Phenotype of plasma cells in multiple myeloma and monoclonal gammopathy of undetermined significance

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Flow cytometry is a useful tool for the analysis of plasma cells in monoclonal gammopathies. The aim of this study was to find possibilities and limits of multicolour flow cytometry in diagnostics of monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM) and to identify parameters that could be used to differentiate between these two disorders. Surface markers CD38 and CD138 were used for identification of plasma cells, CD19 and CD56 further distinguished normal and abnormal plasma cells, respectively. The percentage of circulating plasma cells in peripheral blood was lower in MGUS patients then in MM (p<0,001). In bone marrow, the percentage of residual polyclonal CD19$^+$ plasma cells was higher (p<0,001) and the percentage of malignant monoclonal CD56$^+$ plasma cells was lower (p<0,001) in MGUS than in MM. The relative counts of CD19$^+$ plasma cells permitted unequivocal differentiation of MGUS from all clinical stages of MM. In conclusion, flow cytometry is relatively quick and effective method for analysis of plasma cells thus immunophenotyping can significantly contribute to the differential diagnosis of plasma cell proliferations.

Key words: plasma cells, multiple myeloma, monoclonal gammopathy, flow cytometry.

Multiple myeloma (MM) is a haematological malignancy caused by the transformation of B lymphocytes, their unchecked proliferation and plasmocytic differentiation. The proliferation and accumulation of CD38$^+$CD138$^+$ plasma cells (PCs) occurs predominantly in the bone marrow. Plasma cells with variable maturation status and proliferation activity can be identified in MM using the CD45 marker [1]. Overall survival for all patients is approximately 4 years, with longer survival reported for patients younger than 60 years. Although high-dose chemotherapy followed by autologous peripheral blood stem cell transplantation significantly extends the expected survival, the disease is considered incurable and patients eventually relapse as a consequence of residual disease [2]. Monoclonal gammopathy of unknown/undetermined significance (MGUS) is a precancerous condition that in some patients progresses into MM through a multi-step transformation process [3, 4]. Active MM requiring therapy can be distinguished from MGUS or asymptomatic MM using

the Durie-Salmon criteria or the International staging system [5, 6]. However, the rapidity of progression and the course of the disease are difficult to predict for individual patients. New prognostic parameters are being constantly sought to improve our ability to define the prognosis and optimal treatment strategy for MM patients [7–10].

Multicolor flow cytometry is a sensitive tool for the analysis of PC immunophenotype and identification of normal and neoplastic PC populations. It has been established that normal PCs mostly express CD19 while malignant PCs mostly express CD56 [10–15]. Bone marrow in MGUS patients contains mixture of normal polyclonal CD19$^+$CD56$^-$ PCs and clonal PCs with aberrant CD19$^+$CD56$^+$, CD19$^-$CD56$^+$, or CD19$^-$CD56$^-$ immunophenotype. In contrast, there is an absolute predominance of CD56$^+$ aberrant PCs in MM [14–15]. CD56-negative PCs are found in 20-30% of MM patients and their presence is associated with poor prognosis in patients treated with conventional chemotherapy. However, there seems to be no overall survival difference between patients with CD56-positive versus CD56-negative MM [16]. There is not many information about CD19$^+$ malignant PCs in MM, because these occur rarely [17].

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The pathological populations in peripheral blood (PB) and in bone marrow (BM) are heterogeneous and may contain less mature forms of B lymphocytes. It has been proposed that monitoring of plasma cell subtypes in PB and BM by immunophenotyping for CD38, CD138, and other markers form a valuable part of the diagnostic and prognostic assessment and post-treatment follow-up [18–22].

Therefore, we analysed number and phenotype of PCs to determine whether flow cytometry can be used in differential diagnosis of plasma cell proliferations. Our results show that expression of CD19 and CD56 can identify different subpopulation of PCs and hence flow cytometry is a supplementary method for discrimination between MGUS and different clinical stages of MM cases.

Patients and methods

Patients. A total of 58 patients with MGUS and 156 patients with newly diagnosed, untreated MM were studied from January 2006 to October 2008. Their baseline characteristics are shown in Table 1. The diagnosis of MM or MGUS was made based on the Durie-Salmon criteria [5]. MM patients included 31 Durie-Salmon clinical stage I patients (MM I), 23 stage II patients (MM II), and 102 stage III patients (MM III), MM III consist of 82 IIIA and 20 IIIB patients. This study was approved by the local ethics committee and is in accordance with the Helsinki Declaration of 1975. All patients signed the informed consent. Peripheral blood and bone marrow samples taken during routine planned investigations were mixed with EDTA and processed for flow cytometric analysis on the same day. BM infiltration by PCs was quantified morphologically from the first portion of the same bone marrow aspirate.

Flow cytometry. Peripheral blood and bone marrow samples were incubated with the following fluorescence-labelled monoclonal antibodies (MoAbs): CD38-FITC, CD138-PE, CD45-PC5 or CD45-ECD, CD56-PC5 and CD19-PC7. All MoAbs were purchased from Immunotech (Marseille, France). Lysis of erythrocytes was done using ammonium chloride. Samples were analyzed using the FC500 Cytomics flow cytometer (Beckman Coulter, Hialeah, FL, USA) equipped by 488 nm Argon Ion laser.

The number of acquired cells was 50.000 for PB samples and 250.000-500.000 for BM samples. Two different flow cytometric gating approaches were used for the analysis of BM PCs. First, was performed a 4-color analysis where viable cells were gated in a forward scatter/side scatter plot (live gate) and further analyzed for the expression of CD38 and CD45. The expression of CD19 and CD56 was studied on a gate that contained $CD45^{-/+/++}CD38^{+/++}$ cells. However, using this approach $CD45^+$ PCs with lower expression of CD38 ($CD38^+$) were missed and in some cases it was difficult to the cut-off between the $CD38^+$ and $CD38^{++}$ populations (Figure 1). Therefore, 5-color flow cytometry was used for subsequent studies. Gating was done on the $CD38^{+/++}CD138^{+/++}$ population (PC gate) of viable cells (live gate) which were further analyzed for the expression of CD19 and CD56 and at least 100 plasma cells were acquired from BM samples. CD45 was used as an additional marker (Figure 2). The sequence of setting the live gate and PC gate was interchangeable.

In accordance with the European Myeloma Network (EMN) recommendations, the results are shown as a percentage of $CD19^+$ PCs (normal plasma cells, N-PC) and $CD56^+$ PCs (abnormal plasma cells, A-PC) in the total PC population [22]. For the comparison between MGUS and MM and between the three MM stages we used the N-PC/A-PC ratio (N/A).

Statistics. All results were analysed by Statistica 7.0 from StatSoft, Inc. (2005). The nonparametric Mann-Whitney test was used to analyze the differences between MM and MGUS, and between the MM stages. The level of statistical significance was set at p=0.05. The Kruskal-Wallis ANOVA test was used to detect significant differences between three groups of variables.

Results

Circulating plasma cells in peripheral blood. Circulating $CD38^+CD138^+$ PCs in PB were mostly $CD38^{+/++}$ with variable expression of CD45 and low expression of CD138. These PCs were found in MGUS patients only in 4 of 50 analysed cases but they were always $CD45^+$. In MM were circulating PCs found in 69 of 149 analysed cases and they were almost $CD45^-$ (55 cases). The number of circulating PCs correlated with the stage of MM (Table 2). Circulating PC counts were significantly lower in MGUS patients in comparison to all MM patients (p<0.001), including even individual stages MM I, MM II, and MM III. There was found statistically significant

Table 1: Baseline characteristics of patients.

Parameter	MGUS (n=58)	MM (n=156)
Sex, Males/Females, no.	30/28	76/80
Age – median (range), years	60 (40-80)	66.5 (41-86)
β2m – median (range), mg/l	2.0 (1.2-13.1)	2.8 (1.2-20.5)
Serum MIg – median (range), g/l	12.7 (0.0-33.1)	18.2 (0.0-79.8)

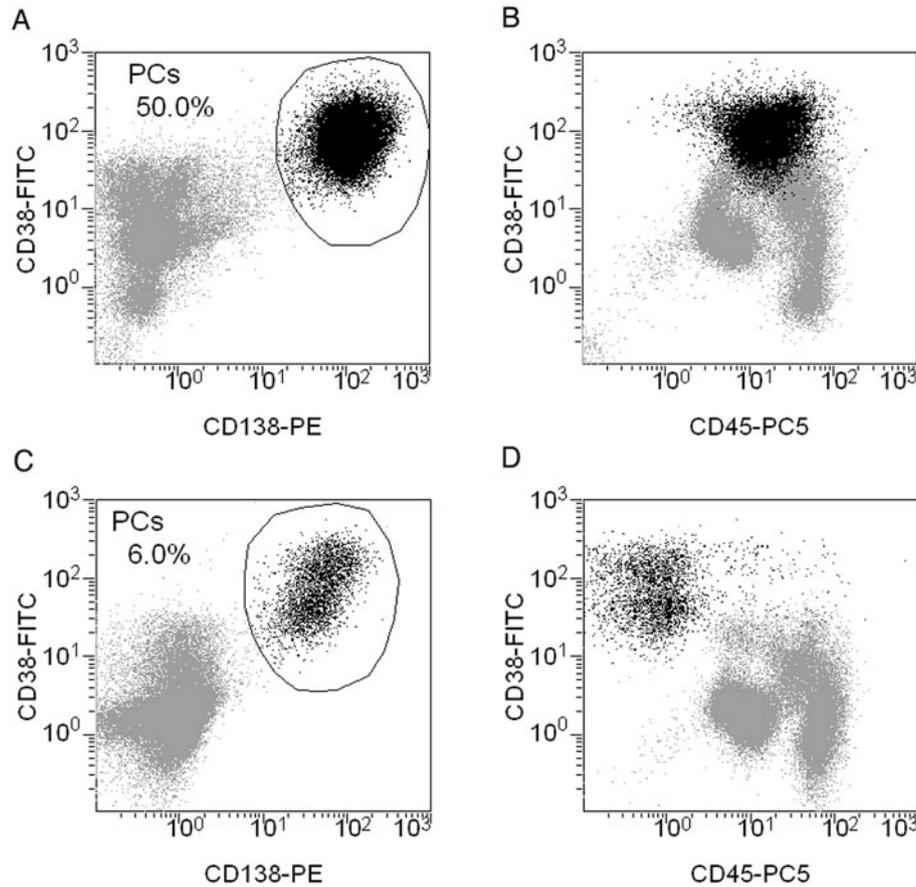Abbreviations: MIg, monoclonal immunoglobulin

Figure 1: Gating for plasma cells. Different gating approaches are illustrated, CD138+ PCs are highlighted. **(A, C) Gating of plasma cells as the CD38+/++CD138+/++ population.** A clearly demarcated population of PCs is separated from other leukocytes. **(B, D) Visualization of plasma cells as the CD38+/++CD45-/+/++ populations. Overlap of CD45+ PCs with other leukocytes is illustrated.**

difference between MM I vs. MM III (p<0.05) (Tables 3 and 4). The overwhelming majority of circulating PCs in MM patients did not express CD19 or CD56 (data not shown).

Bone marrow plasma cells. As expected, morphological evaluation showed significantly lower BM infiltration with PCs in patients with MGUS (median 1.6%; range 0.0-5.2) than in MM patients of any clinical stage (16.0%; 0.0-94.6) (p<0.001) (Tables 3 and 4). When comparing MM clinical stages against each other, the only statistically significant difference was between MM I and MM III (p<0.01).

Flow cytometry detected a population of CD38+CD138+ plasma cells in all investigated patients but the percentage was lower than that determined by morphological analysis. The results of morphological and flow cytometric PC enumeration correlated significantly for all MM clinical stages but not for MGUS, a finding likely due to lower degree of BM infiltration in MGUS.

Again the CD38+CD138+ PC population identified by flow cytometry was smaller in MGUS (0.4%; 0.0-4.2) as compared to any MM clinical stage (5.1%; 0.0-75.3), reflecting the

diagnostic criteria for these two disorders. On comparison of different MM stages, the only significant difference in CD38+CD138+ PC counts was identified between MM I and MM III (p<0.01). Also the population of CD38+CD45- cells was significantly smaller in MGUS patients (0.2%; 0.0-3.8) than in MM patients (3.2%; 0.0-71.2) (p<0.001). The highest percentage of CD38+CD45- PCs was detected in MM III patients, suggesting that a significant proportion of their plasma cells is in the terminal stage of maturation. There was found no significant difference between IIIA and III B MM clinical stages (data not shown). The intensity of staining for CD38 on CD45- PCs was variable, ranging from CD38+ to CD38++. Probably immature proliferating CD45+/++ PCs were simultaneously CD38++ but without analyzing other immunophenotypic markers it was impossible to ascertain whether they represented normal or malignant plasma cells, especially in low infiltration cases.

Expression of CD19 and CD56 by plasma cells. We chose the CD19 and CD56 markers to differentiate between normal and aberrant PC populations. The analysis of expression of these
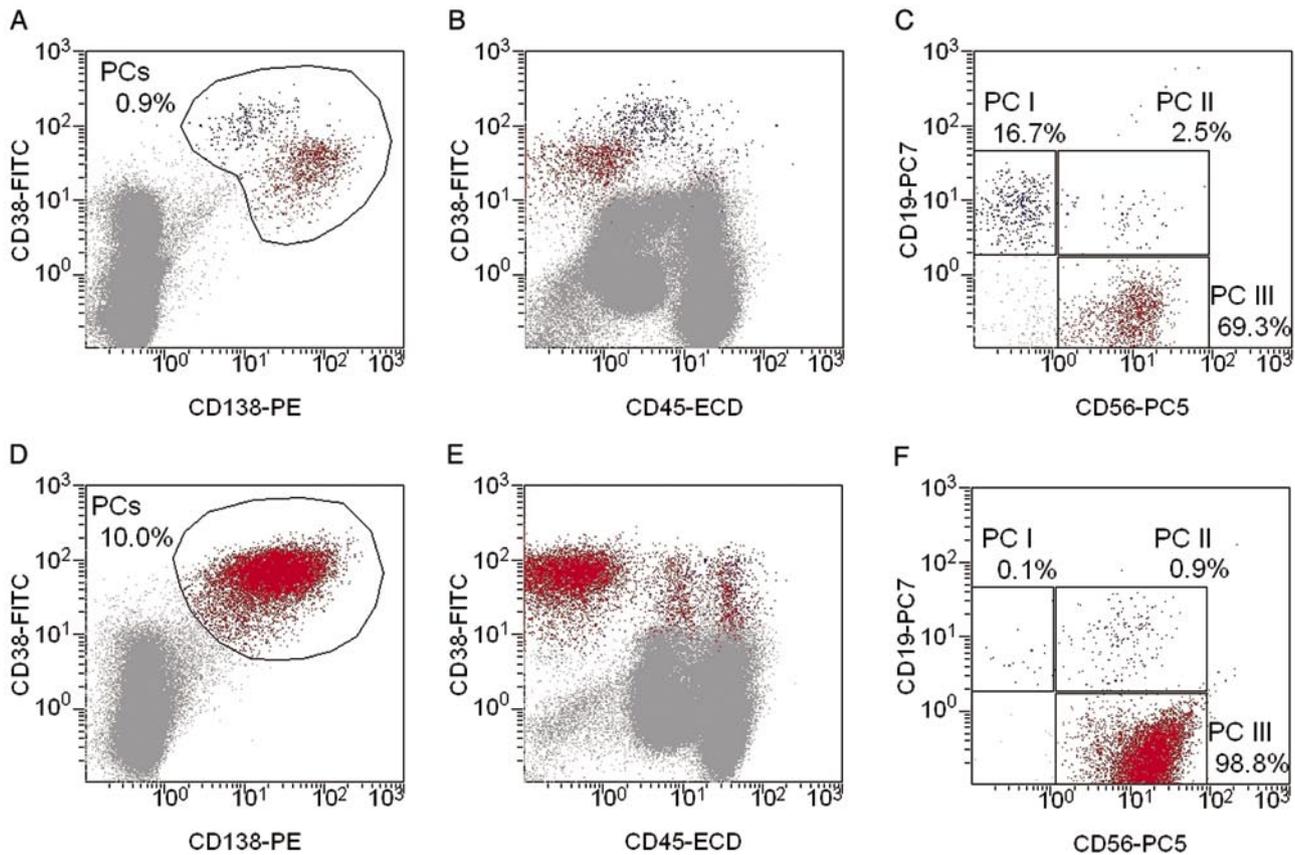
Figure 2: Immunophenotype of bone marrow plasma cells in MGUS and MM. Different visualization of CD38+CD138+ PCs, expression of CD19 and CD56 is illustrated on these PCs. (A, B, C) BM sample of a patient with MGUS. Two populations of plasma cells with different intensity of CD38, CD138, and CD45 expression are found in the leukocyte gate. CD19+CD56- cells (PC I) from the plasmocyte gate are considered physiological plasma cells, CD19+CD56+ (PC II) and CD19-CD56+ (PC III) are clonal neoplastic PCs. (D, E, F) BM sample of a patient with MM. Aberrant CD38++CD45-CD56+ plasma cells are predominant, two subpopulation of CD45+ and CD45++ PCs are visible as well.

markers on CD38+CD138+ PC population in BM showed that percentage of residual normal CD19+ PCs were more frequent in MGUS (22.8%; 1.4-85.9) than in MM of any clinical stage analyzed separately or together (0.6%; 0-79.4). In 2 cases of MGUS patients were numbers of PCs very low and therefore insufficient for analysis of CD19 and CD56. There was found

Table 2: Morphological and flowcytometric assessment. Plasma cell infiltration of bone marrow in MGUS and MM stage I, II, or III. Median values are shown, with range given in parentheses.

Parameter	MGUS (n=58)	MM all stages (n=156)	MM I (n=31)	MM II (n=23)	MM III (n=102)
M-BM	1.6 (0.0-5.2)	16.0 (0.0-94.6)	7.0 (0.0-38.0)	16.0 (0.0-89.0)	21.2 (0.0-94.6)
CD38+CD138+ PB	0.0 (0.0-0.1)	0.0 (0.0-26.5)	0.0 (0.0-0.2)	0.1 (0.0-0.9)	0.1 (0.0-26.5)
CD38+CD45- PB	0.0 (0.0-0.0)	0.0 (0.0-33.1)	0.0 (0.0-0.6)	0.0 (0.0-0.6)	0.0 (0.0-33.1)
CD138+CD38+ BM	0.4 (0.0-4.2)	5.1 (0.0-75.3)	2.6 (0.0-21.3)	4.4 (0.0-44.3)	8.0 (0.0-75.3)
CD38+CD45- BM	0.2 (0.0-3.8)	3.2 (0.0-71.2)	1.3 (0.0-23.2)	2.9 (0.0-39.5)	4.6 (0.0-71.2)
CD19+ PC BM	22.8 (1.4-86.0)	0.6 (0.0-79.4)	1.4 (0.1-54.8)	1.0 (0.0-79.4)	0.4 (0.0-73.6)
CD56+ PC BM	22.2 (1.2-96.8)	92.1 (0.1-100.0)	86.7 (0.5-99.9)	59.3 (0.3-99.7)	94.2 (0.2-100.0)
N-PC/A-PC	1.2 (0.0-58.1)	0.0 (0.0-79.7)	0.1 (0.0-79.7)	0.0 (0.0-15.6)	0.0 (0.0-6.8)

Abbreviations: M-BM, percentage of bone marrow plasma cells by morphological assessment; BM, bone marrow; PB, peripheral blood; N-PC, normal plasma cells; A-PC, aberrant plasma cells, MGUS, monoclonal gammopathy of unknown significance; MM, multiple myeloma; MM I, MM II, MM III, multiple myeloma Durie-Salmon stage I, II or III

Table 3: Differential expression of selected markers in MGUS and MM.

Parameter	MGUS vs. MM all stages	MGUS vs. MM I	MGUS vs. MM II	MGUS vs. MM III
M-BM	p<0.001	p<0.001	p<0.001	p<0.001
CD38+CD138+ PB	p<0.001	p<0.05	p<0.001	p<0.001
CD38+CD45- PB	p<0.001	p<0.05	p<0.01	p<0.001
CD138+CD38+ BM	p<0.001	p<0.001	p<0.001	p<0.001
CD38+CD45- BM	p<0.001	p<0.001	p<0.001	p<0.001
CD19+ PC BM	p<0.001	p<0.001	p<0.001	p<0.001
CD56+ PC BM	p<0.001	p<0.01	n.s.	p<0.001
N-PC/A-PC	p<0.001	p<0.001	p<0.001	p<0.001

Abbreviations: M-BM, percentage of bone marrow plasma cells by morphological assessment; BM, bone marrow; PB, peripheral blood; N-PC, normal plasma cells; A-PC, aberrant plasma cells, MGUS, monoclonal gammapathy of unknown significance; MM, multiple myeloma; MM I, MM II, MM III, multiple myeloma Durie-Salmon stage I, II or III

Table 4: Differential expression of selected markers in the three Durie-Salmon stages of MM

Parameter	MM I vs. MM II vs. MM III	MM I vs. MM II	MM I vs. MM III	MM II vs. MM III
M-BM	p<0.05	n.s.	p<0.01	n.s.
CD38+CD138+ PB	n.s.	n.s.	p<0.05	n.s.
CD38+CD45- PB	n.s.	n.s.	p<0.05	n.s.
CD138+CD38+ BM	p<0.01	n.s.	p<0.01	n.s.
CD38+CD45- BM	n.s.	n.s.	p<0.05	n.s.
CD19+ PC BM	p<0.01	n.s.	p<0.01	n.s.
CD56+ PC BM	n.s.	n.s.	n.s.	n.s.
N-PC/A-PC	p<0.05	n.s.	p<0.05	n.s.

Abbreviations: M-BM, percentage of bone marrow plasma cells by morphological assessment; BM, bone marrow; PB, peripheral blood; N-PC, normal plasma cells; A-PC, aberrant plasma cells, MGUS, monoclonal gammapathy of unknown significance; MM, multiple myeloma, MM I, MM II, MM III, multiple myeloma Durie-Salmon stage I, II or III

predominance of CD19+ PCs over CD56+ PCs in 27 persons with MGUS. At least a proportion of PCs from MGUS patients expressed CD19 or CD56, but there were found also CD19- CD56-/low PCs in 13 cases of MGUS patients.

On the other hand, the CD56+ PC subpopulation prevailed in MM patients of all stages (92.1%; 0.1-100.0). In accordance with published results, the proportion of CD56+ PCs was significantly higher in MM as compared to MGUS (22.2%; 1.4-96.8) (p<0.001) [14–15]. The proportion of CD56+ PCs did not significantly differ between the three clinical stages of MM, and surprisingly, there was no difference between MGUS and MM II. However, we found a statistically significant difference in the N/A ratio between MGUS and MM II (p<0.001). The percentage of residual CD19+ PCs in MM was lower than in MGUS, with the highest counts in MM I (1.5%; 0.1-54.8). There was found significant difference between MM I and MM III in this parameter (p<0.001). In 9 MM patients there was a surprising presence of majority of CD19+ PCs in bone marrow (over 50% of PCs) and these were found mostly in MM

III stage (6 cases). Comparison of IIIA and III B MM clinical stages did not show any significant difference in expression of CD19 and CD56 on PCs (data not shown). As well as in MGUS patients there were also identified pathological CD19- CD56-/low PCs in 45 MM patients, and the number of patients with this finding correlated with increasing stage of MM (8x MM I, 9x MM II, 28x MM III). Circulating PCs were present in majority of these patients.

Discussion

The objective of our study was to evaluate the percentages and the immunophenotype of plasma cells in patients with MGUS and MM. We have compared two gating strategies that used different combinations of markers to maximize the sensitivity of flow cytometric analysis even in patients with low BM infiltration. We have also identified parameters which could be useful for differential diagnosis of plasma cell proliferations.

It has been suggested that circulating PCs appear in the peripheral blood as a consequence of the loss of adhesion to the bone marrow microenvironment. The adhesion is mostly mediated by CD56 which plays a role in homotypic (cell-cell) as well as heterotypic (cell-stroma) interactions [23]. Although the quantity of circulating PCs was low in MM and almost negligible in MGUS, our results confirm the lack of CD56 expression on these cells. The presence of circulating PCs probably reflected not only the size of the tumour mass and the disease activity but also the biologic heterogeneity which can present clinically as a short time to progression or resistance to treatment [24, 25]. This population is also partly formed by recirculating bone marrow PCs [26]. Nowakowski and collaborators suggested that the number of circulating PCs in MM is an independent prognostic factor and that there is an inverse correlation between circulating PC counts and overall survival [25]. Although circulating PCs are considered a marker of advanced disease, we were not able to use this parameter to distinguish between MGUS and asymptomatic MM.

In our hands, PC percentage in the BM based on morphological evaluation was higher than that determined by flow cytometry. It is well known, however, that the first portion of bone marrow aspirate which is used for morphology is always richer in plasma cells than subsequent portions used for other studies, including flow cytometry [22]. The quantification of BM infiltration by either method can differentiate between MGUS and MM. Although results of both methods correlate in MM, the PC percentage determined by flow cytometry should has superior prognostic value [22].

CD19 is a lymphocyte marker already expressed by early pre-B lymphocytes which gradually disappears with plasmocytic maturation [27]. The pathophysiological reason for the absence of CD19 on myeloma cells has not been elucidated but it has been hypothesized that its loss can create a proliferative advantage for the malignant plasma cell clone [28]. We found two PC subpopulations in BM of MGUS patients that could be characterized by the different levels of expression of CD38, CD45, and CD138, and, especially, by the presence or absence of CD19 and CD56. As shown by Ocqueteau *et al.* and Sezer *et al.*, the ratio of immunophenotypically normal CD19+ PCs to all PCs is a unique parameter which permits diagnostic differentiation between MGUS and MM [14, 15]. Based on our results, we can confirm this finding. This parameter is also useful for the evaluation of PCs after treatment [18–21].

The expression of CD56 on PCs with the cut-off of 95% can be used to stratify MGUS patients to two groups with different risk of progression to MM [8]. In our hands, the percentage of CD56+ PC provided a tool for unequivocal differentiation between MGUS and stage I and/or stage III MM but not between MGUS and stage II MM. The N-PC/A-PC ratio was valuable for differentiating between MGUS and MM but not for distinguishing between the three MM stages. The reason for the lack of significance of A-PC parameter is possibly the absence of CD56 on a subpopulation of PCs. There are reports suggesting that the absence of CD56 on PCs is associated with

aggressive course of MM [29]. Before more prospective data of our patients are available, the association of high CD56 expression and/or loss of CD56 expression on PCs with an increased risk of MGUS progression to MM remain hypothetical.

Further characterization of malignant plasma cells is possible by clonality assessment using staining for κ and λ light chains and analysis of other immunophenotypic markers such as CD28, CD33, and CD117, which are expressed on abnormal plasma cells [10, 22, 30]. It has been shown that the presence of aberrant plasma cells is associated with worse outcome and may indicate the need for early primary or salvage treatment [10, 18, 19]. Recently, larger prospective studies were published to validate the use of flow cytometric methods for prognostic assessment and for monitoring of minimal residual disease (MRD) after autologous stem cell transplantation [20, 21].

In summary, CD19+ and CD56+ PC percentages, as well as the N-PC/A-PC ratio are the most important parameters for flow cytometric differential diagnosis of plasma cell proliferations. Our results suggest the potential usefulness of these parameters, together with other markers, also for prospective analysis of monoclonal gammopathies (evaluation of progression, MRD monitoring etc.). Multicolour flow cytometry is a sufficiently sensitive method for analysis and diagnosis of monoclonal gammopathies.

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