) 88B:165–182 (2015)

Original Article

Old and New Immunophenotypic Markers in Multiple Myeloma for Discrimination of Responding and Relapsing Patients: The Importance of "Normal" Residual Plasma Cell Analysis

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Background: Multiple myeloma is an incurable disease characterized by proliferation of clonal malignant plasma cells (CPCs), which can be immunophenotypically distinguished from polyclonal plasma cells (PPCs) by multiparameter flow cytometry (MFC). The utility of PPCs analysis in detecting prognostic and predictive information is still a matter of debate. Methods: we tested the ability of 11 MFC markers in detecting differences in the immunophenotype of CPCs and PPCs among patients in various disease stages; we verified if these markers could be associated with disease stage/response to therapy despite the role of clinical parameters. Results: significant changes in the expression of markers occurred both in CPCs and PPCs. CD58 on PPCs of responding patients was downregulated compared with PPC of relapsing group. Fraction of CD200 expressing PCs was lower in control subjects than in PPCs from MGUS and myeloma groups. CD11a levels of expression on both CPCs and PPCs showed an upregulation in newly diagnosed and relapsing patients versus PCs of controls; CD20 was less expressed on control PCs than on MGUS CPCs and PPCs. CD49d revealed to be advantageous in discrimination of PPCs from CPCs. In our multiple regression model, CD19 and CD49d on CPCs, and CD45, CD58 and CD56 on PPCs maintained their association with groups of patients independently of other prognostic variables. Conclusions: we provide a feasible start point to put in order ranges of expression on PPCs in healthy and myeloma subjects; we propose a new approach based on PPC analysis to monitor the stages of the disease. © 2014 International Clinical Cytometry Society

Key terms: multiparameter flow cytometry; multiple myeloma; monoclonal gammopathies

How to cite this article: Pojero F, Casuccio A, Parrino MF, Cardinale G, Colonna Romano G, Caruso C, and Gervasi F. Old and New Immunophenotypic Markers in Multiple Myeloma for Discrimination of Responding and Relapsing Patients: The Importance of "Normal" Residual Plasma Cell Analysis. Cytometry Part B 2015; 88B: 165–182.

Additional Supporting Information may be found in the online version of this article.

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Received 10 July 2014; Revised 3 December 2014; Accepted 14 December 2014

Published online 19 December 2014 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/cyto.b.21218

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Multiple myeloma (MM) is an incurable neoplastic plasma cell (PC) disorder characterized by proliferation of clonal malignant plasma cells (CPCs) in bone marrow (BM), and presence of monoclonal protein (M protein) in blood and/or urine, associated with organ dysfunction (1). The first stage in the development of MM is the emergence of asymptomatic monoclonal gammopathy of undetermined significance (MGUS). In some of these patients, this progresses to smoldering MM and ultimately to symptomatic MM, with an annual risk of around 1% for patients with MGUS (2). MM accounts for approximately 1% diseases and is the second most common hematologic cancer. The incidence of MM in Europe is 4.5–6.0/100,000/year with a median age at diagnosis of between 63 and 70 years; the mortality is 4.1/100,000/year (3). Treatment differs among autologous stem cell transplantation eligible patients and those who are not candidates (4); responsiveness to treatments varies largely among patients due to the high heterogeneity of MM. The decision of which treatment is best has been a difficult issue in MM. However, due to the introduction of novel drugs (bortezomib, lenalidomide and thalidomide) that have been able to achieve good quality responses, changes in treatment strategies can be seen (5). The importance of reaching complete response (CR) is undoubtedly recognized in the transplant setting (6–8) although it is less clear in the nontrasplant setting. Clinicians may benefit from multiparameter flow cytometry (MFC), since this technology allows to define the immunophenotypic characteristics of CPCs, to study normal/reactive polyclonal PCs (PPCs) of both healthy and MM subjects, to distinguish CPCs from PPC pool (9–14), to evaluate the risk of progression from MGUS to MM (15,16) and the presence of minimal residual disease (MRD) (10,17,18), to identify new therapeutic targets (19–21), and to provide prognostic information. For some markers, immunophenotypic appropriateness and prognostic role have already been explored (CD27, CD56, CD117, CD19, CD45) (22–25), instead other markers have been less investigated (CD20 and CD200) (12,22,26,27) or their utility is still a matter of debate (CD30, CD49d, CD11a and CD58) (28–36). MFC data flank other factors influencing prognosis such as ISS and Durie-Salmon stage at diagnosis, hyper- and hypodiploidy and lactate dehydrogenase levels (37–39). A commonly described experimental approach is based on the study of immunophenotype of CPCs (and sometimes residual PPCs), dividing patients in newly diagnosed subjects and treated/relapsed patients. It is not fully demonstrated if PPCs in BM post therapies may exhibit immunophenotypic variations interfering with MRD detection; similarly it is not clear if disease progression or response to therapy may be predicted analyzing PC ability to downregulate or upregulate surface antigen expression during therapy. In addition, BM PPCs have been demonstrated to be more immunophenotypically heterogeneous than previously understood, and subpopulations of non-neoplastic PCs with an immunophenotype similar to myeloma PCs have been described (40–45). In this article, our primary endpoint was structured as follows: (1) analysis of the expression of CD45, CD19, CD27, CD56, CD117, CD20, CD200, CD49d, CD58, CD11a, and CD30 in MGUS subjects, MM responding and relapsing patients, to test the utility of these markers in detecting CPCs in various clinical scenarios; (2) study of the differences in the immunophenotype of PPCs comparing normal subjects with responding and relapsing patients; (3) detection of variations in expression of the cited antigens on CPCs in groups of new or treated patients, and comparison with PPCs of MRD negative and normal subjects. As secondary endpoint we determined the existence of a correlation between exordium of disease/response to therapy/disease progression and the expression of the listed markers on PPCs and CPCs; then we verified if such a correlation is influenced by most commonly considered clinical and prognostic variables. The inclusive objective is to define the utility of PPCs analysis in MM diagnosis and follow up, and the possibility of elaborating disease stage related MFC panels. In our knowledge, this is the first time that this kind of approach has been used to evaluate the expression of all these markers simultaneously in the same group of subjects.

MATERIALS AND METHODS

Patients and BM Samples

A total of 60 subjects (42 male and 18 female) were included in this study. Control specimens consisted of 10 BM samples from patients who were suspected to have a haematological disease and revealed to be non oncological subjects (Control group). These patients have no history of MM, MGUS or lymphoid/myeloid neoplasm. BM samples of 40 patients with MM and 10 patients with already documented (7 subjects) or newly diagnosed (3 subjects) MGUS submitted to our laboratory for routine analysis were evaluated by MFC and morphology. For every patient clinical chemical and immunological profiles, as well as reference intervals were provided by the U.O. Patologia Clinica – Laboratorio Analisi Cliniche of ARNAS Civico, Palermo (Italy). Clinical data and history for MGUS and MM cases were provided by U.O. Oncoematologia of ARNAS Civico, Palermo (Italy). Disease stage was defined according to Durie-Salmon and ISS staging criteria (46,47). Response to therapy was defined according to Bird et al. (48). Of MM samples, 10 were obtained at presentation (group New), 10 from patients with progressive disease (group Progressive), 10 from patients in partial remission (3 very good partial response, 6 partial response and 1 stable disease – group Therapy) and 10 from patients that achieved stringent CR (group Complete). All MGUS patients were considered as a separate group (MGUS group). Informed consent procedures and forms were proposed to and approved by the ARNAS Civico Medical Ethics Committee. Written informed consent was given by all subjects in line with the Declaration of Helsinki

Table 1
Combinations of Antibodies

	FITC	PE	PC5.5	PC7	APC	APC-Alexa Fluor 750	PB	KO
Tube 1	Cyt κ	Cyt λ	CD38	CD56	CD138	CD27	CD19	CD45
Tube 2	CD20	CD56	CD38	CD117	CD138	CD27	CD19	CD45
Tube 3	CD11a	CD200[a]	CD38	CD56	CD138	CD27	CD19	CD45
Tube 4	CD30[a]	CD58	CD38	CD56	CD138	CD27	CD19	CD45
Tube 5		CD49d[a]	CD38	CD56	CD138	CD27	CD19	CD45

Cyt: cytoplasmic; FITC: fluorescein isothiocyanate; PE: phycoerythrin; PC5.5: phycoerythrin-cyanin 5.5; PC7: phycoerythrin-cyanin 7; APC: allophycocyanin; PB: pacific blue; KO: krome orange.
[a]All antibodies were purchased from Beckman Coulter (Miami, FL), except those with which were purchased from BD Pharmingen™ (San Jose, CA).

Protocol. BM samples were collected in EDTA tubes and processed in one hour since collection.

Multiparameter Flow Cytometry

Details about antibodies and instrument are indicated in on line Supporting Information Tables SI1 and SI2. Specimens were fragmented with a sterile syringe and filtered using a 80μm filter; nucleated cells were enumerated using UniCel® DxH™ 800 Coulter® Cellular Analysis System (Beckman Coulter, Miami, FL) and brought to a final concentration of 10^6cells/100μl with PBS w/o calcium and magnesium (EuroClone, Milan, Italy). Combinations of antibodies used to stain surface and intracellular markers are listed in Table 1. For staining of surface markers, 100μl of each sample were incubated with the opportune combinations of antibodies for 15 minutes in the dark. Erythrocytes were lysed adding 1 mL of VersaLyse™ Lysing Solution and incubating tubes for 20 minutes in the dark. For intracellular staining of kappa and lambda light chains, 50μl of sample were washed 5 times with 2 mL of PBS w/o calcium and magnesium (EuroClone, Milan, Italy), and processed with PerFix-nc (Beckman Coulter, Miami, FL) following instructions. Samples were all acquired with Navios™ Flow Cytometry System, data were collected with Navios v1.0 Software (Beckman Coulter, Miami, FL) and then analyzed with Kaluza® Flow Cytometry Analysis Software v1.2 (Beckman Coulter, Miami, FL). Daily testing of instrument was performed as indicated: standardization of light scatter, fluorescence intensity and optimal hydrodynamic focusing instrument settings were verified using Flow-Set Pro Fluorospheres (Beckman Coulter, Miami, FL); compensation matrix for each combination of antibodies was tested with CYTO-COMP™ Cell Kit (Beckman Coulter, Miami, FL); optical alignment and fluidics were checked using Flow-Check Pro Fluorospheres (Beckman Coulter, Miami, FL). To identify PCs, a combination of CD38, CD138, and CD45 together with side scatter properties was used; the first gate was set on CD38 versus CD138 as suggested (10). Distinction between normal/reactive and clonal PC compartments was performed on the basis of their most frequent aberrant phenotypes (as regards CD38, CD19, CD27, CD56, and CD45); results were confirmed by the presence of clonal restriction in population showing the abnormal phenotype, and the absence of restriction in normal PCs (10,22). The κ:λ ratio was defined as abnormal if < 0.5 or > 3 (49). A minimum of 200 events were collected in the PC gate for each tube; to reach this result, a total of 200,000–2,000,000 events were acquired. For each marker, an internal negative population present within the sample was used to define gates and sample fluorescence background (23,50). Data were measured as percentage of cells presenting the antigen (percentage of positive cells) and Mean Fluorescence Intensity Ratio (MFI ratio). MFI ratio for each fluorochrome-marker conjugate was defined as the geometric mean fluorescence of the positive population normalized for the geometric mean fluorescence of the negative population. Cellular DNA content and ploidy were analyzed by CYCLOSCOPE-MM (Cytognos, Salamanca, Spain) following instructions. DNA index was used to define the presence of aneuploidies as follows: hypodiploidy when the DNA index was <0.95, pseudodiploidy when the DNA index was 0.95–1.05 (excluding those subjects with DNA index of 1, who were indicated as perfect diploid), hyperdiploidy (HRD) when DNA index was >1.05, and near tetraploidy when DNA index was >1.75 (51).

BM Film Staining

BM films were prepared and stained with May-Grünwald-Giemsa staining method as described elsewhere (52,53). All reagents were supplied by Merck Millipore, Darmstadt, Germany.

Statistical Analysis

Continuous non normal data are expressed as median values (range); normal variables are indicated as mean±SD. Baseline differences between groups were assessed by the χ^2 test or Fisher's exact test, as needed for categorical variables. The univariate analysis of variance (ANOVA) was performed for parametric variables, and post hoc analysis with the Tukey's test was used to determine pairwise differences. The Kruskal-Wallis statistic test was performed for nonparametric analysis. The Wilcoxon signed-rank test was used to evaluate intragroup difference. For multiple comparisons the Bonferroni correction was performed. Multinomial logistic

Table 2
General Characteristics of Subjects Included in This Study

		Control	MGUS	Complete	Therapy	New	Progressive		PW comp
Sex	M	100% (10)	70% (7)	40% (4)	80% (8)	30% (3)	100% (10)	Ct, P vs. N	0.02
	F		30% (3)	60% (6)	20% (2)	70% (7)			
Time fd (years)			7 ± 8.1 (7)	3 ± 1.8	1.5 ± 1.5	0 ± 0	4.4 ± 1.8	M vs. T	0.021
								M vs. N	0.002
Subtype	IgA λ		30% (3)			30% (3)			NT
	IgA κ				10% (1)		50% (5)		NT
	IgG λ		30% (3)	20% (2)	30% (3)	60% (6)			NT
	IgG κ		50% (5)	40% (4)	40% (4)	10% (1)	50% (5)		NT
	λ			40% (4)	20% (2)				NT
	κ			20% (2)					NT
D-S Stage	I A			20% (2)		30% (3)	70% (7)	T vs. P	0.003
	II A			20% (2)	50% (5)	50% (5)	30% (3)		
	II B				10% (1)	10% (1)			
	III A			60% (6)	10% (1)			Cp vs. N, P	0.01
	III B				30% (3)	10% (1)			
ISS Stage	I			10% (1)	30% (3)	40% (4)			
	II			90% (9)	40% (4)	30% (3)	80% (8)	Cp vs. N	0.02
	III				30% (3)	30% (3)	20% (2)		
ASCT				60% (6)	50% (5)			Cp vs. P	0.01
								T vs. P	0.032

Continous variables are indicated as mean ± SD; other results are expressed as percentage of cases. The number of subjects is indicated between brackets. *Y*, years; Time fd, time passed from diagnosis of the disease; λ, myeloma secerning only lambda chain; κ, myeloma secerning only kappa chain; D-S stage, Durie-Salmon stage; ASCT, autologous stem cell transplantation; PW comp, pairwise comparison; Ct, Control; M, MGUS; Cp, Complete; T, Therapy; N, New; P, Progressive; NT, not tested. Everytime different pairwise comparison against one group gave the same *P* value, groups that were compared with that group are reported separated by a comma.

regression analysis examined the correlation between various patients groups (dependent variables) and markers measurements (independent variable) in simple and multiple regression models. χ^2 and Likelihood Ratio χ^2 statistics were used in assessing goodness of fit in Regression model. Data were analyzed by the Epi Info software (version 6.0, Centers for Disease Control and Prevention, Atlanta, GA) and IBM SPSS Software 21.0 version (IBM Corp., Armonk, NY). All *P*-values were two-sided and $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of Patients

Characteristics of patients and statistical significant differences as regards sex, stage, and therapies are summarized in Table 2. Examining age, MGUS patients (70.9 ± 5.6 years) were significantly older than Control and Complete subjects (58.3 ± 5.8 and 59.8 ± 8.5 years, respectively; vs. Control $P = 0.008$ and vs. Complete $P = 0.027$). Progressive patients (80.3 ± 4.2 years) also presented a more advanced age compared with Control, Complete, and Therapy (61.9 ± 9.5 years) ($P < 0.0005$ in all cases), and with New subjects (66.3 ± 10.8 years, $P = 0.002$). When MFC analysis was performed, 40% of Complete, 30% of Therapy and 30% of Progressive patients have interrupted treatment from at least 15 days. Lenalidomide was the treatment of choice in 40% of Complete and 20% of Progressive cases. Thalidomide as monotherapy was administered to 20% of Complete subjects. Bortezomib-dexamethasone therapy was taken by 20% of Therapy and 50% of Progressive patients (Progressive vs. Complete $P = 0.032$). In Therapy group, 20 and 30% of subjects underwent bortezomib-cyclophosphamide-dexamethasone and bortezomib-thalidomide-dexamethasone regimens, respectively. Statistical analysis was extended to therapeutic regimens administered to patients before the time of this study. Frequencies of each type of therapeutic regimen was recordered. Significant differences were found for vincristine-melphalan-cyclophosphamide-prednisone (VMCP) therapy, only given in 50% of progressive cases (Progressive vs. Complete and Therapy $P = 0.032$), and bortezomib-thalidomide-dexamethasone (VTD) regimen, used in 20% and 50% of Complete and Therapy patients, respectively, (Therapy vs. Progressive $P = 0.032$). Clinical variables recorded for each group are indicated in Table 3. Patients in each group were also subgrouped depending on presence of serum albumin $\geq$3.5 or $<$3.5 g/dL, serum creatinine levels $<$ or $\geq$2 mg/dL and M protein production rates low, high or comprised between 5 and 7 g/dL (for IgG) and 3 and 5 g/dL (for IgA), just as reported for Durie-Salmon and ISS staging criteria, but in these cases no significant difference emerged (data not shown). In addition, data about presence of eventual comorbidities were recorded. No significant differences were observed

Table 3
Clinical Characteristics of Patients

	Control	Mgus	Complete	Therapy	New	Progressive	PW comp	
CRP > 0.3			20% (2)	40% (4)	40% (4)	100% (10)	P vs.Ct, M P vs.Cp	<0.0005 0.0007
B2M < 3.5	100% (10)	80% (8)	100% (10)	40% (4)	40% (4)		P vs.Ct, Cp P vs.M	<0.0005 0.0007
B2M ≥ 5.5		20% (2)		40% (4)	30% (3)	50% (5)	P vs.Ct, Cp	0.032
3.5 ≤ B2M < 5.5				20% (2)	30% (3)	50% (5)	P vs.Ct, M, Cp	0.032
HB < 8.5					10% (1)			
HB > 10	100% (10)	100% (10)	100% (10)	70% (7)	80% (8)	50% (5)	P vs.Ct, M, Cp	0.032
8.5 ≤ HB ≤ 10				30% (3)	10% (1)	50% (5)	P vs.Ct, M, Cp	0.032
BUN > 25				10% (1)	20% (2)	50% (5)	P vs.Ct, M, Cp	0.032
LDH > 530				20% (2)	20% (2)	50% (5)	P vs.Ct, M, Cp	0.032
S IFE+		100% (10)		100% (10)	100% (10)	100% (10)	Ct, Cp vs.all	<0.0005
U IFE+		50% (5)		80% (8)	30% (3)	80% (8)	N vs.Ct, Cp; P vs.Ct, Cp	0.0007
S AB FLC ratio (range 0.31–1.56)		100% (10)		50% (5)	100% (10)	100% (10)	M vs.Ct, Cp; N vs.Ct, Cp; P vs.Ct, Cp	<0.0005
U AB FLC ratio (range 2.04–10.37)		80% (8)		10% (1)	40% (4)	50% (5)	M vs.Ct, Cp M vs.T	0.0007 0.005

Results are expressed as percentage of cases, the number of subjects is indicated between brackets. CRP > 0.3, C-reactive protein > 0.3 mg/dL; B2M < 3.5, β-2-microglobulin < 3.5 mg/L; B2M > 5.5, β-2-microglobulin > 5.5 mg/L; 3.5 ≤ B2M < 5.5, 3.5 mg/L ≤ β-2-microglobulin < 5.5 mg/L; HB < 8.5, hemoglobin < 8.5 g/dL; HB > 10, hemoglobin > 10 g/dL; 8.5 < HB < 10, 8.5 g/dL<hemoglobin<10 g/dL; BUN > 25, blood urea nitrogen > 25 mg/dL; LDH > 530, lactate dehydrogenase > 530 IU/L; S IFE +, serum immunofixation positive; U IFE+, urine immunofixation positive, S AB FLC ratio, serum abnormal free light chain ratio; U AB FLC ratio, urine abnormal free light chain ratio; PW comp, pairwise comparisons; Ct, Control; M, MGUS; Cp, Complete; T, Therapy; N, New; P, Progressive; all, M, T, N, P. Everytime different pairwise comparison against one group gave the same *P* value, groups that were compared with that group are reported separated by a comma. Different pairwise comparisons giving the same *P* value are reported separated by a semicolon.

for chronic renal insufficiency, hypothyroidism, obesity, thalassemia trait, HCV, and HBV related chronic hepatitis (data not shown). Diabetes interested 20% of Complete, 40% of MGUS and 70% of Progressive subjects (Progressive vs. Control, Therapy and New $P = 0.003$ in all cases). Cardiopathy was a complication in 20% of MGUS and 50% of Progressive patients (Progressive vs. all other groups except MGUS $P = 0.032$). Hypertension was observed in 40% of complete and new, 50% of therapy and 80% of progressive subjects (progressive vs. control and MGUS $P = 0.007$; therapy vs. control and MGUS $P = 0.032$).

PCs Analysis and Intragroup Comparisons between PPCs and CPC

As evidenced by morphology, at the time of diagnosis mean percentage ± SD of PC was 6.4 ± 2.4% for MGUS, 29 ± 7.4% for Complete, 33.5 ± 14.7% for Therapy, and 29 ± 11.7% for Progressive (MGUS vs. complete and progressive $P = 0.001$; MGUS vs. Therapy $P < 0.0005$). Instead, at the time of this study, values changed as follows: 1 ± 0% for Control, 6 ± 2.1% for MGUS, 2.1 ± 1.6% for Complete, 10.4 ± 10.5% for Therapy, 20 ± 7.8% for New, and 29 ± 22.2% for Progressive (Progressive vs.Control, MGUS and Complete, and New vs. Control $P < 0.0005$ in all cases; MGUS vs. New $P = 0.049$; Complete vs. New $P = 0.005$; Therapy vs. Progressive $P = 0.003$). Median percentages of PCs (range) detected by MFC were 1.9% (1.4–2%) for Control, 1.5% (0.4–2.4%) for MGUS, 0.5% (0.05–0.7%) for Complete, 0.9% (0.2–13.5%) for Therapy, 8.7% (1.6–18.8%) for New and 3.8% (1.4–35.2%) for Progressive (Complete vs. Control $P = 0.015$; Complete vs. New and Progressive $P < 0.0005$ in both cases; Therapy vs. New $P = 0.036$). Median values (range) of CPCs of total PCs were 79.5% (64–87.2%) for MGUS, 64.8% (2.8–99.8%) for Therapy, 98.2% (89.5–99.6%) for New and 98.9% (91.3–99.1%) for Progressive (MGUS vs. New $P = 0.008$, vs. Progressive $P = 0.023$). In Complete and Control 100% of PCs were polyclonal, while observed median percentage values

(range) for PPCs in the other groups were: 20.5% (12.8–36%) for MGUS, 35.2% (0.2–97.2%) for Therapy, 1.8% (0.4–10.5%) for New and 1.1% (0.9–8.7%) for Progressive (Control and Complete vs. New and Progressive $P < 0.0005$ in all cases; vs. Therapy $P = 0.018$). In Control and Complete group, 100% of PCs were perfect diploid (Control and Complete vs. all groups $P < 0.0005$). Pseudodiploidy was detected in 20% of MGUS, 10% of Therapy and 10% of New subjects; instead, hypodiploidy interested 20% of New patients. HRD was identified in 80% of MGUS, 90% of Therapy, 70% of New, and 100% of Progressive subjects (Therapy and Progressive vs. Control and Complete $P < 0.0005$ in all cases; Control and Complete vs. MGUS $P = 0.0007$ and vs. New $P = 0.003$). Ranges of expression and median values for each studied marker on CPCs and PPCs as revealed by MFC are indicated in Table 4. Levels of expression for all studied markers expressed as MFI ratio are reported in Table 5. The analyzed markers showed different utility in distinguishing CPCs from PPCs in MGUS, new, therapy and progressive groups, except for CD30 and CD11a, which never showed differences comparing normal and neoplastic populations. Results for intragroup comparisons between CPCs and PPCs in all groups are indicated in Table 4 (ranges of positive PCs), and in Table 5 (MFI ratio).

Definition of Normal Immunophenotype

In order to verify if PPCs are immunophenotipically different from one group to another, we compared the expression of all markers on PPCs from all groups. Results are summarized in Figures 1A–1T. PPCs showed a relevant grade of immunophenotypic heterogeneity among groups.

Differences among Control and Complete PCs and CPCs

To define how CPCs differ from normal/reactive polyclonal PCs found in subjects who do not present signs of clonal expansion, we compared the expression of all markers on PCs in Complete and Control groups with CPCs in MGUS, therapy, new and progressive groups. Results are summarized in Figures 2A–2V.

Regression Analysis

Multinomial logistic regression analysis was used to evaluate the relation existing between the expression of MFC markers (independent variables—considered as both percentage of positive cells and intensity of expression measured by MFI ratio) and disease status defined as the appartenence of each patient to one precise group (dependent variables). Significant results are summarized in Table 6. As reference categories, we chose Control in analyzing PPCs, and MGUS in studying CPCs, since MGUS is considered a "preneoplastic" condition. To test the hypothesis that the significant markers remained informative also considering common prognostic variables we performed a multivariate regression analysis; among parameters showing statistical significant differences in this study, we selected age, sex, Durie-Salmon, and ISS stages at diagnosis, ASCT and HRD, since they were considered highly reliable in determining prognosis, and allowed us to obtain the best fitting models. Other variables did not exhibit explicative power in our model (data not shown). Results are indicated in Table 6.

DISCUSSION

Since the 90's, multiparameter immunophenotyping has been providing relevant information for the diagnosis, classification and monitoring of hematological malignancies. Immunophenotypic differences between normal and neoplastic cells are essential in detecting MRD in myeloma when there are fewer than 5% of PCs by morphologic examination and a very small percentage of PCs to detect clonality by histological examination. Moreover MFC immunophenotyping may be useful to define potential prognostic markers and new therapeutic targets. MFC is perceived as highly dependent on expertise and is regarded to have limited reproducibility in multicenter studies, mostly because of the lack of standardization in data interpretation, analysis, and presentation, and limited evaluation of "new" vs. "classical" markers (54). Since larger complexity of the multivariate data analyses of both major and minor cell populations produced new insights in what is commonly known as "normal" phenotype (41,42), and MM and MGUS present both a strong component of interaction between neoplastic compartment and surrounding cells, including residual PPCs, there is the urgent need to review phenotypic differences among subjects. In this study, we propose an accurate approach to investigate the role of various immunophenotypic markers in discriminating CPCs from PPCs. We clearly demonstrate that PPCs are not equal among patients, showing a great variability in terms of percentage of positive cells and levels of expression of many antigens. Immunophenotypic differences in CPCs between MGUS and MM subjects, as well as changes in the immunophenotype of MM CPCs after therapy have been described (14,18,24,45); here we observe that these changes may also interest PPCs. We focused our attention on some markers involved in cellular adhesion (CD49d, CD11a, and CD58), because a better understanding of quantitative changes in expression of adhesion molecules during the stages of the disease may be fundamental for defining the mechanisms by which the PCs adhere to or detach from the BM niches (32). We also analyzed the expression of CD30, which is present normally on only a very small fraction of activated lymphocytes. CD30 contributes to negative selection of T-cells, and could be an attractive target for therapeutic intervention (55). The classic immunophenotype of myeloma PCs has been described as CD38 bright positive (dimmer than normal PCs), CD138 positive, CD19 negative, CD45 dim to negative and CD56 positive with CD20 and CD117 positivity in selected cases (10,22,23,40). However recent reports demonstrate that PPCs could display a CD20+CD56+CD45-CD19- phenotype (40–42,56). Precedent studies

Table 4
Ranges of Expression for the Studied Markers in All Groups

		% of positive CPCs			% of positive PPCs			
		Median	Min	Max	Median	Min	Max	*P* value
CD45	Control				98.9	54.7	99	
	MGUS	89.2	33.8	97.8	99.1	80.3	100	0.005
	Complete				92.2	76.5	99.9	
	Therapy	26.7	11.1	100	97.6	89.1	100	0.011
	New	87.2	12.7	99.6	98	0	100	
	Progressive	89.9	3.7	98.2	100	78.8	100	0.005
CD19	Control				72.4	58.9	87.4	
	MGUS		0			100		0.002
	Complete				50	33.5	86.6	
	Therapy	0	0	95.3	96.1	13.6	100	0.01
	New	2.8	0	95.6	100	0	100	
	Progressive		0			100		0.002
CD56	Control				4.8	4.7	5.6	
	MGUS	23	4.8	92.9	27.5	24.1	35.7	
	Complete				12	0	18.6	
	Therapy	95.1	0	100	10	0	100	0.05
	New	95.8	4.9	100	10	10	35.3	0.023
	Progressive	96.2	86.7	98.5	25.3	10	26.4	0.005
CD49d	Control				88.4	48.5	100	
	MGUS	48.7	42.9	88.6	75	74.2	91.9	0.012
	Complete				90.8	78.4	100	
	Therapy	77.9	34.6	100	86.5	59.9	100	0.028
	New	74.2	25.3	92.8	73.9	50	100	
	Progressive	74.6	50.8	100	100	45.7	100	
CD30	Control				2	0	3	
	MGUS	1.1	0	3.8	0	0	5.4	
	Complete				0	0	24.5	
	Therapy	0.6	0	68	0	0	12.5	
	New	3.1	0	9.8	3.4	0	37.3	
	Progressive	1	0	43.6	0	0	100	
CD58	Control				96.1	92.2	100	
	MGUS	95.9	94.7	96.4	96.9	92.6	99.2	
	Complete				98.8	83.9	100	
	Therapy	98.4	57.7	100	90.2	82.7	100	
	New	94.1	58.2	100	100	96.5	100	
	Progressive	94.9	59.8	98.2	100	93.5	100	0.005
CD11a	Control				96.6	81.5	98.9	
	MGUS	98.3	97.1	99.3	98.2	89.2	98.8	
	Complete				83.7	64.7	100	
	Therapy	96.4	79.6	100	94.9	93.2	100	
	New	98.6	88.1	100	100	99.7	100	
	Progressive	98.4	20.4	99.3	97	64.7	98.4	
CD117	Control					0		
	MGUS	85.4	15.2	94.7		0		0.005
	Complete					0		
	Therapy	62.6	0	100		0		0.012
	New	21.4	0.3	71.7		0		0.027
	Progressive	49.7	26.2	64.9		0		0.005
CD27	Control				98	97.3	100	
	MGUS	77.7	31.8	100	93.8	83.4	100	0.017
	Complete				97.3	94.2	100	
	Therapy	97.8	0	100	98.2	45.5	100	
	New	80.9	11.6	100	100	28.3	100	
	Progressive	55.4	19.5	100	85.3	72.4	96.5	0.012
CD200	Control				1.3	0	7	
	MGUS	10.6	6.4	42.7	29.7	17.8	65.9	0.035
	Complete				15.5	5.1	100	
	Therapy	53.2	0	88.6	36.3	0	69	
	New	90.4	0.6	99.2	57.5	28.7	69.2	
	Progressive	54.1	17.8	83.4	48.7	11.9	60.7	
CD20	Control					0		
	MGUS	2.4	0	9.2	3.3	0	9.1	
	Complete				4.3	0	26.1	
	Therapy	8.5	0	72.7	9.6	1.9	64.6	
	New	0	0	99.6	0	0	2.7	
	Progressive	0.8	0	4.1	6.6	0	28	0.038

P values referred to intragroup pairwise comparison between CPCs and PPCs are indicated.
Min: minimum; Max: maximum.

Table 5
Levels of Expression of the Studied Markers in all Groups

		MFI Ratio for CPCs			MFI ratio for PPCs			
		Median	Min	Max	Median	Min	Max	*P* value
CD45	Control				12.1	4.7	16.3	
	MGUS	5	4.4	10.5	12	8.4	16.4	0.005
	Complete				11.1	4.3	24.7	
	Therapy	5.2	2.8	25.3	8.1	3.8	23.3	
	New	9.4	3.5	23.5	5.5	1	20.4	
	Progressive	7.6	4.3	9.3	22.3	11	25	0.005
CD19	Control				11	6.2	15.7	
	MGUS		1		9.2	8.6	26.1	0.005
	Complete				8.7	6.7	16.4	
	Therapy	1	1	20.1	8.2	5.2	17.1	
	New	5.3	1	11.6	7.8	1	7.9	
	Progressive		1		9	6.5	16.1	0.005
CD56	Control				16.5	9.4	17.7	
	MGUS	13.1	11	19.3	16.8	8.4	47.2	
	Complete				10.1	1	63.4	
	Therapy	13.9	1	43.4	10.2	1	31.4	
	New	13.3	6.1	19.9	16.7	13	27.3	0.027
	Progressive	15.8	5.9	18	14.4	11.4	19	
CD49d	Control				17.4	11.4	18.2	
	MGUS	14.5	10.1	25.1	19.9	19.1	20.6	
	Complete				21.1	8.5	43.6	
	Therapy	11.8	6.1	39.4	15.3	7	43.4	
	New	11.2	5.5	24.6	13.5	11.1	16.7	0.027
	Progressive	8.2	5	12.5	12.8	6.1	35.1	0.035
CD30	Control				6.5	1	8.2	
	MGUS	9.8	1	10.7	1	1	11.2	
	Complete				1	1	20.7	
	Therapy	4.1	1	39.3	1	1	8.6	
	New	7.3	1	10.9	6	1	12.4	
	Progressive	4.5	1	8.3	1	1	11.9	
CD58	Control				10.2	4	19.5	
	MGUS	7.6	7.2	16.7	11.8	8.5	14.8	
	Complete				12.5	6.6	21.1	
	Therapy	8.9	3.2	30.2	5	3.7	10.5	
	New	6.8	3.7	34.6	11.7	5.3	29.1	
	Progressive	10.3	4	12.2	21.8	7.2	35.1	0.005
CD11a	Control				3.8	3.2	4.6	
	MGUS	4.7	4.3	5.5	4.6	3.7	5.8	
	Complete				4.5	3.3	5.7	
	Therapy	4.6	3.7	18.1	5	3.1	7.6	
	New	6.4	3	15.8	8.1	5.1	17.2	
	Progressive	5.7	2.1	9.2	5.8	4.1	16.5	
CD117	Control					1		
	MGUS	14.4	8.6	14.7		1		0.005
	Complete					1		
	Therapy	7.3	1	23		1		0.012
	New	10.7	6.9	16.3		1		0.027
	Progressive	6	5	11.6		1		0.005
CD27	Control				38.7	19.9	47.3	
	MGUS	37.1	7.9	55.8	46.8	24.5	67.8	0.027
	Complete				42.2	12	55	
	Therapy	29.2	1	62.5	51.6	6	104.2	
	New	11.6	3.6	30.7	21.1	14.5	34.3	
	Progressive	15.7	6.4	44.1	35.8	21	58.4	0.005
CD200	Control				5.2	1	5.8	
	MGUS	7.4	6.3	8.2	8.2	7.7	8.3	0.005
	Complete				8	1.3	12.6	
	Therapy	6.8	1	16.8	8.5	1	12.9	
	New	15.2	7.2	43.4	9.5	9	10	
	Progressive	7.8	4.8	11.6	8.6	5.9	15.4	0.005
CD20	Control					1		
	MGUS	16.9	1	44.2	18.8	1	37.6	
	Complete				6.3	1	36.5	
	Therapy	12.2	1	42.1	14.4	4.4	46.1	
	New	1	1	30.6	1	1	16.9	
	Progressive	16.9	1	32	3.3	1	37.7	

P values referred to intragroup pairwise comparison between CPCs and PPCs are indicated. Min, minimum; Max, maximum.

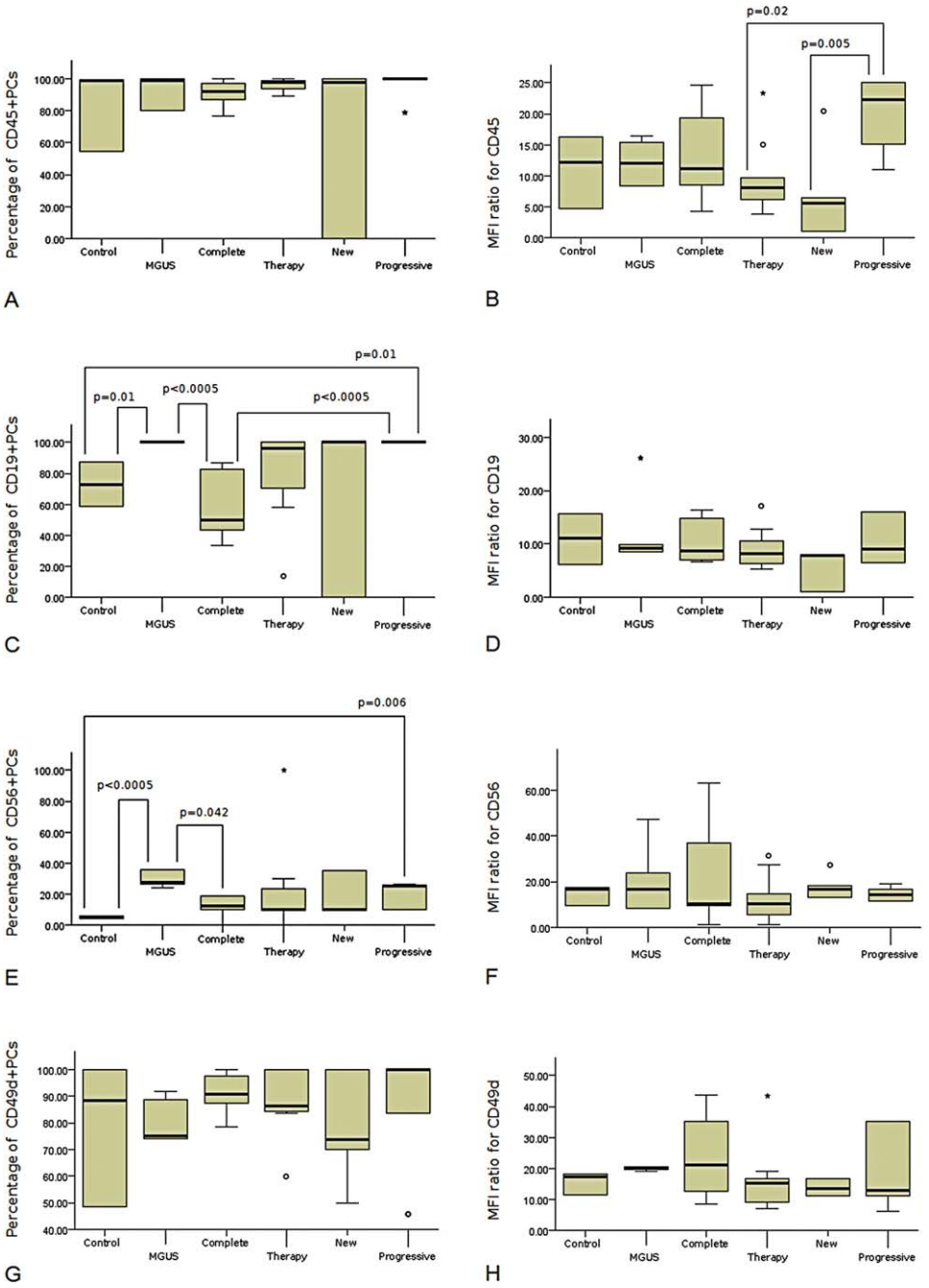

FIG. 1. **A–J**: Immunophenotypic differences in PPCs among groups. Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. •,* = outliers. The following markers exhibited statistically significant differences as regards the mean percentage of positive cells: CD19 ($P<0.0005$), CD56 ($P<0.0005$), CD58 ($P=0.004$), CD11a ($P=0.01$), CD27 ($P=0.01$), CD200 ($P<0.0005$), and CD20 ($P<0.0005$). Also statistically significant differences as regard MFI ratios were demonstrated: CD45 ($P=0.005$), CD49d ($P=0.02$), CD58 ($P=0.011$), CD11a ($P<0.0005$), CD200 ($P=0.001$), and CD20 ($P=0.008$). *P* values of pairwise comparisons are indicated. CD117 is never expressed on PPCs (data not shown). Levels of expression of CD45 were higher in Progressive than in New and Therapy (A,B). The proportion of CD19+ PCs was lower in Control and Complete than in MGUS and Progressive (C,D). Median percentage of CD56+ PCs was lower in Control versus MGUS and Progressive, and in Complete versus MGUS (E,F). Levels of expression of CD49d did not exhibit statistically significant differences at post hoc analysis (G,H). CD30 did not show variability in expression among groups (I,J). **K–T**: Immunophenotypic differences in PPCs among groups. Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. •,* = outliers. *P* values of pairwise comparisons are indicated. CD58+ fraction was littler in Therapy than in New and Progressive; also levels of expression of CD58 were lower in Therapy than in Progressive (K,L). CD11a+ fraction was larger in New than in Complete and Progressive; moreover expression of CD11a was less intense in Control than in Progressive and New (M,N). the proportion of CD27+ PCs was lower in Progressive versus Control and New (O,P). CD200 was expressed by a less extended percentage of PCs in Control than in MGUS, New, Progressive and Therapy. MFI ratio for CD200 was lower in Control than in New, Therapy and Progressive (Q,R). Median percentage of CD20+ PCs was higher in Therapy than in Control and New. Intensity of expression of CD20 was reduced in PCs of Control compared with MGUS and Therapy (S,T). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

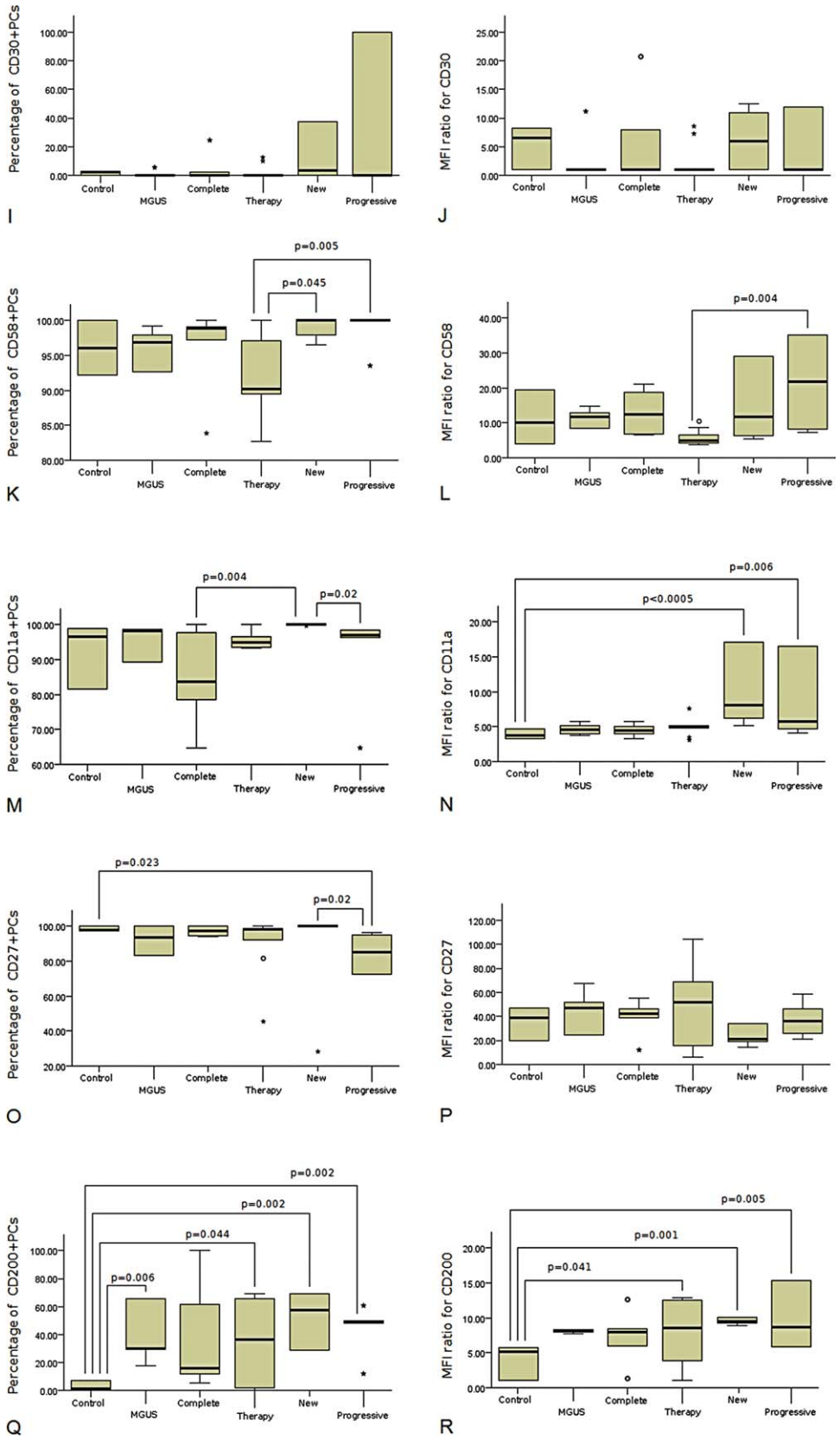

FIG. 1. Continued. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

explored immunophenotype of PPCs only in healthy individuals (42,56), while others reunited in the same category PPCs of healthy and pathological subjects (40,45), causing a loss of information related to possible PPC heterogeneity among different categories of patients. Analyzing our data, it can be easily noted that for CD19, CD45, CD27, and CD117 percentages of positive PPCs reflect the ranges found in precedent studies (10,22,23,40–42,56,57); CD20 was never expressed on PCs in Control group, but the range of expression was broad on PPCs in all other groups. CD200 was expressed on both normal and neoplastic PCs, as previously reported (10,58). CD58, CD49d, and CD11a were never completely absent on PPCs and CPCs, while frequency of CD30+ cells was modest in both plasmacellular compartments in all groups (36). In this study, range of expression for **CD56** (NCAM – Neural Cell Adhesion Molecule) on PPCs essentially did not depart from those described elsewhere (40,42,56), except for an outlier identified in Therapy; however expression close or corresponding to 100% for this antigen has occasionally been reported (32,56). The proportion of CD56+ PCs is constantly described to be low in BM of healthy controls (10,57), however CD56+ PCs may be artificially low in MFC analysis; acting as an anchor, CD56 could make PCs more resistant to BM aspiration (42). Focusing on ranges of expression on PPCs, we observed that CD56+ fraction is reduced in Control than in MGUS and Progressive, and in Complete than in MGUS. This is openly conflicting with report by Pérez-Andrés et al. who described no phenotypic differences for CD56 between PCs from healthy individuals and PPCs from MGUS subjects (45). This discrepancy may be explained considering differences in gating strategies, and in the use of monoclonal antibody clones or fluorochromes between the cited study and ours. As expected, CD56+ CPCs were more numerous than CD56+ PPCs in New, Progressive, and Therapy; the same type of relation was maintained in comparisons with Control and Complete PCs. However, in New, MFIs ratio of PPCs was higher than that of CPCs; to the best of our knowledge, this observation has never been described since the introduction of >4 color FC in analysis of MM patients. On the basis of these results, the expression of CD56 on PPCs should be further elucidated. **CD45** on PPCs is reported to be expressed heterogeneously (10,22,23,40,57); in our study it was effective in distinguishing PPCs from CPCs in MGUS, Progressive and Therapy. Expression of CD45 was more intense on PPCs in Progressive than on PPCs in New and Therapy. Considering that CD45 expression characterizes proliferating compartment of normal, reactive and malignant PCs (13,22), this increased level of expression on Progressive PPCs may depend on the proliferative BM microenvironment to which all PCs of relapsing subjects are exposed (24). **CD19** is downregulated in PCs maturation, and CD19-CD56+ PCs are believed to represent long lived terminal stage PPCs (22,40). CD19 was persistently negative in MGUS and Progressive CPCs. This pattern of expression has already been described in literature (22,40), although cases of CD19+ MGUS cells have been reported (22). Clearly, PCs from Control and Complete exhibited a reduced CD19+ fraction compared to PPCs from MGUS and Progressive. Exact mechanism ruling expression in MGUS and Progressive subjects remains to be explored. **CD27** is strongly expressed on PPCs (9,10,56), but CPCs are reported to be weak or negative (9,10,57); in our study it showed no differences in expression comparing CPCs with Control and Complete PCs, contradicting previous data (9), probably because of discrepancies in methods or the small sample size. We evidenced that CD27+ Progressive PPCs were less numerous than CD27+ PPCs of New and Control. Since this antigen is involved in apoptosis induced by CD27–CD70 interactions (59), it would be interesting to evaluate if other molecules required for programmed cell death show variable expression in PPCs of Progressive patients. **CD117** was never present on normal PCs, thus being highly effective in distinguishing PPCs from CPCs (when present on CPCs). The data about **CD20** in PCs of healthy people are conflicting. Reported ranges vary from a minimum of 0 to maximum of 91% (10,22,40,56,57), but it is universally accepted that low CD20 expression is associated to PC maturity. Expression of CD20 on MM cells is reported to range from 2 to 90% (42). CD20 was a poor antigen in distinguishing CPCs from PPCs, as previously described (56), except in Progressive group, in which proportion of CD20+ PPCs was higher than proportion of CD20+ CPCs. Expression of CD20 on CPCs is associated t(11;14) (Refs. 22) and (23)); since cytogenetic analysis was not performed, we can not say if t(11;14) is present in Progressive CPCs. In

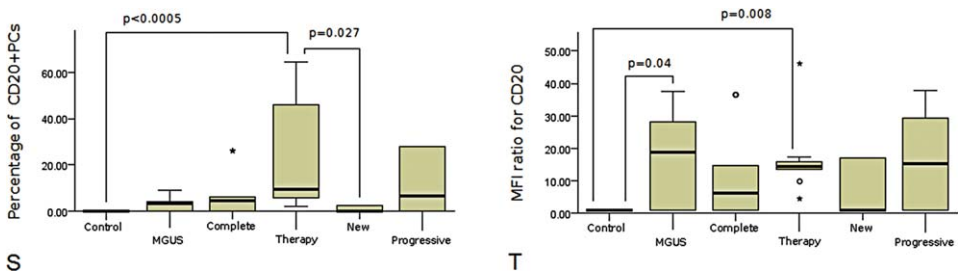

FIG. 1. Continued. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

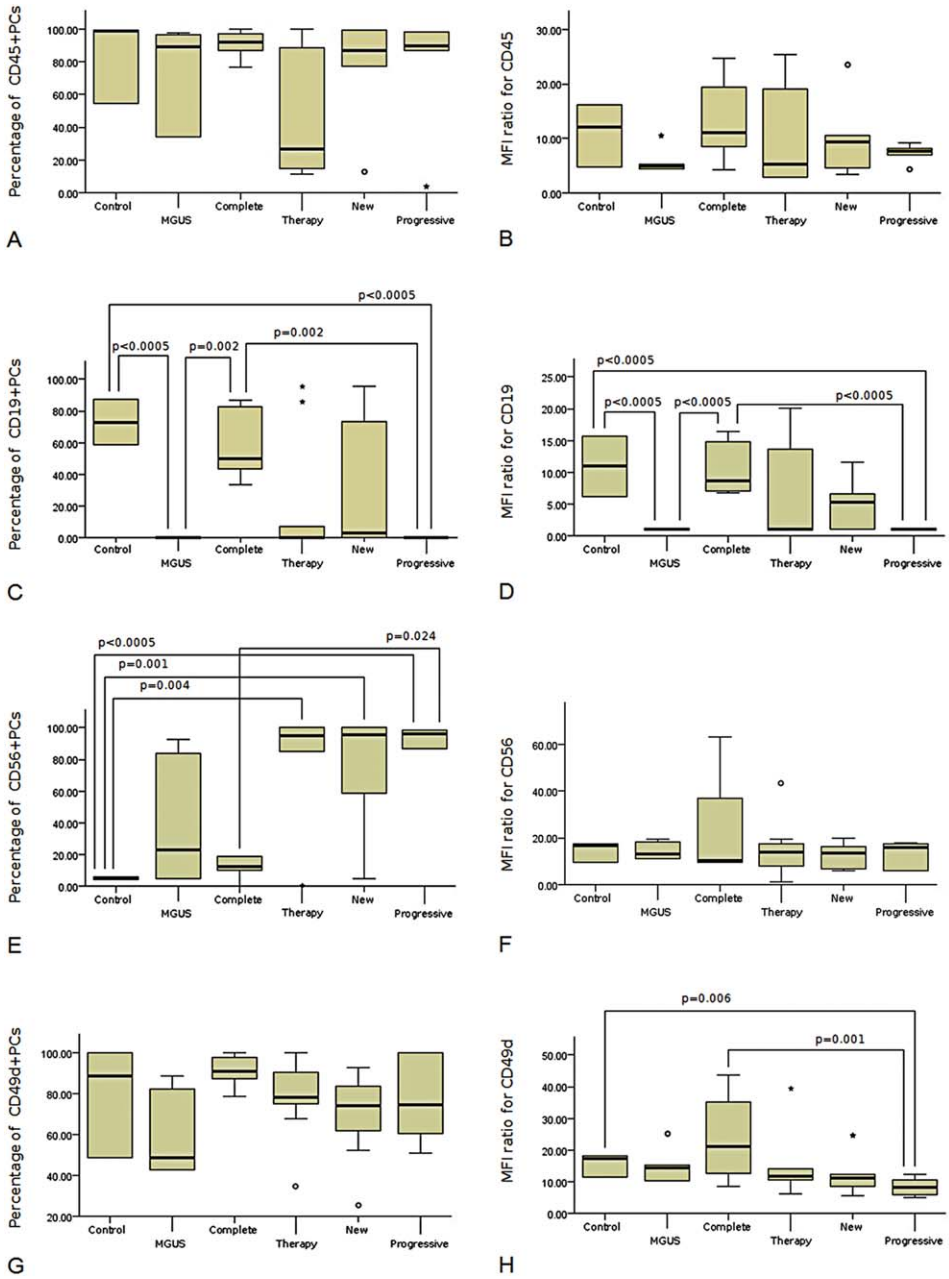

FIG. 2. **A–L**: Results of comparisons among PCs of Control and Complete and CPCs of MGUS and MM groups. Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. •,* = outliers. Kruskal-wallis test revealed that the following markers showed statistically significant differences as regards the median percentage of positive cells among groups: CD19 ($P<0.0005$), CD56 ($P<0.0005$), CD117 ($P<0.0005$), CD200 ($P<0.0005$), and CD20 ($P=0.004$). Moreover, statistically significant differences in intensity of expression emerged among groups for: CD19 ($P<0.0005$), CD49d ($P<0.0005$), CD11a ($P=0.01$), CD117 ($P<0.0005$), CD200 ($P<0.0005$), and CD20 ($P=0.013$). *P* values of pairwise comparisons are indicated. CD45 did not show variability in expression among groups (A,B). Median percentage of CD19+ PCs in Control was higher than in MGUS and Progressive; also CD19+ fraction of PCs was larger in Complete than in Progressive and MGUS. Levels of expression of CD19 were higher in Control and Complete than in MGUS and Progressive (C,D). Proportion of CD56+ PCs was lower in Complete than in Progressive, and in Control than in New, Therapy, and Progressive (E,F). Levels of expression of CD49d were higher in Complete and Control than in Progressive (G,H). CD30 did not show variability in expression among groups (I,J). CD58 did not exhibit any statistical significant difference (K,L). **M–V**: Results of comparisons among PCs of Control and Complete and CPCs of MGUS and MM groups. Box plots indicate the median and 25th and 75th percentiles. Whiskers indicate minimum and maximum values. •,* = outliers. *P* values of pairwise comparisons are indicated. Intensity of expression of CD11a was lower in Control versus Progressive and New (M,N). Since PPCs did not express CD117, the difference of Control and Complete versus all other groups was extremely significant, except versus New ($P=0.16$). MFI ratio measured for CD117+ cells was different comparing Control and Complete versus New, MGUS and Therapy (O,P). CD27 was homogeneous in its expression comparing all subjects (Q,R). CD200+ fraction is less extended in Control than in CPCs of New, Therapy and Progressive. Levels of expression of CD200 were higher in New versus Control (S,T). CD20+ proportion of PCs was lower in Control than in Therapy. Intensity of expression of CD20 was reduced in Control versus MGUS (U,V). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

addition, deeper molecular studies are necessary to explain the reasons why MFI ratio was reduced in Control versus MGUS CPCs and PPCs. Therapy PPCs showed upregulation of CD20 compared with Control and New PPCs, and also proportion of CD20+ CPCs in Therapy is larger compared with Control PCs. Recently a study described the transcriptional effects of bortezomib on human myeloma cell lines, showing a

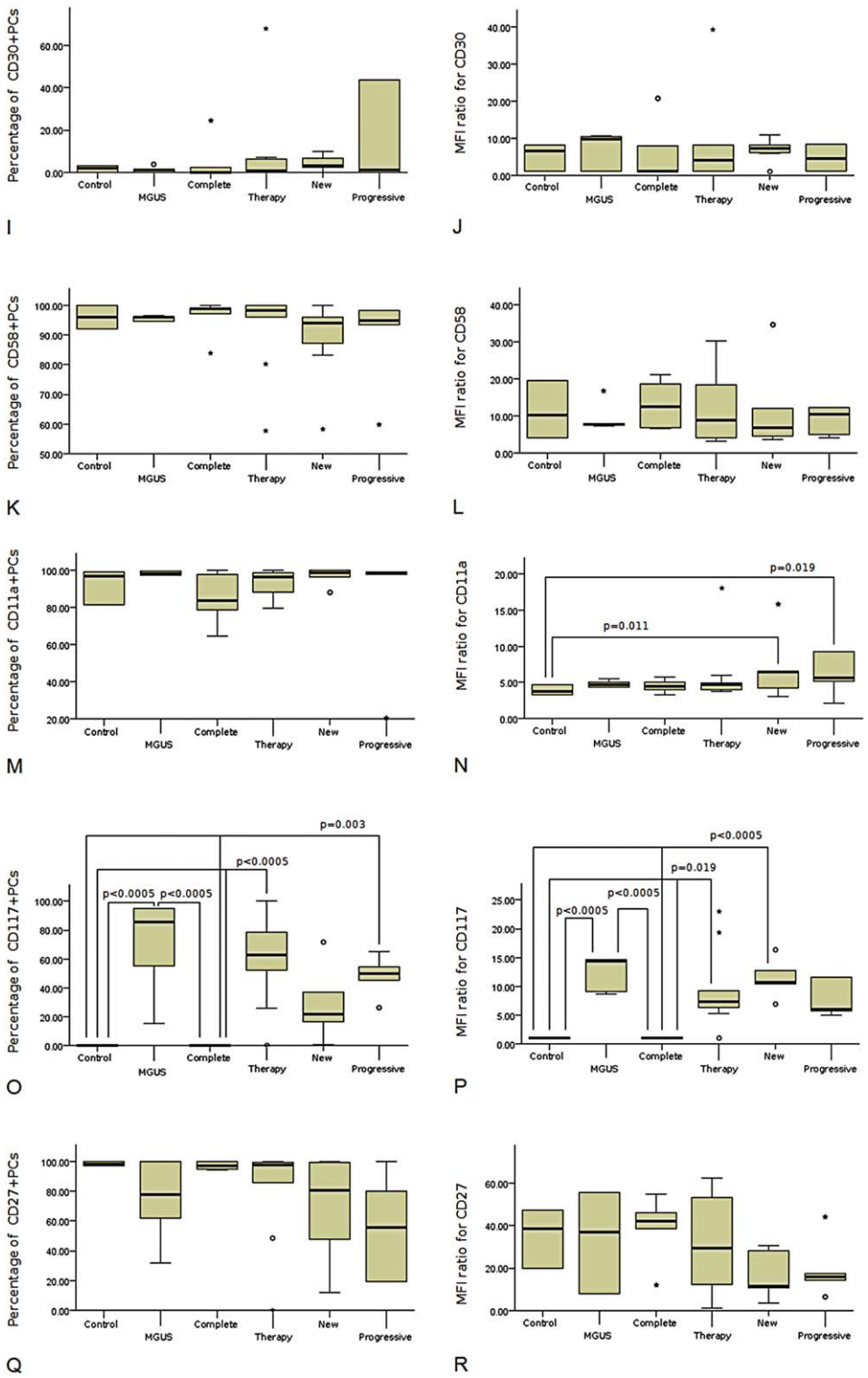

FIG. 2. Continued. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

downregulation of many antigens but no effect on CD20 expression (60). Since in this study 70% of Therapy patients are currently treated with bortezomib containing regimens, we hypotize that instead bortezomib could directly influence expression of CD20 in PCs, although confirmation with larger groups of patients is necessary to confirm or reject this hypothesis. **CD200** is a cell surface glycoprotein expressed on normal B-cells and some T-cell subsets; the expression of CD200 showed to be positive on MM PCs up to 78% (27,61). CD200 on PPCs has been poorly investigated, and no clear range of expression has been defined; however our data match with both papers reporting its absence (26,56) and those indicating positivity (10,58). Evaluating its utility in CPC detection, we observed that in MGUS CD200 was expressed by a higher percentage of PPCs vs.CPCs. In addition, expression was more intense on MGUS and Progressive PPCs vs. MGUS and Progressive CPCs. Defining normal phenotype, Control PCs showed less extended fraction of CD200+ cells vs. MGUS, New, Progressive, and Therapy PPCs, and a dimmer expression vs. MM groups. Comparing Control and Complete PCs with CPCs, once again Control displayed a smaller proportion of CD200+ cells vs. Therapy, New, and Progressive. CD200+ positive cells present reduced immunogenicity compared with normal lymphocytes (27) and absence of CD200 correlates with a better prognosis compared to its presence in MM (12); on this basis, we would have expected a higher expression of CD200 in Progressive patients vs. Complete. Deeper investigation on relapse molecular mechanisms is necessary before excluding a role of CD200 in progression of MM. **CD11a**, the integrin α subunit of LFA-1, has been reported positive as well as negative in literature on MGUS and MM samples (28,30). The ranges of expression on CPCs are really different from one paper to another, ranging from 0% to 100% in newly diagnosed or remitting subjects, and from 0 to ∼80% in relapsing subjects (32), or reported as <30% for CD45+ cells and ≪10% for CD45- cells (34). On PPCs, it has been reported to range from 20 to 100% (32). In our study, CD11a+ proportion of CPCs showed no differences compared to CD11a+ Control and Complete PCs, while levels of expression of CD11a were higher on both CPCs and PPCs of New and Progressive vs.Control PCs; these results diverges from a precedent report demonstrating a downregulation of CD11a in patients in chronic phase, but in that case results might be compromised by the inclusion of newly diagnosed and remitting subjects in the same group (32). Examining PPCs, CD11a+ fraction was expanded in New vs.Complete and Progressive. **CD58**, a CD2 receptor, was previously indicated as present occasionally on PPCs and never expressed on MGUS CPCs (28); in our study, it is positive in a fraction of PPCs and MGUS CPCs close to 100%. This discrepancy may be attributed to differences regarding methods (immunofluorescence microscopy vs. MFC) and choose of control samples. CD58 was more expressed on PPCs than on CPCs in Progressive (considering both proportion of CD58+ cells and MFI ratio). Therapy PPCs showed downregulation of CD58 vs.New and Progressive. These data suggest that CD58 should be considered in further studies to assess its ability in predicting response to therapy or progression of disease when evaluated on non neoplastic population. We did not notice any difference comparing Control and Complete with CPCs, contrary to previous data (28). **CD49d**, the integrin α subunit of VLA-4, has been found strongly expressed by MM PCs (36), while mean percentage of positive normal PCs has been reported ∼75%

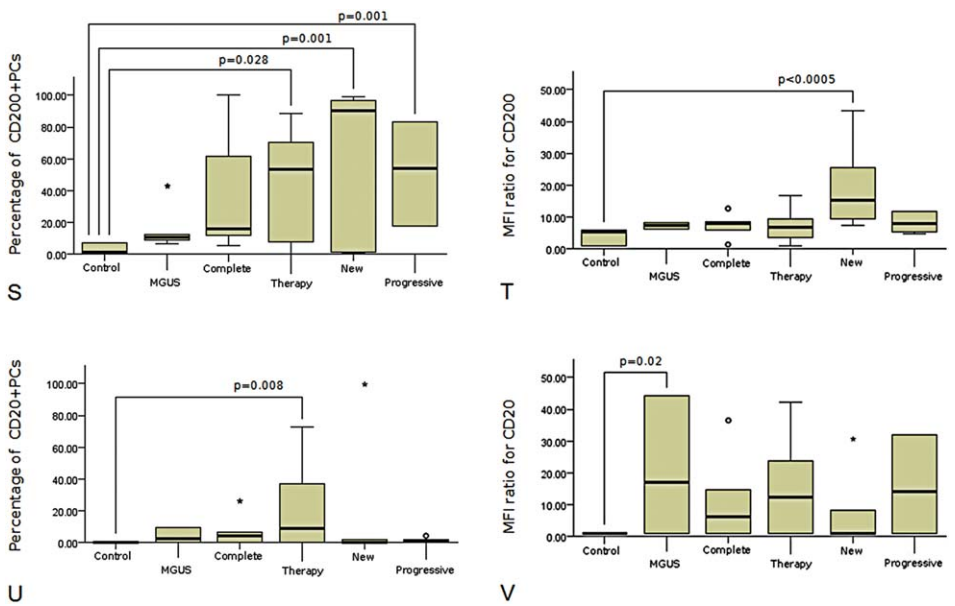

FIG. 2. Continued. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 6
Results of Multinomial Logistic Regression Analysis

		Univariate			Multivariate		
Independent variable	PCs	Exp (B)	95% CI	P value	Exp (B)	95% CI	P value
MFI ratio for CD45	PPCs	1.03	0.88–1.20	0.722[a]			
		1.06	0.91–1.23	0.472[b]			
		0.93	0.79–1.11	0.438[c]			
		0.81	0.63–1.03	0.086[d]			
		1.25	1.06–1.50	0.011[e]	1.23	1.04–1.46	0.015
Percentage of CD19+ cells	CPCs	111.1	111.1–111.1	Na[f]			
		111.6	108.9–114.4	<0.0005[g]	144.9	139.8–150.3	<0.0005
		1		1.0[h]			
MFI ratio for CD19	CPCs	67.6	67.6–67.6	Na[f]			
		64.6	54.9–76.0	<0.0005[g]	61.5	49.6–76.2	<0.0005
		1		1.0[h]			
	PPCs	1.04	0.87–1.22	0.670[a]			
		0.93	0.77–1.14	0.502[b]			
		0.88	0.71–1.11	0.298[c]			
		0.57	0.36–0.89	0.016[d]	0.53	0.26–1.08	0.082
		0.95	0.78–1.15	0.606[e]			
Percentage of CD56+ cells	PPCs	1.51	1.16–1.96	0.002[a]	1.71	1.19–2.42	0.003
		1.33	1.03–1.71	0.029[b]	1.53	1.08–2.16	0.016
		1.48	1.14–1.92	0.004[c]	1.66	1.17–2.36	0.005
		1.43	1.10–1.87	0.008[d]	1.61	1.13–2.29	0.009
		1.46	1.13–1.90	0.004[e]	1.54	1.07–2.21	0.019
MFI ratio for CD49d	CPCs	0.98	0.86–1.09	0.684[f]			
		0.94	0.82–1.08	0.394[g]			
		0.68	0.51–0.92	0.012[h]	0.62	0.38–0.99	0.046
MFI ratio for CD58	PPCs	0.99	0.86–1.13	0.882[a]			
		1.03	0.90–1.16	0.695[b]			
		0.64	0.45–0.93	0.019[c]	0.55	0.37–0.84	0.025
		1.06	0.93–1.22	0.373[d]			
		1.13	0.99–1.27	0.057[e]			
MFI ratio for CD11a	PPCs	4.29	1.10–16.7	0.036[a]	3.19	0.63–15.9	0.158
		4.01	1.03–15.6	0.045[b]	2.41	0.50–11.6	0.273
		5.51	1.38–21.9	0.016[c]	4.82	1.20–19.3	0.027
		10.8	2.64–44.6	0.001[d]	13.7	1.95–95.9	0.008
		10.1	2.48–41.5	0.001[e]	14.1	2.74–72.4	0.02
Percentage of CD117+ cells	CPCs	0.99	0.95–1.02	0.448[f]			
		0.93	0.88–0.97	0.003[g]	0.94	0.89–0.99	0.023
		0.97	0.93–1.01	0.095[h]			
MFI ratio for CD117	CPCs	0.84	0.66–1.06	0.143[f]			
		0.95	0.77–1.17	0.625[g]			
		0.71	0.53–0.94	0.017[h]	0.65	0.42–1.01	0.054
Percentage of CD200+ cells	CPCs	1.04	0.99–1.07	0.057[f]			
		1.05	1.01–1.09	0.008[g]	1.06	1.01–1.11	0.012
		1.04	1.01–1.08	0.032[h]	1.04	0.99–1.09	0.084
	PPCs	1.31	1.07–1.61	0.009[a]	1.36	0.95–1.95	0.089
		1.3	1.07–1.60	0.010[b]	1.36	0.95–1.94	0.089
		1.3	1.06–1.60	0.011[c]	1.36	0.95–1.94	0.091
		1.34	1.09–1.64	0.005[b]	1.38	0.97–1.97	0.073
		1.32	1.08–1.62	0.007[e]	1.63	0.95–2.80	0.075
MFI ratio for CD200	CPCs	1.01	0.78–1.30	0.957[f]			
		1.31	1.02–1.68	0.034[g]	1.38	1.02–1.88	0.038
		1.06	0.83–1.35	0.656[h]			
	PPCs	1.56	1.12–2.16	0.008[a]	2.02	1.19–3.41	0.008
		1.45	1.06–1.98	0.020[b]	1.35	0.88–2.07	0.172
		1.49	1.08–2.07	0.016[c]	1.73	1.11–2.70	0.016
		1.8	1.12–2.65	0.003[d]	2.59	1.25–5.35	0.01
		1.86	1.30–2.65	0.001[e]	2.23	1.28–3.89	0.005

[a]Control vs. MGUS.
[b]Control vs. Complete.
[c]Control vs. Therapy.
[d]Control vs. New.
[e]Control vs. Progressive.
[f]MGUS vs. Therapy.
[g]MGUS vs. New.
[h]MGUS vs. Progressive.

(29). In our study, CD49d showed a more pronounced expression on PPCs than on CPCs in all groups (fraction of positive cells for MGUS and Therapy, MFI ratio for New and Progressive), thus beeing highly useful to refine CPC population when combined with common used marker. Intensity of expression was higher in Control and Complete PCs vs.Progressive CPCs, so it would be intriguing to verify if this marker has a role in drug resistance during progression of disease. As we could not identify a univocous pattern of expression of these three adhesion involved markers, regulatory pathways determining their expression should be explored individually. Continuous efforts are made in MM to improve the sensitivity of immunophenotypic detection of PCs. On the basis our results, we suppose that in future panels of markers specific for the clinical question could be used, with the addition of appropriate MFC markers to the backbone ones (CD38, CD138, CD19, CD45, and CD56); the evaluation of the disease should involve PPCs, in order to follow progressions and explore the opportunity to obtain predictive information. CD49d should be universally present to distinguish CPCs from PPCs; for MGUS diagnosis and monitoring, CD20 and CD200 are suggested to be added; newly diagnosed patients may benefit the addition of CD11a; assessment of disease progression or response to therapy would be more accurate by the introduction of CD27, CD58, CD11a, and CD20. The prognostic value of specific antigenic profiles (CD19, CD117, and CD27) has already been assessed; positive staining for CD19 and absence of CD117 detected on clonal PCs were associated with significantly shorter progression free survival and OS (overall survival) rates of MM patients. Moreover, OS rates were higher in patients with CD27+ MM than in those who were CD27- (57,62,63). The absence (or presence) of CD200 expression in MM cells is predictive for event-free survival independently of ISS stage or β-2-microglobulin serum levels (12). For CD45 and CD56 results from precedent studies are contradictory, and require further investigation (24,57). No information is available about the prognostic value of the other markers used in this study, or about their role in predict response to therapy and disease progression, and their utility in distinguishing disease stages without considering other clinical variables. To see if a correlation exists between the expression of each marker and the attribution of the patients to their own groups, we performed a multinomial logistic regression analysis; results surprisingly revealed that the most abundant associations regarded PPCs, with percentage of cells positive for CD200 and CD56, and levels of expression of CD45, CD19, CD58, CD11a, and CD200 showing statistical significance. In addition, fractions of CPCs positive for CD19 and CD200, and intensity of expression of CD19, CD49d, CD117, and CD200 on CPCs were significant. But when the model was elaborated again including significant prognostic variables (37,38,43), percentage of CD200+ PPCs and levels of expression of CD117 on CPCs and CD19 on PPCs loosed their association, as did levels of expression of CD11a comparing Control with Complete and MGUS PPCs, MFI ratio for CD200 analyzing Control vs. Complete PCs, and percentage of CD200+ CPCs comparing MGUS vs. Progressive. This is not surprising, since other works have already demonstrated relations between variation in expression of MFC markers and BM features (64). Our data comfort the hypothesis that, in assessing the predictive/prognostic values of MFC markers and their utility in following disease steps, immunological-biochemical-hematological profile must not be ignored since it is the "mirror" of the altered BM microenvironment influencing/being influenced by PC phenotype (37,64). However CD19 and CD49d on CPCs, and CD45, CD58 and CD56 on PPCs maintained their explicative power, so they are good candidates for further studies, especially CD19, which was considered a "reliable" antigen in a report exploring relationship between MFC and histological results (56). In conclusion, we have provided a feasible start point to put in order the ranges of expression on PPCs in healthy and myeloma subjects; we propose a new approach based on analysis of PPCs to monitor the stages of the disease. However, the study size is small, and we could not provide a prospective cohort to examine fluctuations in expression of MFC markers related to time and disease progression, just as we could not define a threshold of expression for each marker that allows to clearly attribute each subject to his own group. Confirmation by larger and deeper independent studies, as well as by the elaboration of an adequate Cox proportional-hazard model, is indicated.

ACKNOWLEDGMENTS

Authors declare no conflict of interest. Fanny Pojero, Alessandra Casuccio, and Francesco Gervasi designed the research study; Fanny Pojero and Maria Francesca Parrino performed the research; Giovanni Cardinale contributed clinical information; Alessandra Casuccio analyzed the data; Fanny Pojero, Alessandra Casuccio, Francesco Gervasi, Giuseppina Colonna Romano, and Calogero Caruso interpreted the data; Fanny Pojero and Alessandra Casuccio wrote the article; Francesco Gervasi, Giuseppina Colonna Romano, and Calogero Caruso revised the article; all authors approved the article. Authors would like to thank James Hinchcliffe for reviewing language and syntax of the manuscript, and Filippo Pojero for elaborating artworks.

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