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ORIGINAL ARTICLE

Flow cytometric detection of dyserythropoiesis: a sensitive and powerful diagnostic tool for myelodysplastic syndromes

S Mathis[1,2,7], N Chapuis[1,2,7], C Debord[1], A Rouquette[3], I Radford-Weiss[4], S Park[2,5], F Dreyfus[2,5], C Lacombe[1,2], MC Béné[6], O Kosmider[1,2], M Fontenay[1,2] and V Bardet[1,2]

Several groups have published flow cytometry scores useful for the diagnosis or prognosis of myelodysplastic syndromes (MDS), mainly based on the detection of immunophenotypic abnormalities in the maturation of granulocytic/monocytic and lymphoid lineages. As anemia is the most frequent symptom of early MDS, the aim of this study was to identify markers of dyserythropoiesis relevant for the diagnosis of MDS analyzed by selecting erythroblasts in a whole no-lysis bone marrow strategy by using a nuclear dye. This prospective study included 163 patients, including 126 with cytopenias leading to MDS suspicion and 46 controls without MDS. In a learning cohort of 53 unequivocal MDS with specific markers, there was a significant difference between the coefficients of variation of mean fluorescence intensities of CD71 and CD36 in MDS patients compared with controls. These two parameters and the hemoglobin level were used to build a RED-score strongly suggestive of MDS if $\geqslant 3$. Using the RED-score in the whole cohort, 80% of MDS or non-MDS patients were correctly classified. When combined with the flow score described by Ogata *et al.*, this strategy allowed to reach a very high sensitivity of 88% of patients correctly classified.

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Keywords: myelodysplastic syndromes; flow cytometry; dyserythropoiesis

INTRODUCTION

Morphology remains the gold standard for the diagnosis of myelodysplastic syndromes (MDS). The current WHO (World Health Organization) classification[1] relies on the quantification of blast cells in bone marrow (BM) or peripheral blood and on the evaluation of BM dysplasia to stratify MDS patients. The diagnosis of MDS is straightforward if obvious morphological abnormalities are seen after standard or specific Perls' iron staining, or if specific cytogenetic abnormalities are present. If not, the patient is classified as having idiopathic cytopenia of undetermined significance (ICUS).[2] Over the past 20 years, several groups have developed new approaches in multiparameter flow cytometry (MFC) as diagnostic or prognostic tools in MDS.[3,4] At present, although the respective value of several markers has been acknowledged, there is no consensus about a MFC panel validated to establish robust diagnosis or prognosis in MDS and no single immunophenotypic marker has proven to be able to discriminate accurately between MDS and other pathological conditions leading to cytopenia. Among the different studies published, very few relate specifically to the erythroid compartment. Yet, abnormalities in CD71 (transferrin receptor), CD36 (thrombospondin receptor) or CD235a (glycophorin-A) expression have long been reported in MDS.[5–9] Indeed, several authors have emphasized the specificity and consistency of decreased CD71 expression in MDS patients.[8,10,11]

An important caveat in the quantification of erythroid precursors is the modifications induced by red blood cell (RBC) lysis. Elimination of mature non-nucleated erythroid cells is routinely performed in MFC with various reagents. The latter, however, can induce the lysis of some of the most mature acidophilic or polychromatophilic erythroblasts and/or result in an incomplete lysis of RBC impairing a proper analysis of the most immature cells. An alternative approach to study the erythroid compartment would be to use whole peripheral blood or BM with the addition of a nuclear dye in the MFC panel. To this avail, DRAQ5 or DRAQ7 (Biostatus Limited, Shepshed, UK)[12,13] for instance, allow to discriminate nucleated and non-nucleated cells in MFC.

In this study, we developed a whole BM MFC protocol using the recently developed nuclear dye CyTRAK orange (Biostatus Limited) to gate nucleated cells.[14] This new protocol, avoiding RBC lysis, could enhance the accuracy of the MFC approach as diagnostic tool in MDS. The aim of this study was thus to develop this approach and propose a reproducible and simple MFC tool useful within the diagnostic work-up of patients suspected of MDS.

MATERIALS AND METHODS

This study comprised two stages corresponding to phases I and III of a diagnostic test development process.[15] The aim of phase I is to identify markers able to discriminate between cases (MDS) and control subjects. Phase III is a pragmatic one aiming at developing a diagnostic score using these markers and evaluating its diagnostic properties (sensitivity, specificity and predictive values) in routine practice.

Patients

As described in the flow chart (Figure 1), this prospective study included all patients ($n = 132$) with cytopenias but without circulating blasts (to focus

[1]Service d'Hématologie Biologique, Hôpitaux Universitaires Paris Centre-Cochin, Paris, France; [2]Inserm U1016, UMR 8104, Université Paris Descartes, Hôpitaux Universitaires Paris Centre-Cochin, Paris, France; [3]Unité de Biostatistique et d'Epidémiologie, Université Paris Descartes, Hôpitaux Universitaires Paris Centre-Hôtel Dieu, Paris, France; [4]Service de Cytogénétique, Groupe Hospitalier Necker-Enfants malades, Paris, France; [5]Unité fonctionnelle d'Hématologie Clinique, Hôpitaux Universitaires Paris Centre-Cochin, Paris, France and [6]Service d'Hématologie Biologique, CHU, Nantes, France. Correspondence: Dr V Bardet, Service d'Hématologie Biologique, Hôpitaux Universitaires Paris Centre-Cochin, Paris, France or Inserm U1016, UMR 8104, Université Paris Descartes, Hôpitaux Universitaires Paris Centre-Cochin, 27 rue du Faubourg Saint Jacques, Paris 75014, France.
E-mail: valerie.bardet@cch.aphp.fr
[7]These authors contributed equally to this work.
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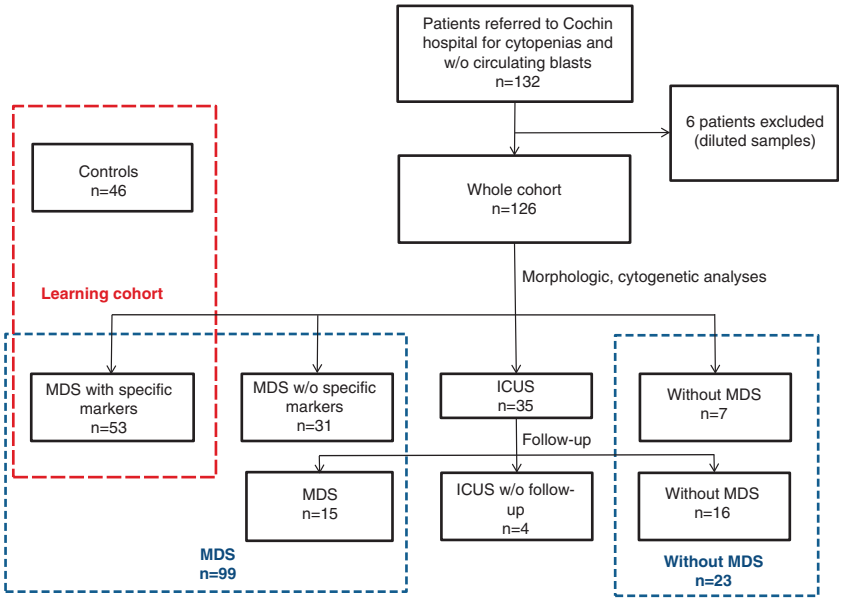

Figure 1. Description of the whole cohort. A total cohort of 163 patients was studied by flow cytometry: 126 patients with cytopenias of unknown etiology and 46 controls. Among the 126 patients, 53 unequivocal MDS patients constituted a learning cohort with the 46 controls. Initially, after morphological and cytogenetic analyses 35 patients were classified as ICUS, after 6-month follow-up, 15 were classified as MDS, 16 as non-MDS and 4 patients were lost, leaving 99 MDS and 23 non-MDS for analyses.

on low-grade MDS), referred to our laboratory for BM investigations between November 2011 and December 2012. Amongst them, six were excluded because of diluted samples (BM purity <80%, as determined by the method of Holdrinet *et al.*,[16]) leaving 126 patients with data available for analyses. BM specimens were collected into heparinized Hank's medium (Eurobio, Courtaboeuf, France) and were processed within 2 h following aspiration.

Cytogenetic analyses were performed in 104 patients and molecular analyses in 15.

Morphological and cytogenetic analyses were used to classify patients as MDS or non-MDS. Patients classified as having an ICUS after these investigations were followed during at least 6 months and then definitely classified as MDS or non-MDS.

From the whole cohort of patients, a learning cohort was constituted with 53 MDS patients in whom the diagnosis of MDS was unequivocal using one or more of the following criteria: abnormal MDS-related karyotype, excess of blasts on BM smear and ring sideroblasts >15%. For the first stage of this study, 46 additional control patients without known MDS referred for BM investigation during the same period for other indications were included to constitute the control group. Twenty-five of 46 controls presented with anemia and 11 with other immune cytopenia (Table 1).

All investigations performed in this study were approved by the Cochin Ethics Committee (Comité Consultatif de Protection des Personnes) and the procedures followed were in accordance with the Helsinki Declaration.

The clinical and biological characteristics of the study population are reported in Table 1.

MFC and morphological studies on BM cells

Staining for MFC was performed on 5 million BM cells after one wash of whole BM in Dulbecco's phosphate buffered-saline (Eurobio). Two combinations of reagents were used. In a first set of 93 patients, cells were stained with CD36-FITC, CyTRAK orange, CD235a-PC7, CD117-APC, CD71-APC-Alexafluor 750 and CD45-Krom Orange according to manufacturer's instructions (Beckman Coulter). In the second set of 70 patients, CD64-PC7 was used instead of CD235a-PC7 and two different conjugates were used, respectively, CD71-APC-Alexafluor 700 and CD117-Brilliant Violet 421. To be sure that the results with the new panel (CD117-BV421, CD71-APC-AF 700) were identical, CD71CV was compared in 46 MDS patients analyzed with the new panel and 45 MDS patients performed with the former one. Although the MDS patients were different, their partition according to the WHO classification was equivalent. All antibodies were purchased from Beckman Coulter (Fullerton, CA, USA) except for CD117-Brilliant Violet 421 obtained from Becton Dickinson (San Jose, CA, USA)

Table 1. Clinical and biological characteristics of the study population

Controls	n = *46*	
Age, years (range)	73 (24–91)	
Sex (female/male)	26/20	
Anemic patients	*25*	*54%*
Anemia associated with renal failure	5	11%
Anemia associated with inflammation, iron deficiency or minor thalassemia	20	43%
Non-anemic patients	*21*	*46%*
Isolated monoclonal gammopathy	10	22%
Immune cytopenia (neutropenia or thrombocytopenia)	11	24%
Cytopenias	n = *126*	
Age, years (range)	73 (43–94)	
Sex (female/male)	68/58	
Anemia	92	73%
Neutropenia	28	22%
Thrombocytopenia	55	44%
Abnormal karyotype	42/104	40%
WHO classification		
RCUD	25	20%
RARS	5	4%
RCMD/RS	38	30%
RAEB-1	8	6%
RAEB-2	8	6%
MDS with del5q	11	9%
CMML	4	3%
Non-MDS	23	19%
ICUS	4	3%
IPSS-R (n = *83*)		
Very low	10	12%
Low	47	57%
Intermediate	13	15%
High	10	12%
Very high	3	4%

Abbreviations: ICUS, idiopathic cytopenia of undetermined significance; IPSS-R, revised International Prognostic Scoring System; MDS, myelodysplastic syndromes; WHO, World Health Organization.

and CyTRAK orange purchased by Biostatus Limited. Data were acquired using a Navios flow cytometer (Beckman Coulter) and analyzed using the Kaluza software (Beckman Coulter). At least 5000 erythroblasts were acquired for each patient to ensure the quality of the analysis.

Geometrical means of fluorescence (GMFI) and their coefficient of variations (CVs) were collected for CD71, CD235a and CD36 for erythroblasts in all patients. For 59 patients, after a first MFC analysis, RBC lysis was performed using Versalyse (Beckman Coulter) according to the manufacturer's instructions and the same data were acquired for erythroblasts and lymphocytes, allowing to compare both conditions. At the same time, cytocentrifuge preparations (Thermo Fisher Scientific, Asnières sur Seine, France) were performed to compare cell morphology after May-Grünwald-Giemsa's staining.

The Ogata MFC-score was calculated for each patient, as previously described.[17,18]

All MFC analyses were performed blinded of morphological or cytogenetics results, and reciprocally.

Cytogenetics

Conventional RHG-banded karyotype was performed on BM cells after 24 h *in vitro* culture and chromosomal abnormalities described according to the International System for Human Cytogenetic Nomenclature (2013).[19]

Molecular analyses

DNA was extracted from BM cells and submitted to gene mutation screening of *TET2*, *ASXL1*, *U2AF35* and *SRSF2* as described previously.[20]

Statistical analyses

For continuous variables, the D'Agostino and Pearson normality tests enabled to investigate data distribution. For parameters with a normal distribution, Student's *t*-test was used to compare mean differences between groups, whereas the Mann–Whitney test was used for parameters with non-Gaussian distribution. For categorical variables, differences between groups were investigated with the χ^2-test or the Fisher's exact test when necessary. All tests used were two-tailed tests and a P-value <0.05 was considered as significant.

In the first stage of this study, the new MFC protocol was tested in a learning cohort of 53 MDS patients and 46 controls to establish significant parameters to discriminate between MDS patients and controls.

In the second stage of this study, baseline characteristics of the patients of the cohort were screened as candidate indicator variables of MDS. Each variable that achieved a significant level of P-value <0.2 was retained as candidate variable. To dichotomize each continuous candidate variable, receiver–operator characteristic (ROC) curves and Youden index were used to determine the cut-off value.[21] A multivariate logistic regression model (backward elimination) was then used to assess the maximum likelihood estimates of the parameter coefficients and the statistical significance of each candidate variable. The good ness-of-fit of the model to the data was evaluated by calculating the Hosmer–Lemeshow statistics.[22] Finally, we assigned points for the score in order to respect the existing ratios between each indicator regression parameters coefficients. The score was then computed for each patient of the cohort and a ROC curve analysis was performed in order to compute the area under the ROC curve and its corresponding 95% confidence interval (CI). In order to estimate the diagnostic power of the cytometric RED-score, specificity, sensitivity, positive predictive value, negative predictive value and their 95% CI were calculated. Analyses were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) and Stata software, version 12.1 (StataCorp LP, College Station, TX, USA).

RESULTS

Comparison of no-lysis and RBC lysis strategies

Results obtained without or after RBC lysis were compared for 59 patients regarding erythroblasts, lymphocytes and granulocytes parameters, including forward scatter, CD71 and CD36 RFI. Forward scatter was drastically and specifically reduced for erythroblasts after RBC lysis (median 152 versus 373, $P<0.0001$) with less severe yet still significant modifications in granulocytic (median 529 versus 492, $P<0.0001$) or lymphocytic (median 284 versus 293, $P=0.005$) lineages (Figure 2a). Erythroblastic morphology was well preserved in whole BM samples, but significantly modified by RBC lysis, as most of the mature forms were reduced to naked nuclei and only the most immature

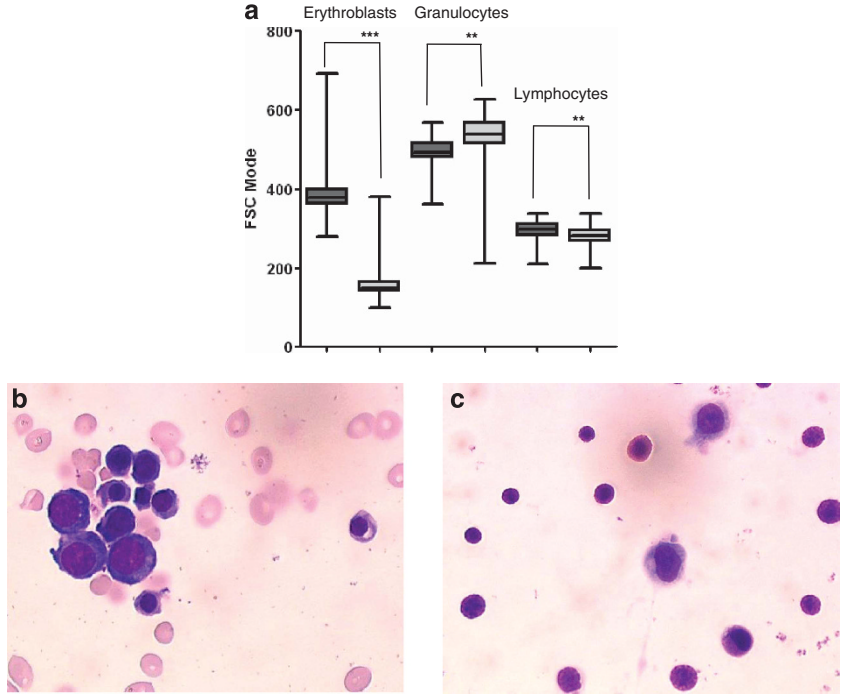

Figure 2. Effect of RBC lysis on different flow cytometric parameters. Panel **a** shows the forward side scatter (FSC) mode measured before and after red cell lysis for erythroblasts, granulocytes and lymphocytes. Panels **b** and **c** show cytocentrifuged preparations of stained cells without or with red cell lysis, demonstrating drastic alteration of morphology. $^{**}P<0.05$ and $^{***}P<0.0001$.

forms such as proerythroblasts or basophilic erythroblasts were still identifiable on smears (Figures 2b and c).

Evaluation of MFC abnormalities in MDS: learning cohort

The analytical strategy of whole BM no-lysis assessment in MFC is depicted in Figure 3. Briefly, after elimination of doublets and debris, nucleated cells were isolated using a CyTRAK orange labeling threshold and erythroblasts were gated using CD36 and CD71 staining.

GMFI and CV values were collected for CD36, CD71, CD117 and CD235a in a learning cohort of 53 MDS patients and 46 controls. There was a significantly lower GMFI for CD36 (median 19.4 versus 29.8, $P<0.0001$) and CD71 (median 45.5 versus 63.7, $P=0.0002$) in MDS patients compared with controls (Figure 4a). Conversely, no significant difference was observed for CD117 and CD235a GMFIs, despite the fact that CD117 expression was abnormally high in 22% of MDS patients (data not shown). CVs were significantly higher in MDS patients than in controls for CD36 (median 73.5 versus 62.8, $P<0.0001$) and CD71 (median 83.5 versus 64.5, $P<0.0001$; Figure 4b). As the statistical significance was better for CV than for GMFI, CD71 CV and CD36 CV were selected as the best tools to discriminate between MDS and controls. For 59 patients (38 MDS and 21 controls), the effect of RBC lysis was measured on CD71 and CD36 CVs (Figures 4c and d). For CD71CV, the difference was still highly significant between MDS and controls after RBC lysis ($P=0.0001$ versus $P<0.0001$) but the gap was reduced in the lysis condition with a difference between the medians of MDS patients and controls of 6% instead of 17% without RBC lysis. The CD36 CV was similar between the two conditions with differences in medians of 57 versus 65, respectively, and a similar level of significance at $P=0.002$. The modification in the panel of antibodies (CD71 conjugate switched from APC-AlexaFluor 750 to APC-AlexaFluor 700) did not induce modifications of the CD71CV in MDS patients (median 82 versus 77, $P=0.07$; Supplementary Figure 1).

Correlation of CD71 CV and CD36 CV with complete blood count and IPSS-R

For 75 MDS patients with an available complete blood count and no history of transfusion, the correlation between CD71 CV, CD36 CV and hemoglobin (Hb), mean corpuscular volume, neutrophils and platelets counts was examined. CD71 CV and CD36 CV were

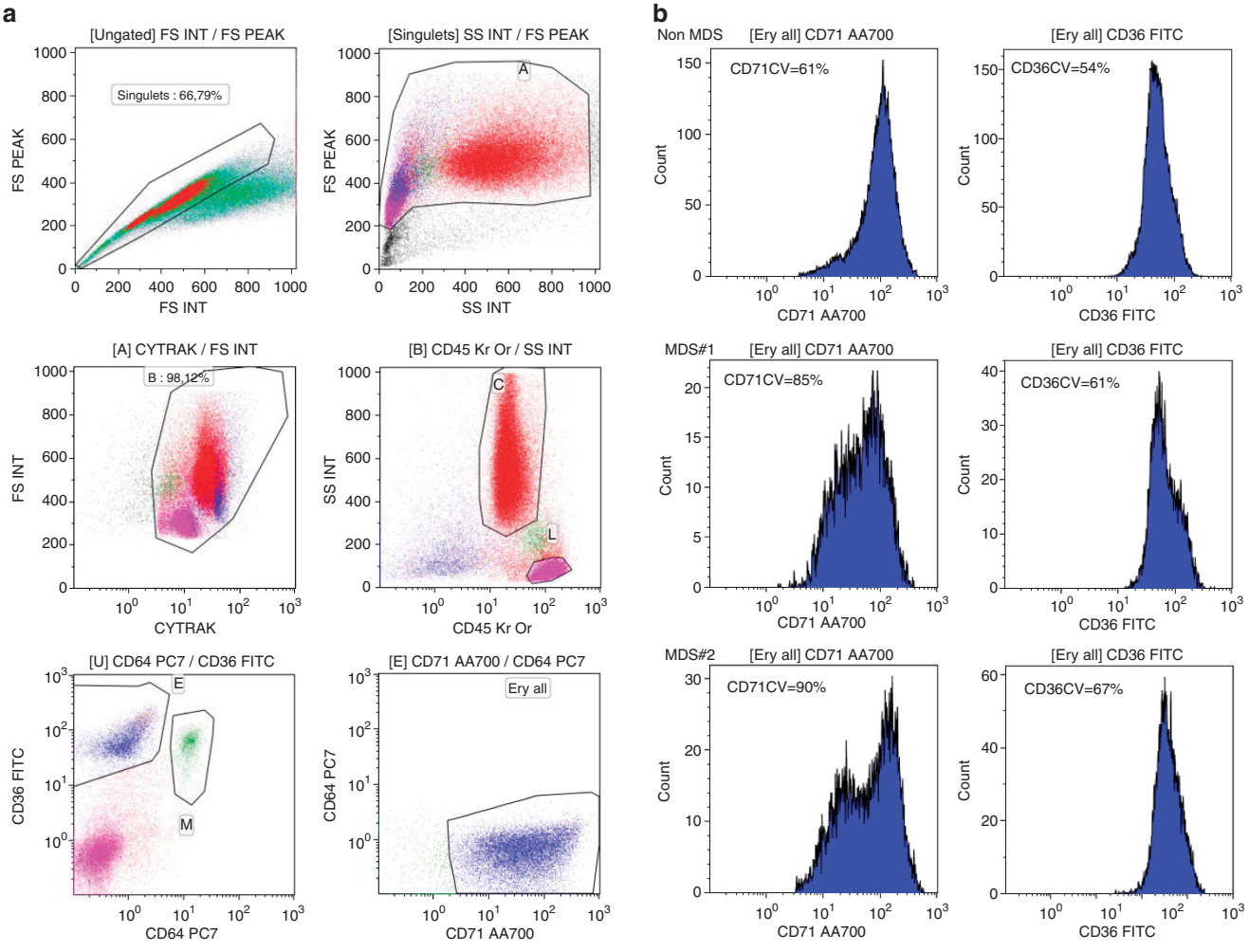

Figure 3. Gating strategy illustrated with two typical MDS patients and a non-MDS patient. The gating strategy is illustrated in part **a** of the figure. After elimination of doublets (forward scatter (FS) peak versus FS bivariate dot plot) and debris (FS versus side scatter (SS) bivariate dot plot), nucleated cells were isolated using CYTRAK Orange in gate B. Granulocytes (C) were eliminated in the SS INT versus CD45 bivariate dot plot. In gate U (B minus C), erythroblasts (in blue) were identified in CD64 versus CD36 (gate E) and CD64 versus CD71 (gate Ery all) bivariate dot plots. Typical patterns of expression of CD71 and CD36 are illustrated in part **b** of the figure. CVs of fluorescence intensity for CD71 and CD36 in erythroblasts are shown in a non-MDS patient, a MDS patient with abnormal CD71CV only (MDS#1) and a MDS patient with both abnormal CVs and two peaks in CD71 fluorescence (MDS#2).

highly negatively correlated with Hb levels ($P = 0.0002$ and $P = 0.0012$, respectively; Figure 5).

As revised International Prognostic Scoring System (IPSS-R) is the most up-to-date prognostic index in MDS patients,[23] the correlation between CD71 CV, CD36 CV and IPSS-R was examined. The latter was available in 83/91 MDS patients who benefited from a karyotypic analysis. No significant correlation was observed between CD71 CV or CD36 CV and IPSS-R or WHO classification, perhaps owing to a low number of high-risk patients in our cohort.

Evaluation of CD71 CV and CD36 CV as a diagnostic tool in MDS: definition of the RED-score

Initially after morphological and cytogenetic examination, 84 patients were diagnosed as MDS, 35 as ICUS and MDS was excluded in 7. For 15 ICUS patients, the four most mutated genes in MDS (*TET2*, *ASXL1*, *U2AF35* and *SRSF2)* were screened but no mutation was found.

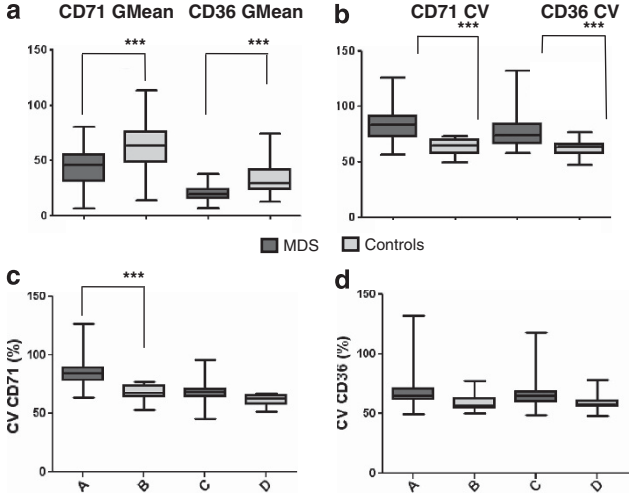

Figure 4. Comparison of CD71 and CD36 expression in MDS patients and controls of the learning cohort. Geometrical means (GMean) for CD71 and CD36 are presented in panel **a**, showing significantly lower values in MDS patients ($n = 53$) versus controls ($n = 46$) for CD71 (median 45.5 versus 63.7, $P = 0.0002$) and CD36 (median 19.4 versus 29.8, $P < 0.0001$). Panel **b** shows CV values for CD71 and CD36. CVs are higher in MDS compared with controls for CD71 (median 83.5 versus 64.7, $P < 0.0001$) and CD36 (median 73.5 versus 62.9, $P < 0.0001$). Panels **c** and **d** show CV values for CD71 and CD36, in MDS patients ($n = 38$) and controls ($n = 21$) without RBC lysis (Lanes A and B, medians 84.6 versus 68.1, $P < 0.0001$ for CD71 and 71.5 versus 63, $P = 0.002$ for CD36) or with RBC lysis (Lanes C and D, median 68.6 versus 62.8, $P = 0.0001$ for CD71, and 65.3 versus 57.3, $P = 0.002$ for CD36). ***P-value is highly significant.

After 6 months follow-up, morphology and cytogenetics led to a definite MDS diagnosis in 99 patients and MDS was excluded in 23 patients. Four patients were lost to follow-up and subsequently excluded.

Screening baseline characteristics of the 122 patients of the cohort revealed four variables significantly associated with MDS diagnosis: CD71 CV ($P = 0.0001$), CD36 CV ($P < 0.0001$), Hb level ($P = 0.0041$) and the Ogata MFC-score ($P = 0.032$).

Using ROC curves and Youden indexes, CV thresholds were subsequently defined as 80% for CD71 CV and 65% for CD36 CV, Hb threshold as 1.5 g/dl under the normal value for each sex and Ogata MFC-score threshold as 2 points, as previously described,[17,18] ROC curves, sensitivity and specificity for the four parameters are presented in Supplementary Figure 1.

These four markers and sex ($P = 0.188$ in the univariate analysis) were entered in a multivariate logistic regression. Following a backward elimination process, only CV71 (regression coefficient (RC) = 2.80, CI 95% = (1.09–4.52), $P = 0.001$), CV36 (RC = 1.89, CI 95% = (0.34–3.44), $P = 0.017$) and Hb level (RC = 1.90, CI 95% = (0.14–3.67), $P = 0.035$) were still significantly associated with MDS diagnosis. The P-value associated with the Ogata MFC-score was equal to 0.095. The Hosmer–Lemeshow statistics was 2.26_{df8} ($P = 0.89$), indicating that the data were adequately fit to a logistic function.

The RED-score was then built using coefficients of logistic regression. The three parameters used were combined as follows: 3 points for a CD71 CV $\geqslant$ 80, 2 points for a CD36 CV $\geqslant$ 65 and an Hb level under 1.5 g/dl from normal values (respective of sex), with a total expected score varying from 0 to 7. A diagnosis of MDS was formulated if the RED-score was $\geqslant 3$. On the whole cohort of 110 patients evaluated (Hb level missing for 12 patients), 71 patients were thereby classified as MDS. Details of RED-score values for the 110 patients are presented in Figure 6. Nineteen percent of MDS patients had an abnormal CD71 only, whereas 26% had an abnormal CD36 only and 43% had both abnormal CD36 and CD71 (Supplementary Figure 2). Among the whole cohort of 110 patients tested, the RED-score yielded a correct diagnosis for 88 patients. Overall, 69 of 89 confirmed MDS patients were correctly diagnosed (sensitivity = 77.5%, CI 95% = (68.9–86.2)), whereas 2 false positive cases were observed (specificity = 90%, CI 95% = (77.9–100)). Positive predictive value and negative predictive value were therefore of 97% (69/71) and 49% (19/39), respectively.

Combination of the RED-score and the MFC-score: toward the perfect algorithm?

An Ogata MFC-score[17,18] was available for 107 patients (15 patients were not eligible because of rituximab treatment or concomitant B-cell lymphoma). ROC curves for the MFC-score and the new RED-score are described in Figures 7a and b, respectively.

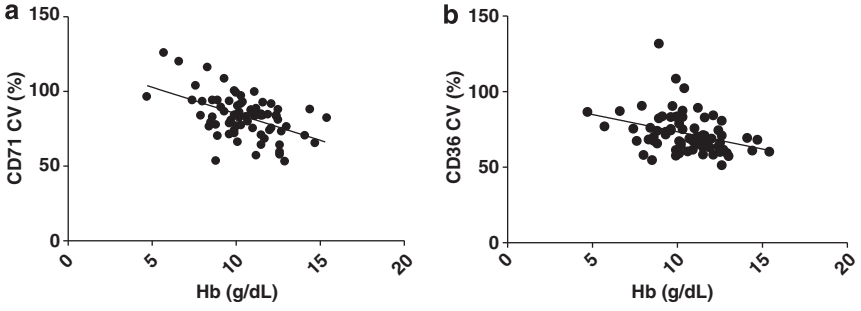

Figure 5. Correlation between CD71 CV, CD36 CV and the level of Hb in MDS patients. Hb level is negatively correlated with CD71 CV (panel **a**, $P = 0.0002$, $r = -0.42$) and CD36 CV (panel **b**, $P = 0.0012$, $r = -0.37$) in MDS patients ($n = 75$).

a

RED-score parameter	Threshold	Points
CD71CV (%)	<80	0
	≥80	3
CD36CV (%)	<65	0
	≥65	2
Hb level (g/dL)	>10.5 (F) or >11.5 (M)	0
	≤10.5 (F) or ≤11.5 (M)	2

b

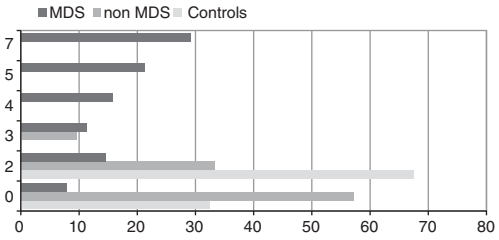

Figure 6. RED-score quantification in the 110 patients of the cohort and the 46 controls. Panel **a** shows the details of the score. Panel **b** shows the results in 89 MDS patients, 21 non-MDS patients and 46 controls as percentage of total for each population.

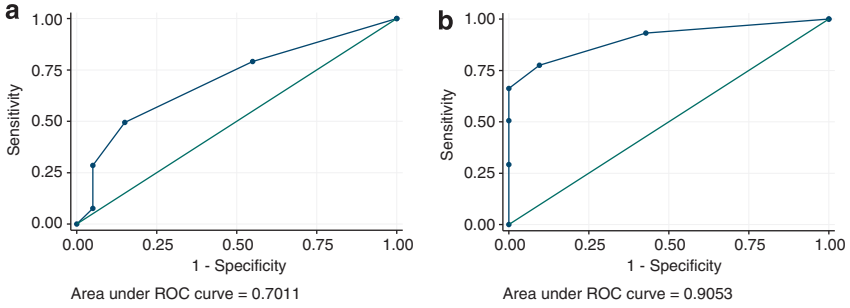

Figure 7. ROC curves for the Ogata MFC-score and the new RED-score in the whole cohort areas under ROC curves for the Ogata MFC-score and for RED-score are 0.7011(panel **a**; 95% CI = (0.5857–0.8165)) and 0.9053 (panel **b**; 95% CI = (0.8524–0.9583)) respectively. With a threshold equal to 3, sensitivity and specificity are 0.77, 95% CI = (0.6886–0.8620) and 0.9048, 95% CI = (0.7793–1), respectively.

In this cohort, 101 patients were tested with both scores. Forty-one of 83 MDS patients displayed an Ogata MFC-score $\geqslant 2$ reflecting the weaker sensitivity of this test. Among the 42 patients with an Ogata MFC-score < 2, a RED-score $\geqslant 3$ was however observed for 32. Conversely, of 15 patients with a RED-score < 3, 5 had an Ogata MFC-score $\geqslant 2$. Interestingly, 73 of 83 (88%) MDS patients had either a RED-score $\geqslant 3$ or an Ogata MFC-score $\geqslant 2$. Comparison of both scores and their combination are detailed in Supplementary Table 1. With this approach, the sensitivity was significantly increased (87.9%), whereas the specificity remained high (88.9%); positive predictive value and negative predictive value were therefore of 97% (73/75) and 61% (16/26), respectively.

DISCUSSION

This study demonstrates that analysis of erythroblasts in no-lysis whole-BM approach is feasible, after exclusion of non-nucleated cells by their absence of staining with CyTRAK orange. This yielded the observation of both a significant decreased fluorescence and broader peaks for CD71 and CD36 in MDS samples compared with control BM. This is likely due to the coexistence of pathological and normal erythropoiesis in MDS patients, where the decreased expression of these markers in the pathological clone induce increased GMFI CVs. CVs are statistical parameters directly available from flow cytometers analysis software. CVs are less affected than GMFI by intrinsic variations between patients or between instruments and provided here the best discrimination between patients and controls.

A preliminary step in our approach was to determine the best analytical process in order to detect with the highest sensitivity differences between MDS patients and controls. As shown here, RBC lysis is deleterious not only for enucleated RBC but also for erythroblasts that have a drastic reduction in size (60%), whereas other nucleated cells are not significantly affected. Indeed, after red cell lysis, erythroblasts are reduced to cells almost devoid of cytoplasm or even to naked nuclei, therefore possibly compromising the detection of surface antigens.

In a first step, comparison of four surface markers of erythroblasts between 53 unequivocal MDS patients and 46 controls allowed to identify CD71 and CD36 CVs as highly sensitive and robust discriminative tools for MDS diagnosis. Using a simple algorithm combining the two CV and the degree of anemia, MFC by itself was able to predict the correct diagnosis in 80% patients of a larger cohort of 110 patients in the second step of the study. Interestingly, both CVs are highly negatively correlated with Hb levels, indicating that this approach will be particularly successful for patients presenting with anemia, who represent the majority of patients with suspected MDS. Indeed, 16 of 20 patients with MDS for whom the RED-score was < 3 did not suffer from anemia. Nine of these patients had an abnormal CD36 CV and could therefore have been diagnosed if they had been anemic. Among the four remaining patients who had anemia, two were ultimately diagnosed with 5q-syndrome. It can be hypothesized that the mechanism of anemia can be different in these patients and more related with erythroblastopenia rather than with dysplasia. Conversely, the two non-MDS patients, classified as false positive with a RED-score $\geqslant 3$, were initially diagnosed with ICUS, had no anemia and mainly suffered from moderate thrombocytopenia. In these patients with slight or absent dysplasia, there was no element for a diagnosis of MDS and they were finally considered as non-MDS patients after 6-month follow-up. It cannot, however, be excluded that they indeed suffered from an early-stage MDS and will develop overt MDS with long-term follow-up. Very recently, the Munich Leukemia Laboratory team demonstrated that with longer follow-up, half of patients with MFC abnormalities and without proof of myelodysplasia by cytomorphology or cytogenetics indeed developed overt disease, enhancing the role of MFC in the early diagnosis of MDS.[24]

When combining the results of the new RED-score with those of the Ogata MFC-score,[17] a very high sensitivity was reached in the diagnosis of MDS by MFC as 88% of patients were correctly classified. We thus believe that MFC can be a useful tool in the diagnosis of MDS, particularly for patients with no obvious dysplasia in cytomorphology. Of note, MFC provided a diagnosis in this series, for the 28% of cases where cytomorphology and

cytogenetics were inconclusive. In such cases, clinical management is influenced by follow-up but also by the degree and type of cytopenia, that is, clinicians are more prone to treat symptomatic anemia than asymptomatic moderate thrombocytopenia. MFC could thus in some cases sustain a MDS diagnosis and help the clinician to choose between supportive care and erythropoietin supplementation. In our experience, patients with cytopenias associated with dysplasia in a context of inflammation, iron deficiency or thalassemia did not show abnormalities of CD71 nor CD36 CV (data not shown). The RED-score could therefore be quite specific for MDS and help in distinguishing MDS from dysplasias of undetermined significance. These results need to be validated through a future multicentre study, which will demonstrate whether this RED-score is easily transposable to another center and which precautions need to be implemented to ensure reproducibility: harmonized photomultiplier settings with standardized beads, use of same fluorochromes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

SM, NC and CD performed the flow cytometric experiments and analyzed the data, AR performed the statistical analysis, IRW performed the cytogenetics, OK performed and analyzed the molecular experiments, SP and FD helped for constituting the cohort of patients and provided patient samples, CL, MCB and MF co-wrote the paper, VB designed the study, analyzed the data and co-wrote the paper.

REFERENCES

1 *WHO. Classification of Tumours of Haematopoietic and Lymphoid Tissues*. 4th edn. (International Agency for Research on Cancer. IARC): Lyon, France, 2008.
2 Valent P, Bain BJ, Bennett JM, Wimazal F, Sperr WR, Mufti G *et al.* Idiopathic cytopenia of undetermined significance (ICUS) and idiopathic dysplasia of uncertain significance (IDUS), and their distinction from low risk MDS. *Leuk Res* 2012; **36**: 1–5.
3 Westers TM, Ireland R, Kern W, Alhan C, Balleisen JS, Bettelheim P *et al.* Standardization of flow cytometry in myelodysplastic syndromes: a report from an international consortium and the European LeukemiaNet Working Group. *Leukemia* 2012; **26**: 1730–1741.
4 van de Loosdrecht AA, Ireland R, Kern W, Della Porta MG, Alhan C, Balleisen JS *et al.* Rationale for the clinical application of flow cytometry in patients with myelodysplastic syndromes: position paper of an International Consortium and the European LeukemiaNet Working Group. *Leuk Lymphoma* 2013; **54**: 472–475.
5 Maynadie M, Picard F, Husson B, Chatelain B, Cornet Y, Le Roux G *et al.* Immunophenotypic clustering of myelodysplastic syndromes. *Blood* 2002; **100**: 2349–2356.
6 Stetler-Stevenson M, Arthur DC, Jabbour N, Xie XY, Molldrem J, Barrett AJ *et al.* Diagnostic utility of flow cytometric immunophenotyping in myelodysplastic syndrome. *Blood* 2001; **98**: 979–987.
7 Malcovati L, Della Porta MG, Lunghi M, Pascutto C, Vanelli L, Travaglino E *et al.* Flow cytometry evaluation of erythroid and myeloid dysplasia in patients with myelodysplastic syndrome. *Leukemia* 2005; **19**: 776–783.
8 Della Porta MG, Malcovati L, Invernizzi R, Travaglino E, Pascutto C, Maffioli M *et al.* Flow cytometry evaluation of erythroid dysplasia in patients with myelodysplastic syndrome. *Leukemia* 2006; **20**: 549–555.
9 Matarraz S, Lopez A, Barrena S, Fernandez C, Jensen E, Flores-Montero J *et al.* Bone marrow cells from myelodysplastic syndromes show altered immunophenotypic profiles that may contribute to the diagnosis and prognostic stratification of the disease: a pilot study on a series of 56 patients. *Cytometry* 2010; **78**: 154–168.
10 Xu F, Wu L, He Q, Zhang Z, Chang C, Li X. Immunophenotypic analysis of erythroid dysplasia and its diagnostic application in myelodysplastic syndromes. *Intern Med J* 2012; **42**: 401–411.
11 Chopra A, Pati H, Mahapatra M, Mishra P, Seth T, Kumar S *et al.* Flow cytometry in myelodysplastic syndrome: analysis of diagnostic utility using maturation pattern-based and quantitative approaches. *Ann Hematol* 2012; **91**: 1351–1362.
12 Allan RW, Ansari-Lari MA, Jordan S. DRAQ5-based, no-lyse, no-wash bone marrow aspirate evaluation by flow cytometry. *Am J Clin Pathol* 2008; **129**: 706–713.
13 Bjornsson S, Wahlstrom S, Norstrom E, Bernevi I, O'Neill U, Johansson E *et al.* Total nucleated cell differential for blood and bone marrow using a single tube in a five-color flow cytometer. *Cytometry* 2008; **74**: 91–103.
14 Edward R. Use of DNA-specific anthraquinone dyes to directly reveal cytoplasmic and nuclear boundaries in live and fixed cells. *Mol Cells* 2009; **27**: 391–396.
15 Sackett DL, Haynes RB. The architecture of diagnostic research. *BMJ* 2002; **324**: 539–541.
16 Holdrinet RS, von Egmond J, Wessels JM, Haanen C. A method for quantification of peripheral blood admixture in bone marrow aspirates. *Exp Hematol* 1980 **8**:103–107.
17 Ogata K, Della Porta MG, Malcovati L, Picone C, Yokose N, Matsuda A *et al.* Diagnostic utility of flow cytometry in low-grade myelodysplastic syndromes: a prospective validation study. *Haematologica* 2009; **94**: 1066–1074.
18 Della Porta MG, Picone C, Pascutto C, Malcovati L, Tamura H, Handa H *et al.* Multicenter validation of a reproducible flow cytometric score for the diagnosis of low-grade myelodysplastic syndromes: results of a European LeukemiaNET study. *Haematologica* 2012; **97**: 1209–1217.
19 Mitelman F. *ISCN: An international system for human genetic nomenclature*. S. Karger: Basel, Switzerland, 2013.
20 Itzykson R, Kosmider O, Renneville A, Morabito M, Preudhomme C, Berthon C *et al.* Clonal architecture of chronic myelomonocytic leukemias. *Blood* 2013; **121**: 2186–2198.
21 Youden WJ. Index for rating diagnostic tests. *Cancer* 1950; **3**: 32–35.
22 Hosmer D, Lemeshow S. *Applied Logistic Regression*. John Wiley & Sons Inc: New York, NY, USA, 1989.
23 Greenberg PL, Tuechler H, Schanz J, Sanz G, Garcia-Manero G, Sole F *et al.* Revised international prognostic scoring system for myelodysplastic syndromes. *Blood* 2012; **120**: 2454–2465.
24 Kern W, Haferlach C, Schnittger S, Alpermann T, Haferlach T. Serial assessment of suspected myelodysplastic syndromes: significance of flow cytometric findings validated by cytomorphology, cytogenetics, and molecular genetics. *Haematologica* 2013; **98**: 201–207.

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