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Expression of the IL-6 Receptor Alpha Chain (CD126) in Normal and Abnormal Plasma Cells in Monoclonal Gammopathy of Undetermined Significance and Smoldering Myeloma

Dalia Abdel-Raouf Salem¹, Neha Korde², David J. Venzon³, David J. Liewehr³, Irina Maric⁴, Katherine R. Calvo⁴, Raul Braylan⁴, Prashant R. Tembhare⁵, Constance M Yuan⁶, Carl Ola Landgren², and Maryalice Stetler-Stevenson⁶

¹Laboratory of Pathology, CCR, NCI, NIH, Bethesda, MD, USA and Clinical Pathology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt

²Myeloma Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York, USA

³Biostatistics and Data Management Section, CCR, NCI, NIH, Bethesda, MD, USA

⁴Hematology, Department of Laboratory Medicine, CC, NIH, Bethesda, MD, USA

⁵Hematopathology Laboratory, Tata Memorial Hospital, Mumbai, India

⁶Laboratory of Pathology, CCR, NCI, NIH, Bethesda, MD, USA

Abstract

IL-6 activity in normal plasma cells (nPCs) and abnormal plasma cells (aPCs) is CD126 (subunit of IL-6 receptor) dependent. We quantified CD126 expression on nPCs and aPCs in monoclonal gammopathy of undetermined significance (MGUS), smoldering myeloma (SMM), and multiple myeloma (MM). CD126 was detected on all nPCs and aPCs indicating that CD126 does not have diagnostic utility. CD126 expression was higher in aPCs than in nPCs in 85% SMM but only 41% MGUS and there was evidence that CD126 was higher in aPCs than nPCs in the SMM ($p=0.048$) but not MGUS ($p=0.96$) patients. There is also a greater association between nPC and aPC CD126 expression in low risk MGUS than observed in high risk MGUS and SMM, suggesting normal regulation of CD126 decreases with disease progression. Future studies need to elucidate the role of bone marrow milieu versus escape from normal CD126 regulation in malignant transformation of clonal plasma cells.

Keywords

MGUS; smoldering myeloma; IL-6 receptor; CD126; Flow Cytometry

INTRODUCTION

Plasma cell dyscrasias (PCD) represent a heterogeneous disease group with a spectrum of clinical manifestations and decreasing survival as patients progress from the asymptomatic monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM) to the symptomatic multiple myeloma (MM) with its low quality of life and greatly reduced life span [1]. MGUS and SMM are viewed as precursor disease states that transform into MM [1–3] and as such have been studied in attempts to elucidate the pathogenesis of MM. Interleukin-6 (IL-6) is a protein that induces the final maturation of activated normal B-cells into plasma cells [4, 5]. IL-6 plays a vital role in MM; not only in myeloma cell proliferation [6], but also in disease progression [7]. IL-6 is a crucial factor for myeloma cell survival [8, 9], conferring chemoresistance against Bortezomib and melphalan [10, 11]. Neoplastic plasma cell survival is dependent on the bone marrow environment. Although IL-6 expression has been demonstrated in some myeloma cell lines, bone marrow stromal cells are the main source of IL-6 [12]. Secretion of IL-6 by bone marrow stromal cells is activated by numerous cytokines and can be stimulated by cellular contact with myeloma cells [13, 14].

IL-6 activity in normal plasma cells (nPCs) and the clonal, abnormal plasma cells (aPCs) of MM is dependent on the IL-6 receptor (IL-6R) complex which consists of two subunits; CD126 (gp80) and CD130 (gp130) [15, 16]. Bone marrow stromal cells can regulate the level of CD126 expression by plasma cells and have been shown to increase CD126 expression in myeloma cell lines, thus effecting IL-6 responsiveness [17]. It is unclear whether elevated CD126 expression is an innate characteristic of malignant plasma cells or if this is secondary to marrow elements or other factors related to malignancy inducing higher levels. IL-6 and its receptor have been considered as therapeutic targets [18–20] and one study found that over-expression of IL-6R was associated with decreased response to thalidomide-dexamethazone treatment in MM patients [21]. The level of expression of the IL-6R receptor in MM and its precursor states of MGUS and SMM therefore garnered interest. Early studies reported differing levels of plasma cell CD126 expression in MGUS and MM compared to normal bone marrow. The aPCs in MGUS and MM appeared to have higher levels of CD126 expression than that observed in nPCs. These studies did not directly measure the level of CD126 expression but relied upon comparative methods, including mean fluorescence intensity (MFI) and Kolmogorov-Smirnov (K-S) analyses [22, 23] due, in part, to suboptimal separation of positive from negative staining, the anti-CD126 antibody clone and fluorochrome utilized, and the limitations of 4 color flow cytometry. We studied a series of patients with MGUS, SMM, and MM to explore the diagnostic potential of CD126. Additionally, to better understand the regulation of CD126 and its role in plasma cell neoplasia, we examined the levels of CD126 expression by both aPCs and nPCs within each bone marrow aspirate from a series of patients with MGUS and SMM where both populations are exposed to the same bone marrow environment. Previously these populations were studied separately, comparing nPCs from normal bone marrows to aPCs from MGUS or SMM marrows. Comparing nPCs from a normal marrow milieu to aPCs from a disease-marrow milieu is not optimal for determining the role of malignant transformation vs. milieu in regulation of CD26 expression. Furthermore, we examined three

different Anti-CD126 antibodies for the best separation of positive from negative cells and instead of examining relative levels of expression (mean fluorescence intensity), absolute levels were quantitated using the QuantiBRITE system (BD Biosciences).

MATERIALS AND METHODS

Case Selection

CD126 expression was evaluated in bone marrow aspirates from 24 patients with MGUS, 56 patients with SMM, 35 patients with multiple myeloma (MM) (diagnosis based on clinical findings, serum monoclonal protein, bone marrow biopsy morphology, CD138 immunohistochemistry of bone marrow biopsy, and bone marrow aspirate flow cytometry and according to the International Myeloma Working Group (IMWG) criteria [24]) and 4 normal bone marrows (based upon morphology, flow cytometry, immunohistochemistry, and clinical history). MGUS patients were assigned risk scores of low, low-intermediate, high-intermediate or high risk of progression to clinically detectable MM according to the Mayo Clinic model [25]. SMM patients were assigned risk scores of low, intermediate, or high risk for progression to clinically detectable MM according to the Mayo Clinic [26] and the Spanish PETHEMA models [27]. Demographic and risk stratification information are summarized in Table I.

Sample Preparation and Flow Cytometric Detection of aPCs:

Specimens were processed within 4 hours of collection according to ICCS/ESCCA consensus guidelines [28] and as previously described [29]. 5×10^6 cells were stained per tube to insure adequate plasma cell acquisition [30–32]. Three anti-CD126 antibodies, namely CD126 PE clone M5 (BD Biosciences), CD126 FITC clone B-R6 (ABCAM), and CD126 PE clone M91 (Beckman Coulter/Immunotech) were evaluated in six PCD bone marrow specimens for optimal staining of nPCs and aPCs. CD126 was studied in patient specimens using a cocktail containing CD19 PerCP Cy5.5, CD20 APC-H7, CD38 v450, CD45 v500, CD56 PE-Cy7, CD81 FITC, CD138 APC and CD126 clone M5 PE. Additional diagnostic cocktails designed in accordance with ICCS/ESCCA consensus guidelines [33] were also utilized (Supplemental Table I for comprehensive panel). For intracellular staining, fix & perm cell fixation and permeabilization kit (Invitrogen, Grand Island, NY) was used after surface staining. All cells were fixed in 0.5% paraformaldehyde and stored at 4°C for up to 4 hours before acquisition. Cellular viability of lysed and washed bone marrow aspirate cells was assessed by staining with the fluorescent dye 7-Amino-Actinomycin D (7-AAD) (BD Biosciences, San Jose, CA) for 10 minutes at room temperature (7-AAD only stains non-viable cells). Specimens were acquired using BD FACSCanto™II (BD Biosciences, San Jose, CA) as previously described [30] with an acquisition goal of at least 3×10^6 total events.

Plasma cells were analyzed as previously described [32] by gating on cells with CD138 positivity, strong CD38 expression and moderate to negative CD45, excluding debris based upon light scatter, according to ICCS/ESCCA consensus guidelines (Figure 1 Supplemental) [33]. Light scatter properties were also examined to exclude debris and doublets. nPCs and aPCs were identified based upon the normal pattern of expression of CD19, CD20, CD27,

CD28, CD38, CD45, CD56, CD81, and CD138 (Figure 1 Supplemental) according to ICCS/ESCCA consensus guidelines [33–37] as well as intracellular light chain expression in select cases [38]. A discrete population of 20 or more aPCs was necessary to define the presence of plasma cell disease.

Quantification of CD126

The antibody bound per cell (ABC) for anti-CD126 (clone M5) (BD Biosciences, San Jose, CA) was determined as previously described [30] using saturating concentrations of antibody and the BD Biosciences QuantiBRITE system (QuantiBRITE standard beads and QuantiCALC software) for fluorescence quantitation. The ABC value represents the mean value of the maximum capacity of each cell to bind the anti-CD126 antibody. QuantiBRITE PE beads are pre-calibrated standard beads containing known numbers of PE molecules bound per bead. QuantiBRITE beads were acquired on a FACSCanto™ II (BD Biosciences, San Jose, CA) on the same day at the same instrument settings as the individual patient specimens. A standard curve comparing the geometric mean of fluorescence to known PE content of the QuantiBRITE beads was constructed using QuantiCALC software. The regression analysis, slope, intercept, and correlation coefficient were determined. aPCs and nPCs were identified based upon immunophenotype and the geometric mean fluorescence of CD126PE staining determined. The measured geometric mean fluorescence can be used to determine ABC values via the QuantiBRITE standard curve. A cut-off of 100 events was required to determine ABC values to achieve adequate precision [30]. The geometric mean fluorescence of unstained cells was determined in 13 specimens and the ABC values used to determine the negative range. ABC values were also determined in 13 specimens on CD126 negative/CD81 negative nucleated red blood cells to assess background staining with the anti-CD126 antibody. Additionally, unstained cells, CD19 PE staining (negative in aPCs studied), and CD117 PE staining (negative in nPCs) were utilized as a negative control for autofluorescence during analysis.

RESULTS

Three different Anti-CD126 antibodies were compared in six bone marrows. The CD126 PE clone M5 had the best separation of positive from negative cells (Figure 1) and was subsequently utilized for quantitation of CD126ABC in all patient specimens. For negative-range determination, the CD126ABC values were as follows: unstained cells (n=13) median 68, min-max 61–87, and mean (S.D.) 69 (8), and nucleated red blood cells (n=13) median 98, min-max 84–115, and mean (S.D.) 100 (8). Therefore, positive expression was defined as CD126 ABC>132 (two S.D. above highest negative values and 4 S.D. above mean).

CD126 expression was clearly demonstrated in the aPCs in bone marrow aspirates from patients with MGUS, SMM, and MM (Figure 2 and Figure 2 Supplemental). In addition, nPCs in aspirates from 17 MGUS and 13 SMM patients with sufficient numbers of nPCs and nPCs in 4 normal bone marrow aspirates were positive for CD126 (Figure 2 and Figure 2 Supplemental). The aPC CD126 ABC levels were determined in MGUS (n=24), SMM (n=56), and MM (n=35) patients. The median CD126 ABC values were 747, 724 and 620 respectively (Table II and Figure 2). There was no clear cut off value for aPC CD126

expression to differentiate MGUS, SMM and MM (Table II) and therefore it is not useful in diagnosis.

We compared the aPC CD126 expression in the pre-malignant MGUS and SMM to determine if levels changed with increasing malignant potential. There was no significant difference between MGUS and SMM in expression of CD126 by aPCs (Wilcoxon rank sum test [WRST], $p=0.60$, median difference (95% CI): 49 (−146, 242)). We also tested the hypothesis that distributions of CD126 ABC levels on aPCs would differ according to either Mayo risk (in MGUS or SMM patients) or Spanish/PETHMA risk (in SMM patients) categories. In MGUS there was no significant difference in aPC CD126 ABC levels between the three Mayo risk groups (0, 1, and 2) (Kruskal-Wallis test [KWT] $p=0.36$, trend test $p=0.34$). There was evidence for a difference in aPC CD126 expression between the three Mayo risk groups (1, 2, and 3) in SMM (KWT, $p=0.033$) and weak evidence of trend ($p=0.27$). Lower aPC CD126 ABC values were also observed in SMM Mayo risk group 3 ($n=3$) compared to combined SMM Mayo risk groups 1 and 2 (both $p=0.019$, WRST) but the sample size in group 3 was small and this result should be interpreted with caution (Table III). There was little evidence for differences between the SMM risk stratification groups using the Spanish risk stratification (KWT $p=0.51$, trend test $p=0.38$).

Aspirates from 30 patients with PCD (17 MGUS, 13 SMM) yielded sufficient numbers of nPCs (100 or greater) for CD126ABC quantification. CD126 positivity was demonstrated in all nPCs and aPCs in MGUS and SMM bone marrow aspirates. We observed that there was no significant difference between the distributions of CD126 ABC values in nPCs present in MGUS compared to nPCs in SMM ($p=0.82$, Wilcoxon's signed rank test [WSRT]). The CD126 ABC values in nPCs differed a great deal in the specimens examined, ranging from 236 to 1380. We compared the CD126 ABC levels between aPCs and the corresponding nPCs in these patients (pair-wise differences between the two levels: aPCs CD126 ABC – nPCs CD126 ABC; and WSRT). CD126 ABC was higher in the aPCs than in the nPCs in 7/17 (41%) of the MGUS patients but in SMM patients 11/13 (85%) demonstrated higher CD126 ABC values in the aPCs compared to the nPCs. Although the CD126 ABC was higher on aPCs than on nPCs in most of the patients with PCD, the difference was not significant across all diagnoses ($p = 0.31$, WSRT, median (95% CI): 70 (−136, 236), $n=30$), and there is no observed cut off value for CD126 expression to define aPCs from nPCs in the same BM specimen. However, in addition to most SMM cases demonstrating higher CD126 expression in aPCs compared to nPCs, there was evidence that the actual level of CD126 expression by all aPC populations was higher than the level that could be observed in nPCs in the SMM patients (WSRT $p=0.048$, median (95% CI): 96 (24, 562)) but there was no evidence of this in MGUS patients ($p=0.96$; median (95% CI): −107 (−327, 236)). This suggests that regulation of CD126 expression in aPC may decrease with disease progression from MGUS to SMM.

We found a weak positive correlation between the level of CD126 expression in nPCs and aPCs in all patients (Spearman's r (95% CI) = 0.32 (−0.09, 0.66), $n=30$, $p=0.085$). In other words, patients with higher CD126 ABCs on their aPCs, tended to have higher CD126 ABCs on their nPCs and vice versa (Figure 3). Grouping the data by Mayo risk stratification resulted in an increased degree of association between the CD126 ABC on the aPCs and the

nPCs ($r=0.74$, $n=8$) in MGUS Mayo risk group 0 (low risk) compared to MGUS risk groups 1 and 2 and SMM (Figure 3). In other words, the levels of CD126 expression by aPC and nPC were more closely associated (higher with higher and lower with lower) in low risk MGUS than in higher risk MGUS and in SMM where CD126 expression on nPCs and aPCs had a lower degree of association. This again suggests that regulation of CD126 expression in aPCs may decrease with disease progression from low risk MGUS to higher risk groups.

DISCUSSION

IL-6 and the IL-6R play a central role in myeloma cell proliferation and survival and as such have been considered as therapeutic targets [18–20]. Bone marrow stromal cells can regulate the level of CD126 expression by plasma cells and have been shown to increase CD126 expression in myeloma cell lines, thus effecting IL-6 responsiveness [17]. However, it is still unclear whether malignant plasma cells have innately elevated CD126 expression or if this is secondary to regulation by marrow elements. As regulation of CD126 expression in the myeloma precursors MGUS and SMM is unknown, we studied the expression of CD126 in nPCs and aPCs in these entities. Previous studies had detected CD126 expression in multiple myeloma but failed to demonstrate CD126 expression by normal plasma cells, leading to the postulation that expression of CD126 may be part of the malignant transformation [22]. Using our technology we were able to measure CD126 expression in all normal plasma cell populations indicating that mere expression of CD126 is not sufficient for diagnosis of myeloma [22]. Kalina et al. have reported changes in fluorescence in the first three hours post specimen fixation [39]. As we simultaneously evaluated nPC and aPC CD126 expression within the same specimen this did not affect our comparison of CD126 expression in these populations. Previous studies, however, studied their expression in different specimens and the time of fixation may have affected their results if there was a non-random distribution of fixation time based upon specimen type. There was also not a clear cutoff value to differentiate normal and abnormal plasma cells or MGUS, SMM and MM, further indicating that CD126 does not have diagnostic utility in the testing performed. All specimens were processed within 4 hours of collection and rapidly analyzed post fixation, with no fixed specimen held longer than 4 hours. Furthermore, they were collected in house, ensuring temperature control. Although Kalina et al. have reported changes in fluorescence in the first three hours post specimen fixation [39] this effect would be random and not a likely cause of the lack of significant differences in the levels of aPC CD126 expression among the three diseases. It is possible that potential differences in soluble CD126 levels in MGUS, SMM, and MM affected our ability to detect significant disease specific aPC CD126 expression, preventing diagnostic utility of this test.

Although there was not a clear cutoff value to differentiate normal and abnormal plasma cells or MGUS, SMM and MM, the results increase our understanding of the regulation of CD126 in normal and malignant plasma cells. We observed a weak positive correlation between the level of CD126 expression in nPCs and aPCs in all patients, i.e. patients with higher aPCs CD126 tended to have higher nPCs CD126 and patients with lower aPCs CD126 tended to have lower nPCs CD126 (Figure 3). This data suggests that in MGUS and SMM the bone marrow milieu, for example interaction with bone marrow stromal cells, affects the level of CD126 expression by clonal plasma cells (aPCs) as it does in nPCs [17].

Although the CD126 ABC was higher on aPCs than on nPCs in most of the patients with PCD, the difference was not significant across all diagnoses, likely due to the wide range of expression of CD126 on normal and clonal plasma cells and the small sample size. In MGUS only 41% of the specimens demonstrated higher CD126 expression by aPCs compared to nPCs (Figure 2 supplemental). In SMM, however, the level of CD126 expression by aPCs was higher than that on nPCs in most cases (85%). There was evidence in the SMM patients ($p=0.048$) that the general CD126 expression by aPCs was higher than that observed in nPC populations but there was no evidence of this in MGUS patients ($p=0.96$). Previous studies have demonstrated significantly higher levels of CD126 expression in myeloma aPCs than in plasma cells from MGUS and normal bone marrows [22, 23]. Our data provides further evidence that aPCs may behave biologically more like nPCs in MGUS compared to SMM and the control of IL-6 activity in aPCs in SMM may more closely approach that observed in myeloma. Since SMM is more likely to progress to plasma cell myeloma than MGUS [1, 27], control of CD126 expression in aPCs appears to change as the malignant potential increases in PCD. These results are also consistent with the theory of Rawstron et al. that neoplastic plasma cells escape stromal cell control of CD126 expression with progression to myeloma [23].

Previous studies detected low level CD126 positivity in 60% to 90% of myeloma plasma cells [22, 23]. In these studies, however, the authors experienced difficulty in detecting CD126 expression on nPCs [22, 23]. Furthermore, the authors acknowledged difficulty in separating positive from negative staining and therefore used MFI and K-S analyses to discern positivity. In the current study, we found that CD126 expression was detectable and quantifiable in both nPCs and aPCs populations in the bone marrow aspirates assessed. This may be due to the quantitative approach where the actual range of fluorescence encountered in negative cell populations was determined, allowing a quantitative determination of positive versus negative. Superior separation of positive from negative staining using the phycoerythrin conjugated M5 clone anti-CD126 antibody may also contribute to the current findings as different antibody clones and fluorochromes were utilized in previous studies.

In conclusion, CD126 expression is detected on the nPCs and clonal aPCs in MGUS, SMM, and MM and does not have diagnostic utility. A weak but positive correlation likely exists between CD126 expression in aPCs and nPCs in the precursor diseases MGUS and SMM consistent with a common regulatory influence. There are, however, some differences between these two precursor diseases. There is an increased incidence of higher CD126 expression on aPCs than on nPCs in SMM (85%) compared to MGUS (41%). Furthermore, there is evidence that general CD126 expression is higher in aPCs compared to nPCs in SMM but not in MGUS. This data suggests that normal regulation of CD126 expression decreases with progression towards disease with greater malignant potential. Further quantitative studies with a larger number of patients are needed to elucidate the possible role of bone marrow milieu versus escape from normal regulation of CD126 expression in malignant transformation of clonal plasma cells in MGUS and SMM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Normal Plasma Cells

Myeloma Cells

Figure 1. Staining of normal plasma cells (nPCs) and abnormal plasma cells (aPCs) using 3 different anti CD126 clones.

a: CD126 PE clone M5, b: CD126 PE clone M91, c: CD126 FITC clone B-R6. Top Row: nPCs in bone marrow with monoclonal gammopathy (CD19 positive, CD20 negative, CD38 bright positive, CD45 positive, CD81 bright, and CD138 positive). Bottom Row: aPCs (CD19 negative, CD38 bright positive, CD45 negative, CD81 negative, and CD138 positive). Negative control histogram to the left and plasma cell histogram to the right. It shows that the best separation between nPCs and aPCs is achieved by CD126 PE clone M5.

Figure 2. CD126 ABC values in aPCs in MGUS, SMM, and MM and in nPCs

CD126 ABC values on the aPCs in bone marrow aspirates from patients with MGUS (n=25, 24 patients-2 aPC populations in same patient), SMM (n=56), and MM (n=35). Normal: nPCs in normal bone marrow aspirates (n=4), MGUS (n=17), and SMM (n=13); n=34 total. Each value is indicated by a circle. Box-and-whisker plots indicate minimum, quartiles and maximum values for a given diagnosis. Values on right y-axis indicate quartiles for all samples combined (25+56+35+34=150).

Figure 3. Comparison of CD126 expression in aPCs and nPCs in same bone marrow specimens in SMM, MGUS and low risk MGUS

Comparison of CD126 ABC levels in normal plasma cells (x-axis) and abnormal plasma cells (y-axis) in bone marrow aspirates that yielded sufficient numbers of nPCs (100 or greater) for CD126ABC quantification. Eighty percent confidence ellipses are shown. Upper Left: Patients with sufficient nPCs (17 MGUS, 13 SMM) (weak positive correlation Spearman's r (95% CI) = 0.32 (−0.09, 0.66), $p=0.085$). Upper Right: All SMM patients. Lower Left: All MGUS patients. Lower Right: Low Risk MGUS. Grouping the data by Mayo risk stratification resulted in an increased degree of association between the CD126 ABC on the aPCs and the nPCs in MGUS Mayo risk group 0 (lower right). ABC; antibody bound per cell, aPCs; abnormal plasma cells, nPCs; normal plasma cells, PCD; plasma cell disease, MGUS; monoclonal gammopathy of undetermined significance, SMM, smoldering multiple myeloma,

Table I.

MGUS and SMM patient demographic data, risk stratification, and immunophenotype of aPC:

		MGUS (n=24)	SMM (n=56)
Age (years, mean $\pm$ SD)		57.7 $\pm$ 12.5	59.8 $\pm$ 9.2
Gender (M/F)		9 / 15	37 / 19
Mayo Risk Group		Low (0), n=11 Low-Intermediate (1), n=8 High-intermediate (2), n=5 High (3), n=0	Low (1), n=22 Intermediate (2), n=28 High (3), n=3 NC, n=3
Spanish Risk Group		NA	Low (0), n=8 Intermediate (1), n=14 High (2), n=34
aPC Immunophenotype	CD19 -	24	56
	CD45 dim or -	24	56
	CD56+	24	42
	CD81 dim or -	23	56
	Monoclonal	24	56

immunophenotype of aPC:

MGUS: monoclonal gammopathy of undetermined significance; n: number of patients; SMM: smoldering myeloma; NC: Not classifiable; NA: not applicable.

Table II.

CD126 ABC on aPCs

Diagnosis	n	Minimum	Lower Quartile	Median	Upper Quartile	Maximum
MGUS	24	175	476	747	1055	1799
SMM	56	181	420	724	984	1746
MM	35	100	381	620	1042	2020

ABC: Antibody binding per cell; aPCs: abnormal plasma cells; MGUS: monoclonal gammopathy of undetermined significance; SMM: smoldering multiple myeloma

Table III.

aPC CD126 ABC in SMM patients according to Mayo risk criteria

Mayo Groups	Risk n	Minimum	Lower Quartile	Median	Upper Quartile	Maximum
1	22	181	423	762	998	1601
2	28	318	456	768	1107	1746
3	3	197	--	250	--	371

aPCs: abnormal plasma cells; ABC: antigen binding per cell; SMM: smoldering multiple myeloma; n: number of patients. 3 patients could not be assigned to a risk group.