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The impact of several phenotypic features at diagnosis on survival of patients with myelodysplastic syndromes

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Multiparametric flow cytometry is a useful co-criterion for diagnostic confirmation of MDS in patients with peripheral cytopenias and a normal karyotype. We examined the impact on patients' survival of several phenotypic aberrancies detected by a small 4-color panel of monoclonal antibodies (MoAbs). Diagnosis of the patients (54) was made by WHO criteria using peripheral blood counts, bone marrow (BM) morphology and karyotype. Flow cytometry was performed at diagnosis, and features obtained were compared to normal BM (24). We could detect 16 alterations: 4 in granulocytic precursors, 4 in monocytes, 6 in CD34$^+$ cells, beside changes in plasmacytoid dendritic cells and basophil precursors. The total number of changes in RAEB was higher (median 8) than in cases with <5% BM blasts (median of 5). Maturation abnormalities in myeloid precursors, increase in basophils and decrease of B-cell precursors showed a similar frequency in all types of MDS, but the number of phenotypic abnormalities in CD34$^+$ cells and monocytes were more common in RAEB. In the survival analysis, WHO type ($p = 0.001$), IPSS ($p = 0.004$), degree of anemia ($p = 0.007$), a higher BM blast percentage in cytology ($p < 0.0005$), higher numbers of total CD34$^+$ cells ($p < 0.0005$), CD34$^+$/CD13$^+$ ($p = 0.0006$) and CD34$^+$/CD13$^-$ cells ($p = 0.0002$), as well as a higher total number of phenotypic abnormalities ($p = 0.004$) were associated to a shorter survival. However, in the multivariate analysis, only IPSS and the number of changes in CD34$^+$ cells (but not the overall number of abnormalities) were independent risk factors for a shorter survival. Our panel was sufficient to confirm the diagnosis of MDS and permitted to detect independent prognostic features.

Key words: *Myelodysplastic syndrom, MDS, flow cytometry, IPSS, survival, CD34$^+$ cells, B-cell precursors.*

The diagnosis of myelodysplastic syndromes (MDS) is based on the presence of persistent peripheral cytopenias, cell atypias in bone marrow (BM) hemopoietic precursors as well as cytogenetics [1, 2]. However, cytogenetic abnormalities, which are an unequivocal proof of clonality are found in only a half of the patients. So, in cases with a normal karyotype and absence of increased BM blasts or conspicuous cell atypias, it may be difficult to assure the diagnosis of MDS.

Flow cytometry has been used for the examination of normal and clonal hemopoiesis assuming that the expression of several lineage and maturation-associated antigens in normal hemopoiesis is very tightly controlled, and that, in clonal disorders, there is a disruption of this process [3–5]. Therefore, the detection of phenotypic abnormalities by flow cytometry has been considered a useful diagnostic tool in MDS.

Several recent studies have underlined its diagnostic and prognostic importance [4–16]. Although three proposals for standardization of flow cytometric analysis in MDS have been published recently [14–16] there has been some difficulty for routine flow cytometry laboratories to adopt this testing, mainly due to the large number of antibodies proposed, the absence of a single pathognomonic profile and the demanding and expert interpretation needed [4].

Yet, there is sufficient evidence that immunophenotyping in MDS is useful for the differential diagnosis between MDS and non-clonal disorders presenting peripheral cytopenias [4, 5, 9,11–13]. The number of alterations detected, increased number and presence of abnormal co-expressions of CD34+ cells and some subsets of immature cells are associated with more aggressive types of MDS, with IPSS and with outcome after BM transplantation [6, 8, 10, 17]. However, the relation between several abnormalities described and survival of patients seldom has been directly examined [18].

In previous works of our group we have shown that B-lymphoid precursors are frequently decreased and phenotypically abnormal [19]. We have also observed that the CD16/CD13 combination was more sensitive than the CD16/CD11b one to disclose myelomonocytic maturation abnormalities [11]. Besides, increase and phenotypic aberrancies in monocytes as well as the increase of activated forms was also very common. The total number of abnormal expressions of individual antigens correlated with WPSS and with survival of the patients

Table 1: Monoclonal antibodies used in the study.

Monoclonal antibodies	*Fluorochromes*	Clone	Source	*Manufacturers*
CD13	APC	WM15	Pharmingen	San Diego, CA
CD14	PE	RMO52	Beckman Coulter	Marseille, France
CD16	FITC	3G8	Pharmingen	San Diego, CA
CD19	FITC	HD37	Dako	Glostrup, Denmark
CD33	APC	WM53	Pharmingen	San Diego, CA
CD34	PE	8G12	Becton Dickinson	San Jose, CA
CD34	APC	8G12	Becton Dickinson	San Jose, CA
CD45	PerCP	2D1	Becton Dickinson	San Jose, CA
CD117	APC	YB5.B8	Pharmingen	San Diego, CA
CD123	PE	9F5	Pharmingen	San Diego, CA
HLA-DR	FITC	AB3	Dako	Glostrup, Denmark

[11, 18]. Furthermore, we have found that SSC ^{int}/CD34^{+} cells, not expressing CD19, CD117 and CD13 were associated with the maturation block occurring during the progression of the disease [17]. In 2006 we introduced in our laboratory routine a small four-color panel of monoclonal antibodies, designed to study specific abnormalities in the myelomonocytic lineage and in the CD34+ cells in the diagnostic work-up of our patients with MDS. Although the panel was small, it permitted to detect up to 16 phenotypic abnormalities.

The aim of the present study was to examine which of these abnormalities had a higher impact on survival of the patients, compared to known prognostic factors such as PB counts, percentage of BM blasts and IPSS.

Materials and methods

Patients and samples. Bone marrow (BM) aspirates from consecutive patients with newly diagnosed MDS from our Intitution (2006-2009) were prospectively examined and compared with normal BM from donors for allogeneic bone marrow transplantation. The diagnosis of MDS was based on PB counts, BM smears stained with May-Grünwald Giemsa and Perls' stains and cytogenetics. The classification of the cases was made by WHO criteria and IPSS. The overall survival of the patients was also scored. All BM samples were obtained after informed consent was given by each person (patient or donor) according to the recommendations of the local Ethics Committee.

Flow cytometry immunophenotypic analyses. The following combinations of monoclonal antibodies (MoAbs) were used: HLA-DR/CD14/CD45/CD33; CD16/CD34/CD45/CD13; CD19/CD34/CD45/CD117 and HLA-DR/CD123/CD45/CD34. The specificity and source of each reagent are listed in Table 1. Immunofluorescence staining was made using a standardized direct lyse-and-wash technique according to the ELN recommendations [16] within 24 hours after sample collection. Immediately after staining, samples were acquired in a FACS Calibur flow cytometer (Becton Dickinson – BD Biosciences) using the CellQuest software (BD Biosciences). Instrument quality control and spectral compensation were performed daily. Information on a minimum of 50000 nucleated BM cells was acquired for each sample. Data analysis was made using the PAINT-A-GATE software (BD Biosciences). The mean fluorescence intensity of each antigen and the mean value for SSC was determined for each cell population.

The several BM cell compartments (progenitor cells, neutrophils, monocytes, lymphocytes) were identified using the CD45 expression and the sideward light scatter (SSC) (Fig 1A). Myeloid precursors, monocytes, progenitor cells, as well as basophils and plasmacytoid dendritic cells were studied.

Maturation pattern of neutrophils was analyzed by their expression of CD13, CD16, CD33 and HLA-DR (Figures 1B and C). Granularity (SSC) of immature myeloid populations was examined.

Monocytes were analyzed in the HLA-DR/CD14 plot. The combination CD16/CD34/CD45/CD33 was used to quantify the CD16^{+} monocytes and to evaluate aberrant expression of CD34.

CD34^{+} cells were separated in the SSC/CD34 dot plot as previously described [17]. The B-cell precursors (CD34^{+}/CD19^{+}) were analyzed as percentage of total nucleated cells and its proportion within the CD34^{+} cells. The precursors of basophils and plasmacytoid dendritic cells were identified in the combination HLA-DR/CD123 (Figure 2).

SSC of the granulocytic population and antigen expressions were considered abnormal, if they were above or below the confidence interval (10%-90%) of the values obtained for the normal cases.

All the features were compared with the values found in normal BM. The cases of MDS were grouped into MDS with <5% BM blasts and MDS with >5% BM blasts (RAEB).

The panel used allowed the detection of 16 antigenic abnormalities:

Granulocytic population (4 abnormalities): hypogranularity, changes of the maturation pattern in the CD13/CD16 and CD33/HLA-DR combinations (increase or decrease in antigen expression, lack of maturation or asynchronous expression),

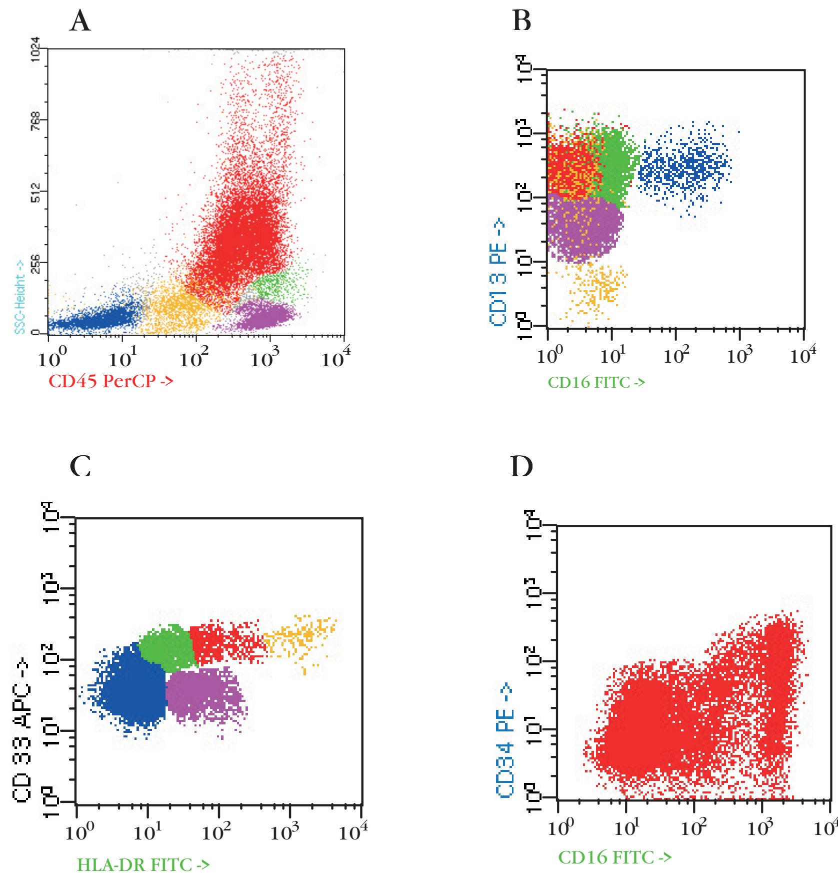

Figure 1: Phenotypic alterations in myeloid precursors in a case of RAEB: A) gating strategy for the separation of several bone marrow cell populations in the SSC/CD45. The myeloid population shows a decreased SSC. B: abnormal maturation pattern in the myeloid series seen in the CD16/CD13 plot. C) a subset of myelocytes showing a decreased expression of CD33 (violet). E) aberrant co-expression of CD34 in neutrophil precursors.

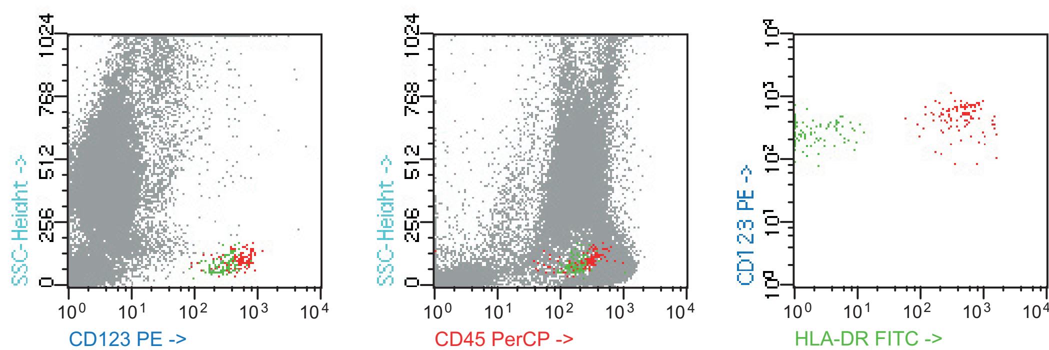

Figure 2: Dot plots showing the strategy for analysis of lymphoid dendritic cells (red) and basophil precursors (green). Normal BM

and aberrant expression of CD34 in more than 10% of the mature granulocytic population.

Monocytes (4 abnormalities): increase in number, increase in CD16$^+$ monocytes, change of maturation pattern (increase or decrease of antigen expression or asynchronous expression of CD33 and/or HLA-DR) and aberrant expression of CD34 in >10% of the monocytes.

Blast population (6 abnormalities): increase of the proportion of cells in the blast gate, increase of CD34$^+$ cells, decrease of B precursor cells (CD34$^+$/CD19$^+$), increase of myeloblasts

CD13+/CD117+/CD34+, increase of non-lymphoid precursors CD34+/CD117- or CD34+/CD13-.

Minor populations (2 abnormalities): increase or decrease of CD123+/HLA-DR+ precursors (plasmacytoid dendritic cells), increase or decrease of CD123+/HLA-DR- precursors (basophils).

Statistical analysis. First, we obtained the mean, standard deviation, median, minimum and maximum and percentiles of all variables in normal BM and in the MDS cases. Percentiles 10% and 90% were used to determine the confidence interval of fluorescence intensities. Values outside the confidence interval were considered abnormal. The differences between normal BM and both groups of MDS for all the variables studied were assessed by the Kruskal-Wallis' test. The correlations among them were studied by the Spearman rank order correlation test. Values were considered significant when $p < 0.05$.

The impact on patients' overall survival of PB counts, percentage of BM blasts in cytology, WHO classification, IPSS and the flow cytometric features were examined in the Cox proportional hazard model. In the multivariate model, only variables with a $p<0.01$ (forward conditional step-wise selection) were analyzed. In order to test the internal stability of the model proposed, it was tested by bootstrap resampling, which consists of creating new data sets of equal size by random sampling of the original data with replacement. In each new bootstrap sample, a patient may be represented once, multiple times or not at all. A new linear regression model (with the same conditions as in the original data set) was calculated for each of these new bootstrap data sets. This procedure is able to test the stability of a mathematical model, detects the most important variables and, moreover, permits to calculate confidence intervals [20, 21]. The Winstat and SPSS 10.0 softwares were used.

Results

We analyzed 24 BMs of normal donors (median age 36 years; 21-63; 12 males and 12 females) and 54 patients. Mean age of the patients was 62 years (23-93); 23 females and 31 males. According to the WHO criteria, 2 patients had refractory anemia (RA), 2 5q- syndrome, 25 refractory cytopenias with multilineage dysplasia (RCMD), 8 RCDM with ring sideroblasts (RCMD-RS), 7 RAEB-I and 10 had RAEB-II. So there were 37 cases with BM blasts <5%, and 17 had RAEB. According to the International Prognostic Scoring System (IPSS), 23 cases were classified as low risk, 20 as intermediate-1, 7 as intermediate-2 and 3 were high risk. In 1 case no mitoses could be obtained for karyotyping.

The distribution of the phenotypic changes among the patients is shown on Table 2. All cases of MDS presented at least 2 alterations. The mean number of changes in patients with BM blasts <5% was 5.0 (2-11) and in RAEB it was 8.0 (4 – 12), ($p = 0.001$). The total number of phenotypic changes was correlated to the percentage of BM blasts in cytology ($r = 0.37$; $p = 0.001$) and the total number of CD34+ cells ($r = 0.39$; $p = 0.002$).

Table 2: Frequency of the several abnormalities detected comparing cases with BM blast <5% and RAEB.

Alterations	BM blasts <5%	BM blasts >5%
	37 cases	17 cases
Granulocytic precursors		
Low SSC	17 (46%)	8 (47%)
Abnormal pattern in CD13/CD16	33 (89%)	13 (76%)
abnormal pattern in CD33/HLA-DR	25 (67%)	12 (70%)
Aberrant expression of CD34	7 (19%)	7 (41%)
Monocytes		
Increase in number	14 (38%)	11 (64%)
Increase in CD16+ monocytes	15 (40%)	6 (35%)
Alteration of CD33/HLA-DR	14 (38%)	9 (53%)
Aberrant expression of CD34	8 (21%)	6 (35%)
Cells in the blast gate		
Increase of CD34+ cells	11 (29%)	14 (82%)
Increase of CD34+/CD13+ cells	10 (27%)	8 (47%)
Increase of CD34+/CD13- cells	3 (8%)	9 (53%)
Increase of CD34+/CD117+ cells	5 (13%)	10 (59%)
Increase of CD34+/CD117- cells	2 (5%)	2 (12%)
Absence / decrease of CD34+/CD19+ among total nucleated cells	16/11 27 (73%)	7/3 10 (59%)
Minor populations		
Decrease of HLA-DR+/CD123+ cells	15 (40%)	3 (17%)
Increase of HLA-DR-/CD123+ cells	10 (27%)	4 (23%)

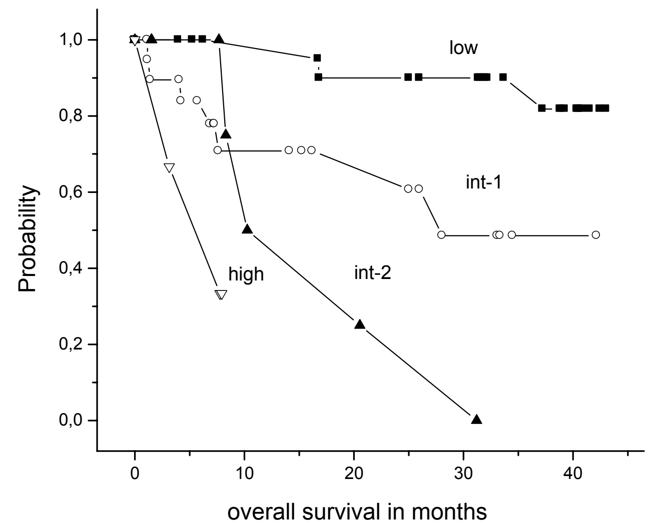

Fig 3 Kaplan-Meier estimates for overall survival of the patients according to IPSS. The four categories of this index were able to separate patients with a significantly different survival (p = 0.002)

Maturation abnormalities in myeloid precursors were very common, regardless of the WHO type and the risk group of MDS (Table 2). Increase in number followed by abnormal antigen expressions was the most common change in mono-

Table 3: Distribution of cells in the blast gate.

	Normal BM	BM blasts <5%	RAEB	P value
Total $CD34^+$ cells	0.9 (0.6 – 2.0)	1.2 (0.2 – 3.4)	7.8 (0.6 -16.0)	< 0.0005
% $CD34^+/CD13^+$ cells	0.3 (0.1 – 0.6)	0.4 (0.06 – 2.0)	3.3 (0.2 – 13.4)	0.005
% $CD34^+/CD117^+$	0.4 (0 – 1.0)	0.6 (0.04 – 3.0)	4.0 (0.4 – 13.6)	< 0.0005
% $CD34^+/CD117^-$	0.2 (0.06 – 0.8)	0.09 (0.02 – 1.5)	0.3 (0.07 – 3.0)	0.016
% $CD34^+/CD13^-$ cells	0.2 (0 – 0.6)	0.15 (0 – 0.6)	1.7 (0.13 - 7.7)	< 0.0005
% $CD34^+/CD19^+$ / total nucleated cells	0.1 (0.04 – 0.5)	0.03 (0 – 0.3)	0.03 (0 - 0.3)	< 0.0005
% $CD34^+/CD19^+$ / total $CD34^+$ cells	20.4 (6.0-31.0)	1.5 (0-37.0)	0.6 (0 – 6.5)	< 0.0005

cytes, which were also equally prevalent in all WHO types. Some alterations were more common in RAEB (Table 3) such as increase of $CD34^+/CD13^+$ cells and $CD34^+/CD117^+$ cells, $CD34^+/CD13^-$ cells and that of $CD34^+/CD117^-$ cells as well as the total number of changes found in $CD34^+$ cells ($p < 0.0005$). Aberrant expression of CD34 in mature granulocytes and monocytes was also more frequent in RAEB.

Some alterations were associated to the IPSS score, such as the number of total $CD34^+$ cells ($r = 0.30$; $p = 0.01$), numbers of $CD34^+/CD13^+$ cells ($r = 0.26$; $p = 0.04$), of $CD34^+/CD13^-$ cells ($r = 0.38$; $p = 0.004$), and the total number of changes in the monocytic series ($r = 0.35$; $p = 0.005$).

Surprisingly, increase or decrease in number of plasmacytoid dendritic cells and basophil precursors was also very common. In normal BM, mean number of dendritic cell precursors was 0.23 (0.08 – 0.37) and that of basophils was 0.14 (0.001 – 0.40). The first ones were decreased in 31% and increased in 19% of the cases, and the last were increased in 30% of the cases. The number of plasmacytoid dendritic cell precursors presented a positive correlation with the percentage of BM blasts in cytology ($r = 0.31$; $p = 0.02$), the number of total $CD34^+$ cells ($r = 0.30$; $p = 0.02$) and that of $CD34^+/CD13^-$ cells ($r = 0.43$; $p = 0.006$). Basophils only showed a positive correlation with the total number of abnormalities found per case ($r = 0.31$; $p = 0.02$).

B-cell precursors were present in all normal BMs. However, decrease or even absence of hematogones was very frequent in MDS. They were absent in 23/37 cases (62%) with BM blasts <5% and in 10/17 cases (58%) of RAEB. The number of $CD34^+/CD19^+$ cells among all nucleated cells showed no correlation with PB count values or with other phenotypic abnormalities. However, their percentage among all $CD34^+$ cells decreased as $CD34^+/CD13^+$ cells increased ($r = -0.33$; $p = 0.01$).

Survival analysis: In the univariate Cox analysis, the following variables were related to a shorter overall survival: WHO type ($p = 0.001$), IPSS ($p = 0.004$), a lower hemoglobin level ($p = 0.007$), a higher BM blast percentage in cytology ($p < 0.0005$), higher numbers of total $CD34^+$ cells ($p < 0.0005$), $CD34^+/CD13^+$ ($p = 0.0006$) and $CD34^+/CD13^-$ cells ($p = 0.0002$), as well as a higher number of abnormalities of monocytes ($p = 0.03$), that of $CD34^+$ cells ($p = 0.002$) and total number of phenotypic abnormalities ($p = 0.004$). Age, PB neutrophils and platelets as well as total number of granulocytic abnormalities had no influence on survival. The number of $CD34^+/CD19^+$ cells among all nucleated cells had a marginal significance ($p = 0.05$).

In the multivariate analysis, comparing the impact of IPSS and the number of each group of phenotypic abnormalities (monocytic, $CD34^+$ cells and the total number) on patients' survival, only IPSS ($B = +1.01$; $p = 0.001$) and the number of abnormalities in $CD34^+$ cells ($B = +0.42$; $p = 0.016$) remained in the model as independent variables for a shorter survival. After bootstrap resampling, IPSS entered in 97%, "number of abnormalities in $CD34^+$ cells" in 71%, "total number of abnormalities" in 18% and "number of monocyte abnormalities" in 11% of the models.

In a second model comparing the survival impact of each of the abnormalities of $CD34^+$ cells examined only the number of $CD34^+/CD13^-$ cells remained in the model as an independent risk factor ($B = +0.64$; $p = 0.0001$).

Discussion

Several recent works have shown the utility of multiparametric flow cytometry for confirmation of the diagnosis of MDS in patients with PB cytopenias with few BM morphologic cell atypias and a normal karyotype [5–16]. In the three recently published reports of working conferences [14–16], although large panels of monoclonal antibodies have been recommended, only some features have been pointed out as more typical and frequent, such as maturation abnormalities in the myelomonocytic cell line, demonstrated especially in the CD16/CD13 and CD16/CD11b combinations, aberrant co-expressions in CD34+ cells, and decrease of B-cell precursors.

In the present study we used a rather simple panel of 4-color monoclonal antibody combinations aiming to specifically examine alterations in the myelomonocytic series and $CD34^+$ cells. This evaluation was applied prospectively in consecutive newly diagnosed patients with MDS from our Institution. We included quantification of basophil and plasmacytic dendritic cell precursors, as they present similar values of SSC and CD45 as myeloblasts, and must be excluded from this cell subset [5]. Using this panel, we were able to detect until 16 alterations. At least 2 abnormalities could be seen in every case examined, thus fulfilling the WHO criteria for this analysis [1].

As expected, the patients with RAEB had a higher number of total phenotypic changes as compared to the patients with

a low number of BM blasts. However, there was an overlap between the groups. Examining the different kinds of abnormalities found, some of them were very frequent, regardless of the type of MDS, such as maturation abnormalities in the granulocytic precursors, decrease in B-cell precursors and changes in number of basophils and dendritic cells. But, increase in number and changes in distribution of the subsets of $CD34^+$ cells as well as expression of CD34 on maturing neutrophils and monocytes were associated to RAEB. Accordingly, these changes were associated to a higher IPSS. This was the rationale for giving a higher weight in the flow scores to abnormalities in $CD34^+$ cells than to myelomonocytic maturation abnormalities in former studies [5, 6]. Among these changes in $CD34^+$ cell subsets, in a previous study [17] we found that especially the increase in $CD34^+/CD13^-$ cells was associated with more aggressive types of MDS. In the present study, we could demonstrate that this feature was also an independent risk factor for a shorter survival. Therefore, this phenotypic variable could be used as predictive of disease progression or indication of BM transplantation in serial measures.

Decrease in hematogones was frequent in all types of MDS. Much emphasis has been given on this feature in the differential diagnosis between MDS, ICUS and cytopenias with normal/reactive BM [5, 9, 12, 22, 23]. Satoh et al have proposed to quantify them as the number of B-cell precursors among all $CD34^+$ cells [12]. In the present study, as expected, this variable was dependent on the number of total $CD34^+$ cells, because, with the increase of the total $CD34^+$ cells due to a rise in myeloblasts, the percentage of hematogones, that was already low, would further decrease. However, when calculated in relation to the total nucleated cells, this feature was equally found in all types of MDS, regardless of the number of BM blasts, showing that this is an early event in the pathogenesis of MDS as has also been pointed out recently by Maftoun-Banankhah et al [23], and also described by our group [18].

Among our patients, several phenotypic variables showed an impact on overall survival, including the total percentage of $CD34^+$ cells, $CD34^+/CD13^+$ and $CD34^+/CD13^-$ cells as well as the total number of phenotypic abnormalities found, together with well-known prognostic variables such as BM blast percentage in cytology, IPSS and degree of anemia. However, in the multivariate analysis comparing well-established prognostic variables with the several types of phenotypic features, only IPSS and the number of abnormalities in the $CD34^+$ cells were independent factors of a bad prognosis. In the present study we did not explore the aberrant co-expressions in hematopoietic precursor cells which have been observed in more aggressive cases [6, 16]. Therefore, the addition of an antibody combination such as CD56/CD7/CD45/CD34 in order to explore these aberrrations could further increase the prognostic value of $CD34^+$ cell abnormalities.

So we can conclude that immunophenotyping in MDS is not only an adjuvant criterion for diagnosis. For this purpose, the finding of maturation abnormalities in the myelomonocytic series and decrease in B-cell precursors are very important.

On the other hand, we were able to show that among phenotypic changes, those detected in $CD34^+$ cells are the most important to predict a shorter survival of the patients. All these changes can be detected using small panels of four-color combinations of monoclonal antibodies, what makes flow cytometric analysis of BM of patients with PB cytopenias easier to perform in laboratory routine.

Multiparametric flow cytometry has been widely used in the diagnosis of several hematologic malignancies [26]. It has been increasingly used also in the diagnosis of MDS, and may provide useful prognostic markers in these clonal disorders, together with molecular markers of proliferation and apoptosis [27, 28].

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