Multicenter validation of a reproducible flow cytometric score for the diagnosis of low-grade myelodysplastic syndromes: results of a European LeukemiaNET study

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ABSTRACT

Background

The current World Health Organization classification of myelodysplastic syndromes is based on morphological evaluation of bone marrow dysplasia. In clinical practice, the reproducibility of the recognition of dysplasia is usually poor especially in cases that lack specific markers such as ring sideroblasts and clonal cytogenetic abnormalities.

Design and Methods

We aimed to develop and validate a flow cytometric score for the diagnosis of myelodysplastic syndrome. Four reproducible parameters were analyzed: $CD34^+$ myeloblast-related and B-progenitor-related cluster size (defined by CD45 expression and side scatter characteristics on $CD34^+$ marrow cells), myeloblast CD45 expression and granulocyte side scatter value. The study comprised a "learning cohort" (n=538) to define the score and a "validation cohort" (n=259) to confirm its diagnostic value.

Results

With respect to non-clonal cytopenias, patients with myelodysplastic syndrome had increased myeloblast-related cluster size, decreased B-progenitor-related cluster size, aberrant CD45 expression and reduced granulocyte side scatter ($P<0.001$). To define the flow cytometric score, these four parameters were combined in a regression model and the weight for each variable was estimated based on coefficients from that model. In the learning cohort a correct diagnosis of myelodysplastic syndrome was formulated in 198/281 cases (sensitivity 70%), while 18 false-positive results were noted among 257 controls (specificity 93%). Sixty-five percent of patients without specific markers of dysplasia (ring sideroblasts and clonal cytogenetic abnormalities) were correctly classified. A high value of the flow cytometric score was associated with multilineage dysplasia ($P=0.001$), transfusion dependency ($P=0.02$), and poor-risk cytogenetics ($P=0.04$). The sensitivity and specificity in the validation cohort (69% and 92%, respectively) were comparable to those in the learning cohort. The likelihood ratio of the flow cytometric score was 10.

Conclusions

A flow cytometric score may help to establish the diagnosis of myelodysplastic syndrome, especially when morphology and cytogenetics are indeterminate.

Key words: myelodysplastic syndrome, diagnosis, flow cytometry.

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Introduction

The pathological hallmark of myelodysplastic syndromes (MDS) is marrow dysplasia, which represents the basis of the World Health Organization (WHO) classification of these disorders.[1] This classification provides clinicians with a very useful tool for defining the different subtypes of MDS and determining individual prognosis.[2,3] The WHO proposal has, however, raised some concern regarding minimal diagnostic criteria. The diagnosis of MDS is straightforward if clearly objective abnormalities, i.e., an increase in blasts and/or ring sideroblasts and/or clonal cytogenetic abnormalities, are present. However, the diagnosis is challenging in low-grade MDS that lack these specific diagnostic markers.[4,5]

Flow cytometry immunophenotyping is a reliable method for quantitative and qualitative evaluation of hematopoietic cells and plays an increasingly crucial role in the diagnosis and management of hematologic malignancies.[1,6] Several studies have evaluated flow cytometry as a potential diagnostic tool for MDS.[7-11] In order to become clinically applicable, flow cytometry analysis should be based on parameters with sufficient specificity and sensitivity, data should be reproducible by different operators, and the results should be easily understood by clinicians.[6,12,13] To date, no single immunophenotypic marker has proven able to discriminate accurately between MDS and other pathological conditions,[13] no consensus exists on which diagnostic parameters are the most appropriate,[12] and published protocols are mainly based on a qualitative analysis of cytometric variables[7,8,11] thus limiting widespread clinical implementation.

The CD34$^+$ cell compartment is peculiarly perturbed in MDS and CD34-related parameters are, therefore, good candidates for the identification of diagnostic markers for these syndromes.[14-16] Consistent immunophenotypic aberrations reported in the CD34$^+$ cell compartment in MDS are: an increase of the myeloid-committed CD34$^+$ population,[10,15,17] a decrease of B-cell progenitors,[15,18] co-expression of stem cell- and late stage-myeloid antigens, expression of lymphoid antigens[7,8,11] and abnormal CD45 expression.[15] We previously reported that data for three of these parameters (percentage of myeloblasts, percentage of B-cell progenitors, and myeloblast CD45 expression) together with the evaluation of side scatter (SSC) on granulocytes are reproducible in many laboratories when measured by methods ensuring little inter-operator variability,[19,20] and when combined are able to differentiate correctly patients with MDS from patients with non-clonal cytopenia.[20]

To be implementable in clinical practice, an extensive validation of this approach is required. We, therefore, conducted a multicenter study in a large population of patients with the aim of defining the reference ranges of these parameters and of developing a reproducible flow cytometric score (FCM-score) that can be used in the diagnostic work-up of patients suspected of having MDS.

Design and Methods

Patients

These investigations were approved by the local Ethics Committees and the procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000.

This retrospective study enrolled 797 patients from six institutions who underwent bone marrow examination because of peripheral blood cytopenia: these patients comprised 417 with low-grade MDS (defined as MDS patients with <5% of bone marrow blasts) and 380 pathological controls with non-clonal cytopenias.

The clinical procedures were performed in accordance with the "European LeukemiaNET proposal for standardized diagnostic and prognostic procedures in adult MDS".[21] For each case with suspected MDS, the following differential diagnoses were considered: B12/folate deficiency, chronic liver disease, excessive alcohol intake, exposure to heavy metals, anemia of thyroid dysfunction, anemia of chronic disorders, anemia associated with renal failure, anemia associated with iron deficiency, idiopathic hypoplasia/aplasia, drug-induced cytopenias, cytopenia associated with marrow infiltration, autoimmune cytopenia, common variable immunodeficiency, human immunodeficiency virus infection; paroxysmal nocturnal hemoglobinuria, and other hematopoietic stem cell disorders.

Bone marrow was aspirated from all patients enrolled in the study and subjected to immunophenotypic and cytogenetic analysis; when appropriate a bone marrow biopsy was also performed. The diagnosis of MDS was based on the 2008 WHO criteria.[1] Accordingly, morphological definition of marrow dysplasia required the presence, in the appropriate clinical setting, of at least 10% of the cells of at least one myeloid bone marrow lineage (erythroid, granulocytic, megakaryocytic) showing unequivocal dysplastic features. In cases suspected to be refractory cytopenia with unilineage dysplasia, if there was no evidence of clonality by genetic studies, the patients were observed clinically for 6 months before a diagnosis of MDS was made.[1] A category called "MDS without specific markers" was defined, which included subjects with refractory cytopenia with unilineage or multilineage dysplasia without ring sideroblasts and without chromosomal abnormalities;[20] the diagnosis of this condition is particularly difficult in clinical practice.[1,4,22] The WHO-classification based Prognostic Scoring System (WPSS) was employed to define prognosis.[23]

The study population was divided into two groups (by a 2:1 random splitting balanced by age, sex, WHO subgroups and contribution from each institution), including a "learning cohort" whose analysis was aimed at defining the FCM-score for the diagnosis of MDS, and a "validation cohort" in which the diagnostic value of the score was to be confirmed. The clinical characteristics of the study population are reported in Table 1.

Flow cytometry studies on bone marrow cells

Methods for processing and handling samples were in accordance with the "European LeukemiaNET recommendation for standardization of cytometric procedures in MDS".[12] Bone marrow specimens were collected into a heparinized syringe and stained using a whole-blood lysis technique (ammonium chloride) and direct conjugated monoclonal antibodies. All four parameters presented in this paper were analyzed using this single sample of cells stained with the CD34/CD45 antibody combination. Antibody staining was performed as follows: 5–8×10^5 nucleated cells were placed into each tube and stained with phycoerythrin-conjugated anti-CD34 and peridin chlorophyll-conjugated anti-CD45 (purchased from Becton Dickinson, San José, CA, USA or Beckman Coulter, Fullerton, CA, USA). Data were acquired using a FACSCalibur cytometer (Becton Dickinson) in three laboratories, a FACSCanto cyometer (Becton Dickinson) in two laboratories and an Epics XL flow cytometer (Beckman Coulter) in one laboratory. Daily instrument quality controls, including fluorescence standardization, linearity assessment, and

spectral compensation, were performed to ensure identical operation from day to day.

At least 100,000 cell events were acquired. Investigators (who were blinded to the patients' clinical and laboratory features) analyzed immunophenotypic data with CellQuest software, Diva software (Becton Dickinson) or EXPO32 ADC software (Beckman Coulter).

The analytical methods involved have been previously extensively described.[15,19,20] Briefly, on the forward scatter (FSC)-*versus*-side scatter (SSC) display, we defined "all nucleated cells" (P1, Figure 1A), and cells with relatively low SSC were gated (P2) and then plotted on a CD45 *versus* CD34 display (Figure 1B). Next, CD34$^+$ cells with intermediate CD45 expression were gated (P3) and then plotted on a CD45 *versus* SSC display (Figure 1C). In the CD45/SSC dot plot, we identified the cell population consisting predominantly of B-progenitor cells which formed an easily recognizable cluster that had the lowest SSC and relatively low CD45 expression (P5, called "B-progenitor-related cluster" in this paper).[15,17,19] Other CD34$^+$ cells (P4) showing more SSC and a wider distribution of CD45 expression consisted predominantly of myeloblasts (called "myeloblast-related cluster" in this paper).[15,17,19] The phenotypes of CD34$^+$ cells in both myeloblast-related and B-progenitor-related clusters (Figure 1C1) was determined: in all 30 cases analyzed, CD34$^+$ cells in the myeloblast-related cluster expressed myeloid antigens (such as CD33, CD13, CD117 and CD15) but not CD10, while CD34$^+$ cells in the B-progenitor-related cluster expressed CD10 but not myeloid antigens (Figure 1C2 and 1C3).

Meanwhile, all cells were plotted on a CD45 *versus* SSC display (Figure 1D) and lymphocytes (P6, defined as CD45^{high}SSC^{low} cells) and granulocytic cells other than myeloblasts (P7, defined as CD45^{high}SSC^{high} cells, and called granulocytic cells in this paper) were gated. SSC peak channel values (SSC channel number where the maximum number of cells occurs) and CD45 mean fluorescence intensity (MFI) of both fractions were computed using the software (Figure 1E and 1F, respectively).

The four analyzed parameters are: (i) percentage of cells in the myeloblast-related cluster in all nucleated cells (called "myeloblast-related cluster size"); (ii) percentage of cells in B-progenitor-related clusters in all CD34$^+$ cells (called "B-progenitor-related cluster size"); (iii) the lymphocyte to myeloblast CD45 ratio (Ly/Mbl CD45 ratio, defined as the MFI of CD45 on lymphocytes ÷ MFI of CD45 on CD34$^+$ cells in myeloblast-related clusters) and (iv) the granulocyte to lymphocyte side scatter peak channel ratio (Gra/Ly SSC ratio, defined as the SSC channel number where the maximum number of granulocytes occurs ÷ SSC channel number where the maximum number of lymphocytes occurs).

The reliability of the adopted gating strategy was previously reported,[15,19,20] and was verified in addition in 30 patients enrolled in this study (*Online Supplementary File S1*).

Table 1. Clinical characteristics of the learning and validation cohort.

	Learning cohort		Validation cohort		P
Low-risk MDS	**281**		**136**		
Age, years (range)	69	(18-95)	66	(29-95)	ns
Sex (female/male)	118/163		58/78		ns
Diagnosis (WHO, 2008)					
RCUD	55	20%	26	19%	ns
RARS	30	11%	20	14%	
RCMD/RS	178	63%	83	61%	
MDS with del5q	14	5%	5	4%	
MDS-U	4	1%	2	1%	
MDS with specific markers*	166	59%	73	54%	
MDS without specific markers	115	41%	63	46%	
Transfusion dependency	104/212	49%	55/108	51%	ns
Karyotype	251/281	89%	122/136	90%	ns
good risk	162	64%	73	60%	ns
intermediate risk	59	24%	41	33%	
poor risk	30	12%	8	7%	
WPSS	202/281	72%	102/136	75%	ns
very low, low, intermediate, high, very high	36,72,51,43,0		18,31,29,24,0		ns
Controls	**257**		**123**		**P**
Age, years (range)	62	(18-89)	62	(23-79)	ns
Sex (female/male)	119/138		58/65		ns
Diagnosis					
Idiopathic or iatrogenic hypoplasia**	21	8%	15	12%	ns
Anemia associated with chronic disease	57	22%	22	18%	
Anemia associated with iron and/or B12/folate deficiency	31	12%	8	7%	
Anemia associated with renal failure	6	2%	7	6%	
Hemolytic anemia	10	4%	5	4%	
Cytopenia associated with marrow infiltration	14	5%	14	11%	
Cytopenia in transplant recipients	3	1%	-	-	
Infective cytopenia	6	2%	5	4%	
Immune cytopenia	65	26%	27	22%	
Idiopathic thrombocytopenic purpura	45	18%	20	16%	

**Definition of specific markers of dysplasia included the presence of ring sideroblasts and/or chromosomal abnormalities. **Including chemotherapy-induced cytopenia. RCUD: refractory anemia with unilineage dysplasia; RARS: refractory anemia with ring sideroblasts; RCMD-RS: refractory anemia with multilineage dysplasia with or without ring sideroblasts; MDS-U: MDS unclassified.*

Statistical analysis

An analysis of variance was performed to test each variable for differences between MDS and controls and among different subgroups of patients. An association between ordinal and continuous variables was tested by linear regression models. A general linear model was applied to estimate the effect of both continuous and categorical variables in a multivariate setting. For each cytometric variable, the receiver-operator characteristic (ROC) curve method was adopted to choose a cut-off value to discriminate between MDS and controls. In the case of CD45 ratio, which has a Gaussian distribution and has been reported to be both aberrantly decreased and increased in MDS,[19] a reference range of the cytometric parameter in controls was calculated using the mean ± two standard deviations (SD). The weight of each parameter in the diagnosis of MDS was tested by a multivariable general logistic regression model. In order to estimate the diagnostic power of the cytometric score, the following parameters were calculated: specificity, sensitivity, positive predictive value [PPV, defined as the number of true positives/(number of true positives + number of false positives)] and negative predictive value [NPV, defined as the number of true negatives/(number of true negatives + number of false negatives)]. To define the utility of the clinical implementation of the FCM-score, the likelihood ratio, defined as sensitivity/(1 – specificity), was employed.[24] All analyses were performed using Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA).

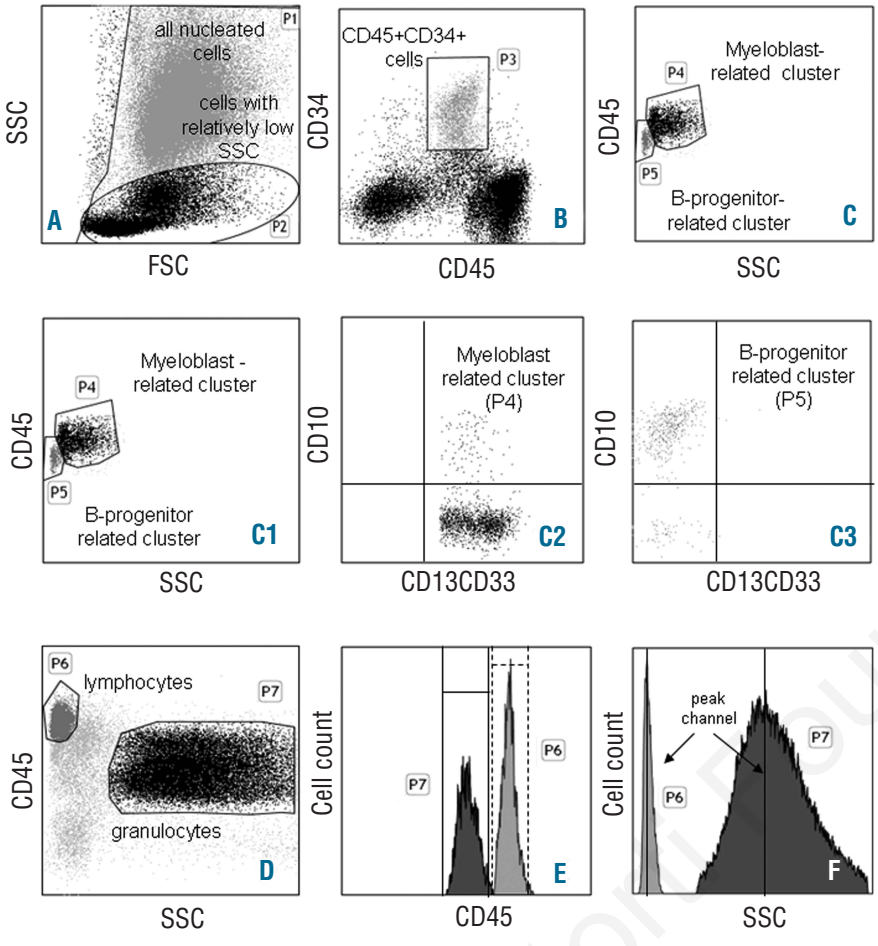

Figure 1. Analysis of four parameters from a sample of cells stained with CD34 and CD45 antibodies. (A) All nucleated cells (P1) and cells with relatively low SSC (R2). (B) Cells in R2 in panel A were displayed on a CD34-*versus*-CD45 plot, and CD34$^+$ cells with intermediate CD45 expression were gated (P3). (C) Cells in the P3 gate were plotted on a CD45-*versus*-SSC display. In the CD45/SSC dot plot, we identified a cell population consisting predominantly of CD34$^+$ B-cell progenitor cells which formed an easily recognizable cluster that had the lowest SSC and relatively low CD45 expression (P5, called B-progenitor-related cluster).[15,17,19] Other CD34$^+$ cells (P4) showing more SSC and a wider distribution of CD45 expression consisted predominantly of myeloblasts (called the myeloblast-related cluster).[15,17,19] (C1-C3) Phenotype of CD34$^+$ cells in the myeloblast-related and B-progenitor-related clusters. CD34$^+$ cells in the myeloblast-related cluster (P4) expressed myeloid antigens but not CD10, while CD34$^+$ cells in the B-progenitor-related cluster (P5) expressed CD10 but not myeloid antigens. (D) All nucleated cells were plotted on a CD45-*versus*-SSC display and lymphocytes (P6, defined as CD45^{high}SSC^{low} cells) and granulocytic cells other than myeloblasts (P7, defined as CD45^{high}SSC^{high} cells, and called granulocytic cells in this paper) were gated. (E) CD45 expression of lymphocytes (P6 gate) and CD34$^+$ cells in the myeloblast-related cluster (P4 gate). Mean fluorescence intensity (MFI) of CD45 of both fractions was computed. (F) SSC of lymphocytes (P6 gate) and granulocytic cells (P7 gate). SSC peak channel values (SSC channel number where the maximum number of cells occurs) of both fractions were computed using the software.

Results

Flow cytometry analysis of bone marrow cells

The learning cohort consisted of 281 patients with a definitive diagnosis of MDS and 257 controls with non-clonal cytopenia (Table 1). No significant effect of demographic factors on the expression of cytometric variables in either the MDS or control patients was noted (*data not shown*).

Myeloblast-related cluster size in bone marrow samples from MDS patients was significantly increased compared to the size in samples from controls (median 1.57, range 0.03-4.49 *versus* 0.9, range 0.01-3.11, respectively; $P<0.001$) (Table 2 and *Online Supplementary Figure S1*). Among MDS patients, myeloblast-related cluster size was significantly increased in subjects with multilineage *versus* unilineage dysplasia (median 1.95, range 0.8-4.49 *versus* 1.02, range 0.03-4.41; $P<0.001$), in transfusion-dependent *versus* transfusion-independent patients (median 1.87, range 0.9-4.49 *versus* 1.19, range 0.03-4.3; $P=0.01$) and in patients with poor risk *versus* good and intermediate risk cytogenetics (median 2, range 0.03-4.3, *versus* 1.24, range 0.3-4.41; $P=0.004$). A positive correlation was found between myeloblast-related cluster size and WPSS risk (Spearman's rank correlation: r=0.48, $P=0.01$).

MDS patients had a significantly decreased B-progenitor-related cluster size with respect to controls (median 1.6, range 0-74.1 *versus* 10.7, range 0-54.1; $P<0.001$) (Table 2 and *Online Supplementary Figure S1*). Focusing on the population of patients with MDS, B-progenitor-related cluster size was significantly decreased in subjects with multilineage *versus* unilineage dysplasia (median 1, range 0-74.1 *versus* 2.55, range 0-62.4; $P<0.001$) and in transfusion-dependent *versus* transfusion-independent patients (median 1, range 0-74.1 *versus* 2.01, range 0-37.5; $P=0.04$).

Mean Ly/Mbl CD45 ratio was not significantly different between MDS patients and controls (Table 2). However, it was noted that this parameter was distributed differently between the two groups (non-Gaussian *versus* Gaussian distribution), and a considerable proportion of MDS patients (81 subjects, 29%) had a CD45 ratio value outside the range of the mean ± 2 SD in controls. In detail, 36 patients had a Ly/Mbl CD45 ratio below the lower limit of the reference range in controls (median value 3.3, range 1.8-4), while 45 subjects had a Ly/Mbl CD45 ratio above the upper limit of the reference range (median value 8.57, range 7.5-19.2)

Finally, the Gra/Ly SSC ratio was found to be reduced in MDS patients compared to in controls (median 6.46, range 2.05-18.5 *versus* 7.98, range 4.40-15.96; $P<0.001$) (Table 2 and *Online Supplementary Figure S1*). Considering patients affected by MDS, the Gra/Ly SCC ratio was significantly

Table 2. Values of cytometric variables (expressed as median and ranges) in patients in the learning cohort.

Control patients

Center	Patients (n)	Myeloblast-related cluster size (%)	B-progenitor-related cluster size (%)	Ly/Mbl CD45 ratio	Gra/Ly SSC ratio
1	34	1.05 (0.2-2.8)	9.5 (0-44.5)	6.45 (3.5-9.62)	7.9 (6.4-15.8)
2	36	0.87 (0.5-2.57)	6.9 (0-44.8)	6.6 (3.77-8.72)	8.35 (5.06-19.6)
3	34	1.09 (0.01-3.11)	8.9 (0-54.1)	6.83 (5.17-11.2)	7.58 (4.4-12.7)
4	83	0.8 (0.04-1.8)	12.3 (0.1-47.1)	5.02 (1.7-7.9)	7.7 (4.7-13.21)
5	33	0.9 (0.4-2)	13.57 (1.3-33.8)	6.79 (4.01-8.11)	7.67 (5.48-12.33)
6	37	1.07 (0.26-2.91)	9.54 (0-36.8)	6.24 (4.55-10.68)	8.29 (5.71-15.96)
Total	257	0.9 (0.01-3.11)	10.7 (0-54.1)	6.1 (1.7-11.2)	7.98 (4.40-15.96)

MDS patients

Center	Patients (n)	Myeloblast-related cluster size (%)	B-progenitor-related cluster size (%)	Ly/Mbl CD45 ratio	Gra/Ly SSC ratio
1	35	1.4 (0.1-4)	6.4 (0-74.1)	6.7 (3.3-19.1)	6.4 (3.7-12.6)
2	37	1.53 (0.43-4.49)	0.5 (0-10.3)	7.32 (3.49-15.1)	6.66 (2.05-12.2)
3	31	1.89 (0.54-4.09)	0.19 (0-28)	7.38 (3.19-14.67)	5.99 (4.16-12.63)
4	121	1.8 (0.03-4)	1.1 (0.1-34.9)	6.68 (1.8-11.2)	5.7 (2.2-13.2)
5	30	1.2 (0.3-3.92)	2.6 (0.9-39.1)	6.4 (3.29-10.58)	7.2 (3.81-18.5)
6	27	1.15 (0.34-3.83)	0.61 (0-34.4)	7.39 (4.21-19.24)	7.22 (5.12-12.63)
Total	281	1.57 (0.03-4.49)	1.6 (0-74.1)	5.72 (1.8-19.24)	6.46 (2.05-18.5)

decreased in subjects with multilineage *versus* unilineage dysplasia (median 6.08, range 2.05-18.5 *versus* 7.55, range 3.2-13.7; $P<0.001$), in transfusion-dependent *versus* transfusion-independent patients (median 6.81, range 2.2-18.5 *versus* 7.82 range 2.05-15.96, $P=0.005$) and in patients with poor *versus* good and intermediate risk cytogenetics (median 6.77, range 2-18.5 *versus* 7.21, 2.05-15.8; $P=0.004$). A negative correlation was noted between Gra/Ly SSC ratio and WPSS risk (Spearman's r= -0.41, $P=0.02$).

We then focused on the population of control patients. Both myeloblast-related cluster size and Gra/Ly SSC ratio were significantly reduced in patients with idiopathic/iatrogenic hypoplasia with respect to other pathological conditions (P values ranging from 0.006 to 0.049 and from 0.02 to 0.07, respectively). The Ly/Mbl CD45 ratio was significantly lower in patients with cytopenia associated with marrow infiltration than in those with other pathological conditions (P values ranging from 0.01 to 0.08). No significant difference was noted in B-progenitor-related cluster size between the clinical entities considered in the study (*data not shown*).

Definition and validation of the flow cytometric score for the diagnosis of low-grade myelodysplastic syndromes

For all cytometric parameters, the threshold values to discriminate MDS from other conditions were determined in the learning cohort. We first investigated the differences in the values of cytometric variables among the laboratories participating in the study. We fitted a multiple regression model on control patients of the learning cohort, including age, sex and laboratory as covariates. Demographic factors had no impact on the values of immunophenotypic variables (*data not shown*). No significant association was found between the variable "laboratory" and myeloblast-related cluster size ($P=0.10$), B-progenitor-related cluster size ($P=0.53$) or Gra/Ly SSC ratio ($P=0.60$). A lower Ly/Mbl CD45 ratio was found in control patients from one laboratory [which enrolled the great majority of patients (11/14 cases) with cytopenia associated with marrow infiltration] (P values ranging from 0.001 to 0.08), while Ly/Mbl CD45 ratio values were comparable between the remaining five laboratories (more detailed results are reported in Table 2).

Table 3. Calculation of the flow cytometric score (FCM-score) for the diagnosis of low-risk MDS.

Cytometric Parameter	Cut-off values	Regression coefficient	Variable weighted score#
Myeloblast-related cluster size (%)*	≥2	2.59	1
B-progenitor-related cluster size (%)**	≤5	1.87	1
Lymphocyte to myeloblast CD45 ratio	≤4 or ≥7.5	1.76	1
Granulocyte to lymphocyte SSC ratio	≤6	2.31	1

**In all nucleated cells; **in all CD34⁺ cells; #a diagnosis of MDS is formulated in the presence of a FCM-score value ≥2.*

Defined cut-off values for each cytometric parameter were as follows: myeloblast-related cluster size ≥ 2% [area under the ROC curve (AUC) 0.675±0.023; $P<0.001$), B-progenitor-related cluster size ≤5% (AUC 0.712±.0.022; $P<0.001$), Gra/Ly SSC ratio ≤6 (AUC 0.720±0.0221; $P<0.001$) and Ly/Mbl CD45 ratio ≤4 or ≥7.5 (based on mean ±2 SD in controls) (Table 3).

The FCM-score was defined by combining the four parameters in a general logistic regression model: based on

regression coefficients (i.e. myeloblast-related cluster size 2.59, B-progenitor-related cluster size 1.87, Ly/Mbl CD45 ratio 1.76 and Gra/Ly SSC ratio 2.31) a value of 1 was assigned to each variable and a diagnosis of MDS was formulated in the case that the value of the FCM-score was 2 or more (Table 3).

We assessed the diagnostic power of the FCM-score in the learning cohort (Table 4). We first considered 166 MDS patients with specific markers of dysplasia (i.e. ring sideroblasts and/or abnormal karyotype). A correct diagnosis was obtained in 123/166 cases (sensitivity 74%). We next analyzed 115 MDS patients without specific markers: 75

Table 4. Application of the FCM-score in the learning and validation cohorts.

Learning cohort	FCM-score							
	0	1	2	3	4	positive cases	sensitivity (%)	specificity (%)
Low-risk MDS	33	50	107	67	24	198/281	70%	
Specific markers of dysplasia*								
MDS with specific markers	17	26	58	49	16	123/166	74%	
MDS without specific markers	16	24	49	18	8	75/115	65%	
WHO category								
RCUD	8	12	26	6	3	35/55	64%	
RARS	7	5	13	5	0	18/30	60%	
RCMD/RS	15	31	61	51	20	132/178	74%	
MDS with del5q	1	1	6	5	1	12/14	86%	
MDS-U	2	1	1	0	0	1/4	-	
Transfusion-dependency								
Yes	10	16	33	34	11	78/104	75%	
No	14	26	48	16	4	68/108	63%	
Cytogenetic risk								
Good-Intermediate	29	44	84	46	18	148/221	67%	
Poor	1	2	11	13	3	27/30	90%	
Controls	132	107	16	2	0	18/257		93%
Idiopathic or iatrogenic hypoplasia**	6	7	7	0	0	7/20		35%
Anemia***	42	57	5	0	0	5/104		95%
Cytopenia associated with BM infiltration	6	5	1	2	0	2/14	86%	
Cytopenia in transplant recipients	0	3	0	0	0	0/3		-
Infective cytopenia	2	4	0	0	0	0/6		-
Immune cytopenia****	76	31	3	0	0	3/110		97%

Validation cohort	FCM-score							
	0	1	2	3	4	positive cases	sensitivity (%)	specificity (%)
Low-risk MDS	16	26	57	32	5	94/136	69%	
Specific markers of dysplasia*								
MDS with specific markers	6	14	25	24	4	53/73	73%	
MDS without specific markers	10	12	32	8	1	41/63	65%	
WHO category								
RCUD	3	5	11	6	1	18/26		69%
RARS	5	4	8	3	0	11/20		55%
RCMD/RS	7	16	33	23	4	60/83		72%
MDS with del5q	1	1	3	0	0	3/5		-
MDS-U	0	0	2	0	0	2/2		-
Transfusion-dependency								
Yes	3	11	23	15	3	42/55		74%
No	8	11	21	12	1	35/53		64%
Cytogenetic risk								
Good-Intermediate	12	26	47	27	2	76/114	114	67%
Poor	0	1	4	2	1	7/8	8	-
Controls	45	68	7	3	0	10/123		92%
Idiopathic or iatrogenic hypoplasia**	5	8	2	0	0	2/15		87%
Anemia***	12	26	3	1	0	4/42		90%
Cytopenia associated with BM infiltration	6	6	1	1	0	2/14		86%
Infective cytopenia	3	2	0	0	0	0/5		-
Immune cytopenia****	19	26	1	1	0	2/47		96%

**Definition of specific markers of dysplasia included the presence of ring sideroblasts and/or chromosomal abnormalities. **Including chemotherapy-induced cytopenia. ***Including anemia associated with chronic disease, anemia associated with iron and/or B12/folate deficiency, anemia associated with renal failure and hemolytic anemia. ****Including idiopathic thrombocytopenic purpura. RCUD: refractory anemia with unilineage dysplasia; RARS: refractory anemia with ring sideroblasts; RCMD/RS: refractory anemia with multilineage dysplasia with or without ring sideroblasts; MDS-U: MDS unclassified.*

cases were correctly classified (sensitivity 65%). Overall 198/281 MDS patients were correctly diagnosed (sensitivity 70%). Considering patients stratified according to WHO subgroups, 35/55 (64%) cases of refractory cytopenia with unilineage dysplasia, 18/30 (60%) cases of refractory anemia with ring sideroblasts, 12/14 (86%) cases of MDS with del5q and 132/178 (74%) cases of refractory cytopenia with multilineage dysplasia were correctly classified by the FCM-score (Table 4). Among 36 subjects with MDS associated with marrow hypocellularity and/or moderate-to-severe fibrosis, 26 (72%) were correctly classified.

There were 18 false-positive cases among 257 controls (specificity 93%) (Table 4). False positive controls included seven patients with idiopathic or iatrogenic marrow hypoplasia, five patients with isolated anemia (associated with chronic disease or with combined iron and B12/folate deficiency), three with cytopenia associated with marrow infiltration, two with idiopathic thrombocytopenic purpura and one with immune cytopenia (Table 4).

Based on the FCM-score cut-off value, the PPV and NPV were 92% and 74%, respectively, without significant differences between patients with and without specific markers of dysplasia. The likelihood ratio was 10, without significant differences between patients with and without specific markers of dysplasia.

No significant effect of sex and age on the FCM-score value was found in either the MDS patients or control population (*data not shown*). In MDS patients, a high FCM-score (3 or 4) was found to be significantly associated with the presence of multilineage dysplasia (P=0.001), with transfusion-dependency (P=0.02) and with poor risk-cytogenetics (P=0.04), resulting in a higher WPSS risk (P=0.002) (Table 4). A single immunophenotypic abnormality was frequently reported in patients with non-clonal cytopenia (107/257 cases, 42%). The most common immunophenotypic aberrations included small B-progenitor-related cluster size (34% of cases) and aberrant CD45 ratio (15%), while reduced Gra/Ly SSC and increased myeloblast-related cluster size were present in smaller proportions of cases (8% and 5%, respectively).

The diagnostic value of the FCM-score was tested in an independent cohort of 259 patients, including 136 subjects with MDS and 123 controls with non-clonal cytopenia. When the clinical characteristics of the two cohorts were compared, no significant differences were found (Table 1).

A correct diagnosis was obtained in 53/73 patients with specific markers of marrow dysplasia (i.e ring sideroblasts and/or abnormal karyotype, sensitivity 73%) and in 41/63 patient without specific markers (sensitivity 65%). Overall 94 of 136 MDS patients were correctly classified (sensitivity 69%), while ten false-positive cases were noted among 123 controls (specificity 92%) (Table 4). False-positive controls included two patients with idiopathic marrow aplasia, four patients with isolated anemia (2 cases due to chronic disease, 1 case due to B12 deficiency and 1 case due to extravascular hemolysis), two patients with cytopenia associated with marrow infiltration and two patients with idiopathic thrombocytopenic purpura (Table 4).

The PPV and NPV were 90% and 73%, respectively, without significant differences between patients with and without specific markers of dysplasia. The likelihood ratio was 8.7, without significant differences between patients with and without specific markers of dysplasia.

Discussion

Here we have presented the results of a multicenter validation of a simple FCM-score for the detection of marrow dysplasia, based on four highly reproducible parameters from a single bone marrow cell aliquot stained with CD34/CD45 antibodies. The advantage of the proposed score is that it can be easily implemented in many laboratories and does not require an expensive antibody panel. We focused our analysis on subjects with low-grade MDS (most without specific markers of dysplasia such as ring sideroblasts and/or an abnormal karyotype), who are notoriously difficult to diagnose in clinical practice.[4,5] As controls, we enrolled patients with non-clonal cytopenia who represent the main population that must be differentiated from MDS.[1] The results of the analyses were confirmed in an independent cohort of patients.

The CD34$^+$ cell compartment is peculiarly perturbed in MDS[14] and CD34-related parameters are, therefore, good candidates for the identification of diagnostic markers for these disorders.[11,17]

Clonal transformation in MDS occurs at the level of a CD34$^+$ committed stem cell which can give rise to red cells, platelets, granulocytes and monocytes and has a competitive advantage over the normal stem cell compartment.[25] Accordingly, the proportion of CD34$^+$ cells is significantly higher in patients with MDS than in healthy subjects, and the great majority of cells are committed to the myeloid lineage.[10,15,17] A significant down-regulation of B-cell lineage-affiliated genes was observed in CD34$^+$ cells isolated from patients with low-grade MDS with respect to in those from healthy controls and patients with non-clonal cytopenia,[14,18] and a reduction in stage I hematogones is one of most consistent immunophenotypic findings in MDS patients.[12,15,20] Different immunophenotypic methods are available to detect B-cell progenitors and myeloblasts.[17,26] We decided to analyze two CD34$^+$ populations enriched in B-cell progenitors and myeloblasts defined on the basis of CD45 and SSC characteristics. The reliability of our gating strategy was previously reported,[15,19] and was verified in the present study. Importantly, when comparing different methods to define B-cell progenitors and myeloblasts, the analysis based on SSC and CD45 characteristics was found to have the highest efficiency in discriminating patients with marrow dysplasia with respect to patients with non-clonal cytopenia, and to be highly reproducible by different FCM operators.[19,27]

Several other immunophenotypic abnormalities on MDS CD34$^+$ cells have been reported, including asynchronous co-expression of stem-cell and late-stage myeloid antigens and abnormal expression of lymphoid markers.[7,8,15] Most of these parameters do not have adequate reproducibility[20] with the exception of the Ly/Mbl CD45 ratio that ensures acceptable inter-observer variability by adjusting data on target cells with those on lymphocytes in the same sample.[19,20]

The pathological hallmark of myelodysplastic CD34$^+$ precursors is a defective capacity for self-renewal and differentiation, leading to ineffective hematopoiesis.[14] Flow cytometry was found to be highly sensitive in detecting abnormal maturation of the myeloid lineage associated with ineffective hematopoiesis.[7,8,11] The most frequently used approach to evaluate myeloid dysplasia by immunophenotyping is pattern recognition analysis, which is based on a qualitative evaluation of a deviation

from the normal antigen expression pattern.[7,8,11] Although this approach is a good tool for expert operators,[28] it has several weak points and no robust reproducibility data in the setting of MDS are available.[12,29] In the present study we tested the diagnostic utility of granulocyte SSC ratio, which was associated with defective maturation of myeloid precursors and marrow dysplasia in several studies.[7,11,20] Among different SSC-related parameters, the Gra/Ly SSC peak channel ratio was found to be highly reproducible[19] and the most useful for discriminating MDS patients from pathological controls.[20]

Overall, increase in CD34$^+$ myeloblast-related cluster size, reduction of B-progenitor-related cluster size, aberrant Ly/Mbl CD45 ratio and reduction of Gra/Ly SSC ratio reflect MDS-specific biological features, have little inter-observer variability and their expression is correlated with disease severity (confirming a strong relationship with the myelodysplastic phenotype). We therefore combined these four parameters in a score (FCM-score) that was able to discriminate low-grade MDS from non-clonal cytopenias, and was suitable for implementation as an additional test in the diagnostic work-up of these disorders.

This conclusion was mainly supported by the results of the likelihood ratio test. The likelihood ratio is commonly used in medicine to define a test's accuracy and its clinical "implementability".[24] It is defined as the ratio of the probability of a given test result (e.g., "positive" or "negative") in a patient with disease to the probability of that result in a patient without disease.[24] With a likelihood ratio of 10 obtained with the present FCM-score, in a patient with a 50% pre-test probability of having MDS that is positive for the diagnostic test, the post-test probability of having the disease increases to more than 90% and the test can, therefore, be considered for clinical application. We would like to emphasize the importance of the diagnostic strategy using the likelihood ratio, because immunophenotypic data in low-grade MDS are usually not absolutely specific.[7,11] On the other hand, a negative FCM score value does not exclude the diagnosis of MDS, and in cases in which morphology, cytogenetics and flow cytometry are indeterminate, clinical observation for 6 months followed by a new bone marrow evaluation is advisable.[1]

Given its simplicity, the results of the immunophenotypic analysis by our FCM-score can be easily understood by clinicians. We clearly defined the reference ranges of the FCM-score variables in a large control population of patients with the major clinical conditions that need to be differentiated from MDS when making the patients' diagnosis.[1] The parameters included in the FCM-score appeared to be stable across the different laboratories involved in the study.

Interestingly, the FCM score consistently helped to establish the diagnosis of MDS in patients without specific markers of marrow dysplasia (such as ring sideroblasts and/or clonal chromosomal abnormalities). Patients with MDS without specific markers represent a significant proportion of the whole MDS population (42% in the present study), and the concordance for morphological recognition of dysplasia in these patients is usually poor in clinical practice,[4,22] The introduction of more objective markers for defining marrow dysplasia in these patients may result in a significant improvement of the clinical implementation of the WHO classification.[1,2]

As underlined before, a critical point for the morphological evaluation of marrow dysplasia is that it may be hampered by the presence of hypocellularity, fibrosis or an inadequate specimen collection.[30] The FCM-score allowed correct classification in the great majority of patients. In fact, dilution can influence "all nucleated cells" assessed by flow cytometry, thus causing falsely low CD34$^+$ myeloblast-related cluster size and may increase the proportion of mature circulating neutrophils, thus affecting the Gra/Ly SSC ratio. On the other hand, when marrow samples contain a substantial number of CD34$^+$ cells, even if diluted with peripheral blood, our method provides accurate data for both B-progenitor-related cluster size and Ly/Mbl CD45 ratio.[15,20]

The WHO classification has emerged as an important prognostic tool[2] and interesting data have also been reported on the ability of the WHO classification to guide therapeutic decision-making.[31] We observed that the value of the FCM-score reflected the degree of marrow dysplasia. In this context, the possibility of distinguishing patients with unilineage dysplasia from those with multilineage dysplasia by flow cytometry with higher sensitivity and reproducibility with respect to morphology might have important clinical implications.

Overall our results indicate that immunophenotyping may help to establish the diagnosis of MDS, especially when morphology and cytogenetics are indeterminate.

Authorship and Disclosures

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