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Original Article

Multiparametric Flow Cytometry in Newly Diagnosed Multiple Myeloma Patients: An Italian Monocentric Experience

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Abstract. Multiple myeloma (MM) is a heterogeneous malignancy characterized by the proliferation of abnormal plasma cells in the bone marrow. Multiparametric flow cytometry (MFC) plays a role in the work-up of the disease in view of the aberrant expression of surface antigens. Our study aimed at describing the antigenic profile detected by MFC in a series of newly diagnosed MM patients to correlate the level of expression with other features of the disease. Between April 2018 and June 2022, 84 consecutive MM patients were studied at presentation. CD56 and CD117 were commonly detected, while CD45, CD28, CD20, CD19, CD13 and CD33 were less recurrent. CD20 expression was associated with the type of secretory MM (p=0.041) and with a higher disease burden (p=0.038). CD28 positivity correlated with a lower platelet count at baseline (p=0.005) and with a lower rate of complete response (p=0.038). Furthermore, CD28 positivity and a lower CD138 expression tended to associate with the high-risk chromosomal translocations t(14;16) and t(4;14). The results of this study indicate that in the diagnostic work-up of MM, MFC may help to identify different patient subsets and improve risk stratification. These observations need to be validated in larger series of patients with a longer follow-up.

Keywords: Multiparametric flow cytometry; Multiple myeloma; Fluorescence in situ hybridization; Markers; Karyotype; Chromosomal translocations.

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Introduction. Multiple myeloma (MM) is a heterogeneous disorder characterized by the expansion of clonal plasma cells (PCs) in the bone marrow (BM), often associated with a detectable monoclonal immunoglobulin in the serum and/or urine. The European Myeloma Network has underlined the clinical utility of multiparametric flow-cytometry (MFC) analysis in the diagnostic work-up and follow-up of MM patients.[1,2] The prerequisite of MFC in MM is to discriminate within the whole PC compartment between

normal and aberrant clonal PCs.

PCs are considered end-stage B cells, lacking surface expression of the most common markers of the B-cell lineage, such as CD22, CD20, and surface membrane immunoglobulins. Clonal PCs show a heterogeneous expression of CD19, CD45lo, and CD56−/lo, together with high amounts of CD38, CD138, and c(cytoplasmic)VS38.[3,4] Their identification is favored by the concomitant expression of other surface antigens, such as CD28, CD20, CD33, CD13, CD117, and CD56.[5,6] Specific panels of antibody combinations have been designed, and the definition of clonal PCs is established due to the variable association of these antigens with cytoplasmic immunoglobulin k or λ chain staining. The prognostic impact of the immunophenotypic profile of clonal PCs has been suggested based on the results of a large series of transplant-eligible newly diagnosed MM patients treated with high-dose chemotherapy followed by autologous stem cell transplantation (ASCT). The expression of CD19 and CD28, as well as the absence of CD117 on clonal PCs, have been associated with a shorter time to progression.[7]

The aim of our study was to assess the immunophenotypic characteristics of clonal PCs on a consecutive series of newly diagnosed MM patients managed at our Center and to investigate the possible correlation between the aberrant phenotype, the clinical characteristics of the disease, and cytogenetic abnormalities.

Materials and Methods. Between April 2018 and June 2022, we analyzed BM samples from 84 consecutive newly diagnosed MM patients managed at the Hematology Center of the Sapienza University of Rome by flow cytometry. Informed consent was obtained from all individual participants, and the study was in accordance with the ethical standard of the institutional national research committee and the 1964 Helsinki Declaration.

Bone marrow samples in sodium citrate were required for each patient, and MFC was performed after erythrocyte-lysis. The samples were quickly processed, considering that down-regulation of CD138 expression has been demonstrated on aged PC samples. MFC immunophenotyping study was conducted using an 8-12 color combination of the following monoclonal antibodies (CD45/CD38/CD138/CD19/CD20/CD28/CD56/CD117/CD13/CD33/cVS38/cIgkappa/cIglambda), using the FACSCanto II/FACSLyric flow cytometers and the PAINT-A-GATE/FACSDIVA software. Specifically, the antigen expression was considered positive if more than 10% of PC displayed a level of expression. The specific antibody combinations of each staining tube with markers and their respective fluorochromes are summarized in **Table 1**.

Table 1. Specific antibody combinations.

8-COLOR FLOW CYTOMETRY							
FITC	**PE**	**PerCP**	**PECy7**	**APC**	**APC-Cy7**	**V450**	**V500**
CD38	CD56	CD19	CD33	CD138	CD20	CD3	CD45
CD38	CD28	CD19	CD56	CD138	CD20	CD3	CD45
cVS38	CD117	CD19	CD13	CD138	CD20	CD3	CD45
cIg Lambda	cIg kappa	CD19	HLA-DR	CD138	CD20	CD3	CD45

12-COLOR FLOW CYTOMETRY											
FITC	**PE**	**PerCP**	**PECy7**	**APC**	**APC-Cy7**	**R718**	**V450**	**V500**	**BV605**	**BV711**	**BV786**
CD38	CD28	CD13	CD56	CD117	HLA-Dr	CD20	CD19	CD45	CD138	CD3	CD22
cVS38	CD28	CD33	CD56	cIgKappa	cIg Lambda	CD20	CD19	CD45	CD138	CD3	HLA-DR

For the intracytoplasmic staining of cIg kappa, cIg lambda, and cVS38, a BD fixation and permeabilization KIT was used, followed by labeling with specific antibodies (BD Intrasure KIT).

As recommended, the combination of CD38 and CD138 was used to identify PCs in MM. For an optimized exclusion of other non-PC populations potentially contaminating the CD38hi CD138+ PC gate, CD45 was simultaneously stained, in addition to sideward (SSC) and forward (FSC) light scatter. Within this population, the Ig light chain Kappa/Lambda ratio was used for discriminating between clonal aberrant cells and their normal counterparts. In rare events, at least 500,000 total cells were acquired with a plasma cell identification cluster of at least 50 cells.

Fluorescence in situ hybridization (FISH) was performed on purified PCs by immune-magnetic separation using anti-CD38 microbeads.

Patients' characteristics were summarized using cross-tabulations for categorical variables or utilizing median and range for continuous variables. Non-parametric tests were performed for comparisons between groups: Chi-Squared and Fisher Exact test in case of categorical variables, Mann-Whitney and Kruskal-Wallis test in case

Table 2. Patients' baseline characteristics.

	N=84 (%)
Median age, years (range)	61.3 (27.9 – 88.2)
<65/≥65	54/30
Gender	
Male, n (%)	52 (62)
Female, n (%)	32 (38)
Type of MM	
Classic	76 (91)
Micro-molecular	6 (7)
Non secreting	2 (2)
Type of heavy chain	
G	50 (59)
Non-G	31 (37)
No heavy chain	3 (4)
Type of light chain	
k	60 (71)
λ	22 (26)
k + λ	2 (3)
CRAB	
Ca^{++} ≥12 mg/dl	5 (6)
Cr >2 mg/dl	7 (8)
Hb <10 g/dl	27 (32)
Osteolytic bone lesions	69 (82)
Bone marrow plasma cells infiltration, median % (range)	30 (10-90)
ISS, n (%)	
I	32 (38)
II	20 (24)
III	26 (31)
NA	6 (7)
High-risk FISH cytogenetics, n (%)	
No	67 (80)
Yes	12 (14)
NA	5 (6)
High-risk FISH cytogenetics + ampl/gain1q, n (%)	
No	45 (54)
Yes	34 (40)
NA	5 (6)
R-ISS, n (%)	
I	22 (26)
II	39 (47)
III	16 (19)
NA	7 (8)
ASCT eligible	
Yes, n (%)	71 (85)
No, n (%)	13 (15)
Overall response rate (ORR)	70 (83)
VGPR or better	43 (51)
CR or better	19 (23)

*NA: not available

of continuous variables. All analysis was performed using R software (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

Results. Fifty-two newly diagnosed MM patients were males and 32 females. The median age was 61 (28-88); 30 patients were 65. Patients' risk stratification was based on the International Staging System (ISS) and revised ISS (R-ISS).[8] Thirty-eight % of patients were ISS I, and 46% were R-ISS II. Fifteen % of patients showed high-risk cytogenetic abnormalities, according to R-ISS [del17p, t(4;14) and t(14,16)]. The number of high-risk patients increased (43%), including those harboring amp1q and gain1q.

Eighty-five % of patients were considered eligible for ASCT. The median BM PCs observed by conventional cytomorphology staining from bone marrow aspirate was 30% (10-90%). As expected, the median of BM PCs detected by MFC was lower (8%, range 0.1-94%), probably due to a dilution effect. The main therapeutic regimens used were bortezomib-based combinations, such as VTd (bortezomib, thalidomide, and dexamethasone) in transplant-eligible patients and VMP (bortezomib, melphalan, and prednisone) in transplant-ineligible patients. The overall response rate of the entire cohort was 85%. The baseline clinical characteristics and the frontline induction treatments of the 84 MM patients are summarized in **Tables 2** and **3,** respectively.

BM clonal PCs from a minority of patients in our cohort presented early B-cell maturation antigen expression, such as CD19 (2%). CD20 and CD45 were detected in 17% and 52% of the clonal PCs, respectively. In 69% of cases, BM PCs showed a bright CD56 surface expression. Among the remaining patients, 1% showed a reduced reactivity for CD56, while CD56 was completely negative in the other cases (30%). CD117 was detected in 42% of clonal BM PCs, while CD28 and CD33 were detected in 15% and 5% of clonal PCs,

Table 3. Patients' baseline induction therapy

Transplant eligible patients	N=71 (84%)
Induction therapy	
VTd	59 (83)
D-VRd	3 (4)
D-VTd	6 (9)
VRd	3 (4)
ASCT	
Single	37 (52)
Tandem	11 (16)
No ASCT*	23 (32)
Maintenance	
Lenalidomide	43 (61)
Lenalidomide plus anti-CD38	3 (4)
No therapy**	25 (35)
Transplant ineligible patients	**N=13 (16%)**
Therapy	
VMP	8 (62)
Rd	2 (15)
Kd	2 (15)
VRd	1 (8)

*No ASCT: 9 pts waiting for ASCT; **No therapy: waiting to start maintenance therapy; VTd: bortezomib, thalidomide and dexamethasone; D-VRd: daratumumab, bortezomib, lenalidomide and dexamethasone; D-VTd: daratumumab, bortezomib, talidomide and dexamethasone; VRd: bortezomib, lenalidomide and dexamethasone; ASCT: autologous stem cell transplantation; VMP: bortezomib, melphalan and prednisone; Rd: lenalidomide and dexamethasone; Kd: carfilzomib and dexamethasone

respectively.

When considering unusual antigens on the surface of aberrant PCs (CD28, CD20, and CD45), we observed that the expression of CD28 was mutually exclusive compared to CD56 ($p<0.001$). In addition, the presence of CD20 was associated with the absence of CD28 ($p=0.048$). We then investigated the correlation between CD28, CD20, and CD45 expression on clonal PCs with the patient's characteristics and response to treatment. Expression of CD28 on clonal PCs was associated with a significantly lower median number of platelets at baseline [$192x10^3$ vs. $218x10^3$ ($p=0.005$)], even if this difference was not clinically relevant, and with a significantly reduced percentage of MM patients achieving a complete response [25% vs. 66% ($p=0.038$)]. Focusing on high-risk chromosomal aberrations, t(14;16) tended to associate with CD28 expression ($p=0.079$), while t(4;14) tended to associate with a lower median value of CD138 mean fluorescence intensity (MFI) [974 vs 1745 ($p=0.58$)]. We also observed that CD20 expression on clonal PCs (18% of all patients) was associated with the type of secretory MM compared to non-secretory MM ($p=0.041$). Furthermore, patients with CD2O expression showed a higher median level of serum monoclonal protein at baseline compared to patients lacking CD20 [3.86 g/dl vs 2.42 g/dl ($p=0.038$)] (**Table 4**).

Table 4. Correlations among clinical features of our MM cohort and aberrant antigen expression on clonal PCs. Correlations with statistically significance or tending to it are highlighted.

Clinical features	CD20+ *vs* CD20-	CD28+ *vs* CD28-	CD45+ *vs* CD45-
μe Hb at baseline	p=0.760	p=0.360	p=0.076
μe ANC at baseline	p=0.800	p=0.440	p=0.730
μe Plts at baseline	p=0.200	**p=0.005**	p=0.730
del(17p)	p=0.99	p=0.580	p=0.990
t(4;14)	p=0.580	p=0.580	p=0.660
t(14;16)	p=0.091	**p=0.0790**	p=0.990
amp/gain(1q)	p=0.770	p=0.360	p=0.300
μe CM at baseline	**p=0.038**	p=0.170	p=0.100
CR after induction therapy	p=0.540	p=0.038	p=0.270

μe: median value; Hb: hemoglobin; ANC: granulocytes; Plts: platelets; CM: M protein; CR: complete response.

Discussion. Despite the relatively limited sample size, these data on a consecutive series of newly diagnosed MM patients confirm that the antigenic surface profile of MM PCs is highly variable, in line with the characteristic heterogeneity of the disease. These results are in agreement with previously published data[1,2] and confirm the accuracy, reproducibility, and utility of flow cytometry to dissect within clonal PCs in MM patients at presentation. In particular, CD20 and CD28 were the two surface antigens that showed the greatest correlation with high tumor burden features in our series and could, therefore, help identify upfront MM patients with a likely aggressive evolution. CD28 is a T-cell costimulatory receptor, usually associated with a rapidly evolving disease and resistance to frontline therapy.[9] In our cohort, CD28 expression correlated with the absence of CD56 ($p<0.001$). The neural cell adhesion CD56 antigen is a membrane glycoprotein, usually expressed on the surface of neoplastic PCs.[10] Even if the role of CD56 in the evolution of MM is highly debated, a recent study has postulated that its absence could be associated with a lower degree of maturation of the neoplastic cells and unfavorable prognostic parameters but not with outcome.[10] Thus, based on the results of our study, clonal PCs with CD28 positivity and absence of CD56 might identify a subset of patients with baseline unfavorable disease characteristics. This observation is supported by the evidence that CD28 expression correlates with both a low platelet count ($p=0.005$) and with the high-risk t(14;16) chromosomal abnormality ($p=0.079$).[11] In addition, this subgroup of patients achieved significantly lower complete response rates ($p=0.038$) compared to patients with CD28 negativity. An extended cohort of patients and a prolonged follow-up are warranted to have more significant and relevant data about the prognostic role of CD28 antigen expression on PC. MM patients with CD20 positivity had higher levels of serum monoclonal component ($p=0.038$), confirming that the aberrant expression of this antigen could define a more aggressive MM subset.

Interestingly, our analysis shows that t(4;14) correlates with a lower median CD138 MFI. CD138 is usually highly expressed on the surface of MM cells; it mediates cell adhesion, and its loss may contribute to the dissemination of the disease out of the BM.[12] Therefore, lower levels of expression of this protein may define a condition with high-risk aberrations.

In conclusion, our study confirms the high heterogeneity of MM patients. In this setting, MFC represents a simple, reproducible, and cost-effective tool that could help to identify MM subsets at diagnosis and improve risk stratification. A larger series of cases with

a prolonged follow-up is warranted to confirm these preliminary observations and corroborate the clinical correlations.

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