Analysis of erythroid maturation in the nonlysed bone marrow with help of radar plots facilitates detection of flow cytometric aberrations in myelodysplastic syndromes

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Abstract
Background: Accumulating data support the role of flow cytometry (FCM) in diagnostic work-up of myelodysplastic syndromes (MDS). Changes in erythropoiesis are less documented than in granulopoiesis. However, most studies were performed on bone marrow samples (BMSs) after red blood cell lysis. We have established a FCM protocol for erythropoiesis, following a no-lysis approach and live gate acquisition of nucleated cells using DNA dye DRAQ5.

Methods: The ERY tube consisted of CD36, CD71, CD105, CD117, CD13, and CD45. Comparison with cytomorphological differential counts was carried out in a learning cohort of 80 BMS. To detect aberrations, we analyzed 208 BMS from 135 patients and five normal donors, divided into three cohorts: MDS (n = 68), nonclonal cytopenia (n = 43), and normal controls (n = 29). Radar plot (RP) was created for an overview of normal and aberrant patterns.

Results: The proportion of erythropoiesis in the ERY tube showed better agreement with the cytomorphology, compared to FCM panels on lysed BMS. We confirmed that aberrations in coefficient of variation (CV) of CD36 fluorescence intensity (p < .001), mean fluorescence intensity of CD36 (p = .012), and CV of CD105 (p < .001) can distinguish between MDS and nonclonal cytopenia. RP facilitated evaluation of erythropoietic maturation patterns and aberrant patterns were identified in 85% of MDS patients.

Conclusion: This study provides evidence that a no-lysis approach and RP analysis allow a more reliable evaluation of erythropoiesis and erythroid dysplasia, supporting the integration of FCM erythroid panels in the standard work-up of MDS.

Keywords: erythropoiesis, erythroid dysplasia, flow cytometry, lysis, myelodysplastic syndrome
The differential diagnosis between cytopenia due to myelodysplastic syndromes (MDS) and nonclonal bone marrow (BM) pathology remains a great diagnostic challenge in hematopathology. The WHO 2017 classification (Swerdlow, Campo, Harris, Jaffe, & Pileri, 2017) mandates the integration of clinical data, cytological and pathomorphological examination, and cytogenetic/molecular genetic analyses of blood and BM specimens in MDS work-up. Still, half of MDS patients have no detectable cytogenetic abnormalities by conventional karyotyping (Haase et al., 2007) and 10–20% lack somatic mutations or present with mutations seen in the elderly population with no hematological symptoms (Jaiswal et al., 2014; Steensma et al., 2015).

Despite published elaborate technical and reporting guidelines (Porwit et al., 2014; Van De Loosdrecht et al., 2013), the role of flow cytometry (FCM) in the diagnosis of MDS is described by the WHO as an optional ancillary method (Swerdlow et al., 2017). Several scoring systems mainly based on phenotypical aberrations of the granulocytic/monocytic lineages have been published (Aanei, Picot, Tavernier, Guyotat, & Campos Catafal, 2016; Della Porta et al., 2012; Kern et al., 2016; Kern, Haferlach, Schnittger, & Haferlach, 2010; Malcovati et al., 2005; Matzen, Raaschou-Jensen, & Kallenbach, 2018), but there is some overlap of the described aberrations between MDS and nonclonal cytopenia. The diagnostic power of the scoring systems could be reinforced by a more comprehensive integration of the aberrations of the erythroid lineage (Brodersen et al., 2015; Cremers et al., 2017; Della Porta et al., 2006; Malcovati et al., 2005; Mathis et al., 2013; Westers et al., 2017; Xu et al., 2012).

Knowledge of the normal process of differentiation and maturation of erythropoiesis is essential for the detection and understanding of FCM aberrations in dyserythropoiesis. Previous FCM studies have already defined the immunophenotypic patterns seen during the normal maturation of the erythroid lineage with help of the following markers: CD34, CD117, CD105, CD235a, CD36, and CD71 (Wangen, Eidenschink Brodersen, Stolk, Wells, & Loken, 2014).

The main erythroid lineage associated marker aberrations described in MDS are the increased coefficient of variation (CV) of CD36 and CD71 fluorescence intensity, in combination with a decrease of the mean fluorescence intensity (MFI) of CD36 and CD71, and a decrease or increase of the proportion of immature CD117+ erythroid progenitors (Brodersen et al., 2015; Mathis et al., 2013; Westers et al., 2017; Xu et al., 2012). However, in most of the published studies, various lysis protocols have been applied for the removal of red blood cells (RBCs) before the remaining erythroid precursors were analyzed. Although the lysis protocols were reported not to affect marker expression significantly (Wangen et al., 2014), they do affect light scatter parameters (Wangen et al., 2014; Westers et al., 2017) and lead to an unavoidable destruction of varying amounts of erythroblasts, which may in turn lead to an underestimation of erythropoiesis and an overestimation of other cell compartments, including CD34+ blast cells. There are, to the best of our knowledge, no definitive data in the literature concerning the comparison between lysed and nonlysed samples in the evaluation of the erythroid cell compartment and the proportions of various cell populations.

The aim of this study was to establish an optimal protocol for evaluation of the erythroid lineage differentiation, with which we can reliably define quantitative and qualitative properties of the various erythropoietic cell subpopulations without any substantial loss of erythroid precursors by avoiding lysis (ERY tube). For this purpose, we used markers of different stages of erythroid maturation (CD36, CD71, CD105, CD117) combined with the nucleic acid dye DRAQ5 that selectively binds to nucleated cells (Table S1). Thus, with a live gate set on DRAQ5-positive cells, the erythroid precursors can easily be separated from RBCs, platelets, and cell fragments/debris. DRAQ5 expression also allows for evaluation of cell proliferation within various cell populations.

For comprehensive identification of normal erythroid maturation patterns, we created a radar plot (RP), which provided a combined overview of all applied marker expressions and all identified subpopulations in one plot. We then focused on recognizing deviations from the normal patterns of erythroid maturation in MDS patients as well as in patients with cytopenia of other causes, in order to identify aberrations that could help to differentiate between these conditions.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design

The initial purpose of the ERY tube was to establish an optimal assessment of erythropoiesis by FCM, comparable to the gold standard, that is, the morphological differential count and to avoid the destruction of erythroblasts due to lysis. To this end, we used a learning cohort of 80 BM specimens referred to our department between February and April 2017, with various diagnoses (Table S2) and with representative BM material for both FCM and cytological analysis. Fifteen of these specimens were also analyzed using the ERY tube with and without lysis.

The next step was to investigate which aberrations can discriminate between cytopenia of nonclonal causes and MDS by using the ERY tube. Besides the ERY tube, screening and/or more extensive flow cytometric panels including myeloid markers were also applied as a part of the routine diagnostic work-up (Table S1).

We analyzed a total of 208 BM samples from 135 patients referred between February 2017 and October 2018 for investigation of cytopenia. BM samples from five healthy volunteers were also included. The patients were divided into three cohorts (Table 1): normal controls (NC, \( n = 29 \)), patients investigated for nonclonal cytopenia, designated as hospital controls (HC, \( n = 43 \)) and patients with MDS (MDS, \( n = 68 \)) diagnosed according to the revised fourth edition of the WHO classification of myeloid neoplasms 2016 (Swerdlow et al., 2017). Differential counts were performed on 500 BM cells (by D. V.) without knowledge of the FCM results or the final diagnosis.
For 64 of the 68 MDS patients and for 32 of the 43 HC patients, there were available cytogenetic data, by conventional karyotyping performed at the Department of Clinical Genetics, Lund University Hospital. Molecular genetic analyses were available for 28 MDS and 6 HC patients. The molecular analysis included massive parallel sequencing (MPS) of 54 genes related to myeloid malignancies, performed with the TruSight Myeloid Sequencing panel (Illumina, CA).

Ethical approval for the study was granted by the Regional Ethical Review Board of Lund, Sweden (Nr 223/2017).

2.2 | Patients

The NC cohort included 20 males and 9 females, mean age 55 (range 19–85); criteria of inclusion for this group were normal blood count, morphologically normal hematopoiesis and no previous history of treatment for malignancy. Five specimens were from healthy volunteers (age 19–35) and six were specimens from patients investigated for focal or systemic symptoms (splenomegaly, lymphadenopathy, bone lesions, and weight loss) initially suspicious for lymphoproliferative diseases but without evidence of such after completed medical investigation. Eighteen patients underwent BM biopsy for lymphoma staging. The prerequisites for inclusion of the lymphoma staging patients in the normal cohort were the absence of morphological and FCM evidence of BM involvement by lymphoma and normal blood counts (Table 1).

The hospital controls’ (HC) cohort included 43 patients (22 males, 21 females, mean age 65, and range 18–85) with cytopenia of non-clonal causes, where MDS was not confirmed after completed medical investigation (including genetic analyses for 32 of the 43 patients). The HC patients did not develop MDS during observation time of at least 1 year (Table 1).

The MDS cohort consisted of 68 patients (43 males, 25 females, mean age 72, and range 27–92, Table 1). All the MDS patients had at least one diagnostic specimen before any treatment and 15 of them had one to six follow-up specimens taken under treatment (including chemotherapy and/or BM stem cell transplantation). We further subdivided the MDS cohort into a low risk, LR-MDS (52% of MDS patients, including all the MDS categories with <5% blasts,) and high risk, HR-MDS subgroup (48%, including MDS with excess of blasts [MDS-EB1 and MDS-EB2]).

2.3 | Flow cytometric analysis

BM samples were stained using ERY-panel as described in detail in the Supplementary Data S1. Acquisition was performed on a Navios flow cytometer (Beckman Coulter, Miami, FL) with live gate set on DRAQ5 positive cells (see Supplementary Data S1). Analysis was performed using the Kaluza Analysis Software (Beckman Coulter). The gating strategy is explained in Figure 1. The erythroid

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**TABLE 1** Clinical characteristics and diagnoses or reasons of bone marrow investigation in the three cohorts

| Normal controls NC (n = 29) |  |
|-----------------------------|------------------|
| Age, years, mean (range)    | 55 (19–85)       |
| Sex (M:F)                   | 20:9             |
| Hb, g/L, mean (range)       | 142 (130–158)    |
| WBC, x10^9/L, mean (range)  | 9.0 (3.1–7.5)    |
| Plt, x10^9/L, mean (range)  | 226 (148–331)    |

| Reasons of investigation of the BM |  |
|-----------------------------------|------------------|
| Staging of lymphoma               | 18 (62%)         |
| Focal or systemic symptoms* tested for lymphoproliferative disease | 6 (21%) |
| Healthy volunteers                 | 5 (17%)          |

| Hospital controls (n = 43)         |  |
|-----------------------------------|------------------|
| Age, years, mean (range)          | 65 (18–85)       |
| Sex (M:F)                         | 22:21            |
| Hb, g/L, mean (range)             | 106 (44–153)     |
| WBC, x10^9/L, mean (range)        | 4.2 (0.2–14.1)   |
| Plt, x10^9/L, mean (range)        | 161 (5.0–427)    |

| Diagnostic groups of hospital controls |  |
|----------------------------------------|------------------|
| ACD                                    | 14 (32%)         |
| Anemia due to nutrient deficiency      | 2 (4%)           |
| Hemolytic anemia                       | 2 (4%)           |
| AA/PRCA                                | 4 (9%)           |
| Cytophenias associated with toxicity/ medication other than chemotherapy | 8 (18%) |
| Isolated neutropenia                   | 4 (9%)           |
| ITP                                    | 2 (4%)           |
| Anemia of unknown reason              | 9 (20%)          |

| MDS patients (n = 68)                |  |
|--------------------------------------|------------------|
| Age, years, mean (range)             | 72 (27–92)       |
| Sex (M:F)                            | 43:25            |
| Hb, g/L, mean (range)                | 98 (50–154)      |
| WBC, x10^9/L, mean (range)           | 3.9 (1.5–6.4)    |
| Plt, x10^9/L, mean (range)           | 134 (9–335)      |

| Diagnostic groups of MDS patients according to WHO 2016 |  |
|--------------------------------------------------------|------------------|
| MDS - SLD                                              | 1 (1%)           |
| MDS-MLD                                                | 13 (19%)         |
| MDS-RS-MLD                                             | 15 (22%)         |
| MDS-EB1                                                | 20 (29%)         |
| MDS-EB2                                                | 11 (16%)         |
| t-MDS                                                  | 5 (7%)           |
| MDS with isolated del(5q)                            | 3 (4%)           |

Abbreviations: AA, aplastic anemia; ACD, anemia of chronic disease; ITP, immunologic thrombocytopenic purpura; MDS-SLD, MDS with single lineage dysplasia; MDS-MLD, MDS with multilineage dysplasia; MDS-RS-MLD, MDS with ring sideroblasts and multilineage dysplasia; MDS-EB, MDS with excess blasts; t-MDS, therapy-related MDS; PRCA, pure red cell aplasia.

*Bone lesions, lymphadenopathy, splenomegaly, weight loss.
maturation analysis using the ERY tube is illustrated in Figure 2. In order to visualize the maturation patterns in an integrated way, we constructed a multivariate RP combining all the markers of the ERY tube (Figure 2g), allowing for an overview of the erythroid maturation. The same RP was used to analyze all samples. Optimizing process of RP is described in detail in the Supplementary Data S1 and illustrated in Figure S1. The analysis template was created in the Kaluza (BC) software to ensure uniformity of the analysis. Analysis was performed manually with only minimal adjustment of the gates and without further adjustments of the axes of the RP plot (Figures 1 and 2).

Using the DRAQ5 property of stoichiometric binding to DNA, the fraction of proliferating cells within erythropoiesis could be assessed (Figure S2).

2.4 | Statistical analysis

The agreement of the percentage of erythropoiesis in the ERY tube and in the other FCM panels with that obtained by morphology (regarded as gold standard) was investigated with Bland Altman plots of agreement.

The normal reference ranges of the FCM parameters were defined as the mean ± two SD of the values observed in the normal cohort. Thus, marker expression with MFI or CV or population proportions higher or lower than 2SD from mean were considered as abnormal.

The comparison of the different flow cytometric parameters between the three groups (NC, HC, and MDS) was done with the nonparametric Kruskal–Wallis H test. When the Kruskal–Wallis H

**FIGURE 1** Gating strategy in the ERY tube. (a) The starting point in the analysis of the ERY tube is to gate all the nucleated cells, defined as DRAQ positive, eliminating negative cells and debris. Erythropoiesis is defined by a two-step gating: (b) In the first step, the CD36+/CD45−/dim population is gated. We start with CD36 since its expression precedes that of CD71 (Wangen et al., 2014). Since the most immature erythroid precursors are CD45dim, overlapping partially with the granulocytic precursors, we use a broad gate toward CD45dim. (c) Eventual granulocytic precursors included in this gate are subsequently excluded by gating on the CD71+CD13− cells. (d) All the non-erythroid cells ("Not Ery") are plotted in a CD13/CD45 plot, where granulopoiesis is defined as the CD45dim population with heterogeneous CD13 expression and the lymphocytes as the CD13−CD45bright population [Color figure can be viewed at wileyonlinelibrary.com]
test was statistically significant (i.e., \( p < .05 \)), the analysis was completed with post hoc test (Dunn's procedure with a Bonferroni adjustment) for the pairwise comparisons. The paired samples t-test was used for the comparison of the population proportions and marker expressions in the paired lysed and nonlysed samples analyzed with the ERY tube.

The association of the aberrant FCM patterns with different clinical and other FCM parameters (MDS entities, presence of ring sideroblasts, presence of megaloblastic anemia, the Comprehensive Cytogenetic scoring system CCSS, Ogata score; Della Porta et al., 2012) was tested with the chi-square test of independency.

The statistical analyses were performed with the SPSS software (IBM SPSS statistics 25.0, Chicago, IL).

3 | RESULTS

3.1 | Proportion of erythropoiesis by flow cytometric analysis and morphological differential count

Differential count was performed on 500 cells in adequate BM aspirates in a learning cohort of 80 BM specimens with various diagnoses (Table S2). The percentages of erythropoiesis, granulopoiesis, and of the CD117+ granulocytic precursors were obtained in the ERY tube (nonlysed bone marrow) and in the corresponding screening or myeloid FCM panels (Table S1) using lysed BM samples.

Greater agreement in the proportion of erythropoiesis was observed between the morphological differential count and the ERY tube than the FCM myeloid panels using lysis (Figure 3), where the percentage of erythroblasts was regularly underestimated at a statistically significant level (morphology vs. FCM panels using lysis \( p < .05 \); Figure 4a). The difference in frequency of erythropoiesis was not significant between morphology and the ERY tube \( p = .305 \). Conversely, granulopoiesis and the early granulocytic precursors were usually over-represented in the lysed BM specimens compared to the morphological assessment (Figure 4b,c). The difference was statistically significant \( p < .05 \) between morphology and FCM panels using lysis for both the percentage of granulopoiesis and the early granulocytic precursors. Comparing the ERY tube with morphology, the difference was not significant regarding the early granulocytic precursors \( p = .632 \) but turned out significant regarding the proportion of granulopoiesis \( p < .05 \). Similar results were observed when these comparisons were performed in all samples and for each of the three cohorts separately (Figure S3, data shown only for erythropoiesis).

In contrast, the early CD117+ erythroid compartment was preserved in the FCM panels after lysis or even over-represented compared to the ERY tube (Figure S4). This finding was consistent in all cohorts and indicates that the discrepancy in the percentage of erythropoiesis in the panels using lysis depends on the destruction of the more mature erythroblasts.
3.2 | Quantitative and phenotypical differences between lysed and nonlysed samples analyzed with the ERY tube

To better illustrate how lysis affects the percentage of erythropoiesis we analyzed 15 BM specimens with various diagnoses with the ERY tube without lysis and after lysis. There was a consistent decrease of the fraction of erythropoiesis \( (p < .001; \text{Figure S5a}) \) and an increase in the fraction of granulopoiesis \( (p < .001; \text{Figure S5b}) \) in the lysed samples compared to the nonlysed ones. Fractions of the CD117\(^+\) myeloid precursors and lymphocytes were similar in the lysed and nonlysed samples \( (p > .05) \).

We also investigated the effect of lysis on scatter parameters and marker expression. In the lysed samples, most erythroblasts showed a strongly diminished side and forward scatter, ending up outside the area that usually defines the live gate (Figure S5c). In the lysed samples, we also observed significantly lower values for the MFI and CV of CD36 (both \( p < .001 \)), MFI and CV of CD71 (MFI \( p = .031 \), CV \( p < .001 \)) and for MFI and CV of CD117 (MFI \( p = .003 \), CV \( p < .001 \)). The MFI of CD105 showed statistically significant difference between the lysed and nonlysed samples while CD105 CV did not differ \( (MFI p = .007) \).

3.3 | Quantitative and phenotypic aberrations in erythropoiesis discriminating MDS patients from patients with nonclonal cytopenia

Based on the frequent morphological finding of expansion and left shift of the erythroid compartment in the BM of MDS patients, we
first explored quantitative parameters, including the proportion of the erythropoiesis, of the immature precursors and the proliferating erythroblasts (Table 2). The percentage of erythropoiesis was increased in MDS patients compared to NC ($p = .044$). The early CD117$^+$ erythroid precursors were decreased in MDS patients compared to NC ($p = .001$) and not significantly different by comparison to HC ($p > .05$, Table 2).

No statistically significant difference was observed in the next stadium of early precursors (CD117$^-$CD105$^+$) between MDS, HC, and NC. When the proliferating fraction (defined as DRAQ5$^{bright}$, Figure S2) of the total erythropoiesis was compared between the three cohorts, lower proliferation was observed in the MDS patients compared to NC ($p < .001$).

The most frequent phenotypical aberrations detected in the erythroid lineage in the MDS group compared to both NC and HC was an increase of CD36 CV (MDS vs. NC $p < .001$, MDS vs HC $p < .001$) seen in 29% of MDS patients ($n = 20$) and a decrease of CD36 MFI (MDS vs. NC $p = .004$, MDS vs. HC $p = .012$) in 25% of MDS patients ($n = 17$). When we compared the NC and HC groups with the low- and high-risk MDS subgroups separately, these differences proved significant only compared to the HR-MDS subgroup (for CV CD36 HR-MDS vs. NC $p < .001$; for MFI CD36, HR-MDS vs. HC $p = .003$), but not to the LR-MDS subgroup.

There was no statistically significant difference in CD71 CV or MFI in MDS compared to NC and HC (Table 2). The results remained nonsignificant when the comparison was done within the LR- and HR-MDS subgroups separately.

The diminished expression of CD36 with or without diminished CD71 expression, leads to a distinct aberrant pattern (Figure 5c) was observed in 25% of the MDS patients ($n = 17$) and leading to a distinct aberrant pattern (Figure 5c) was a diminished expression of CD105 in the CD117$^+$ early erythroid precursors, accompanied by an increase in the CV of CD105 in this population. This pattern was observed only in 4% of the HC ($n = 2$), significantly less often than the MDS group ($p < .001$). Regarding the total fraction of the CD105$^+$ erythroid precursors (irrespective of CD117 expression), there was a statistically significant difference in CD105 CV between HC and the whole MDS group ($p < .001$; Table 2), and between HC and the HR-MDS subgroup ($p < .001$) but not between HC and the LR-MDS. The CD105 MFI for the total CD105$^+$ precursor population did not show any significant difference between HC and the whole MDS cohort or HR-MDS subgroup. However, it was significantly different between HC and the LR-MDS subgroup ($p = .031$). There were also some highly aberrant cases with a complete loss of CD105 expression in the CD117$^+$ early erythroid compartment (Figure 5d).

3.4 Diminished expression of CD36 on nucleated red cells reflects asynchronous maturation in MDS

We wanted to further investigate the finding of the “aberrant subpopulation,” with diminished expression of CD36 with or without a diminished expression of CD71, which was observed in a large number (66%) of MDS specimens (Figure 6c,d), and also in 5 of 43 HC, mostly with megaloblastic changes. We assumed therefore that these
erythroblasts have mature cytoplasm with low CD36 and CD71 expression on the cell surface, similar as reticulocytes, but still carry a nucleus. This population may represent the phenomenon of asynchronous maturation typical of megaloblastic changes. To further investigate this interpretation, we analyzed 27 whole, nonlysed BM specimens (11 NC and 16 MDS; Figure S6) with the ERY tube and during acquisition the gate was set using forward scatter instead of DRAQ5-positive events. Thus, we collected all cells in the nonlysed specimen including RBCs. In a normal BM, the expression of CD36 diminishes gradually in the nucleated erythroid cells (NEC) and could predict MDS with 85.3% specificity and 81.7% sensitivity. This pattern was observed in five HC patients (11%). Interestingly, four of these samples were from patients with megaloblastic anemia due to B12 deficiency or toxic effect of methotrexate. This pattern showed a statistically significant difference of incidence between MDS and HC patients (Table 2). There were also statistically significant differences between LR- and HR-MDS subgroups. This pattern was observed in 25% of the MDS patients (n = 17) and in 19% of the HC patients (n = 8), with no statistically significant difference between the two groups (p = .946). There was no overrepresentation of any specific diagnosis in the HC patients showing this pattern.

The third and most common pattern, “the aberrant subpopulation” pattern (Figure 6c) was characterized by the presence of an aberrant erythroid subpopulation with diminished expression of CD36 with or without simultaneous diminished expression of CD71. This was the dominant pattern seen in 66% of the MDS patients (n = 45), and in 19% of the HC patients (n = 21). This pattern was observed in five HC patients (11%). Interestingly, four of these samples were from patients with megaloblastic anemia due to B12 deficiency or toxic effect of methotrexate. This pattern showed a statistically significant difference of incidence between MDS and HC patients (Table 2) and could predict MDS with 85.3% specificity and 81.7% sensitivity. There were also statistically significant differences between LR- and HR-MDS subgroups and the HC cohort (p < .001 for both comparisons).

3.5 | Aberrant patterns of erythroid differentiation in MDS visualized in radar plots

Radar plots presentation of erythropoiesis in BM samples from the MDS group showed frequent deviation from normal maturation patterns (85% of all MDS patients).

We identified three main patterns: The first one, left shift or “tail-heavy” (Figure 6a), was seen in 20% of MDS patients (n = 14), where the early precursors (CD117+ and CD117-CD105+) were increased as compared to the normal reference ranges. The same pattern was observed in three HC patients (7%). The incidence of this pattern did not differ significantly between MDS and HC (p = .266).

The second pattern, right shift or “heads-heavy” (Figure 6b), was characterized by a decrease of the early precursors. This pattern was observed in 25% of the MDS patients (n = 17) and in 19% of the HC patients (n = 8), with no statistically significant difference between the two groups (p = .946). There was no overrepresentation of any specific diagnosis in the HC patients showing this pattern.
3.6 | Aberrant erythropoietic FCM patterns in MDS in relation to morphological dyserythropoiesis and other FCM aberrations

Of the 68 MDS patients, none had a completely normal erythropoiesis on cytological evaluation. Thirteen MDS patients (19%) had only slight cytomorphological changes in the erythroid lineage (<10% of erythropoietic dysplasia, thus nonsignificant according to WHO criteria), despite concomitant significant dysplasia in the granulocytic or megakaryocytic lineage and/or blast excess. Of these 13 patients, nine did show aberrant FCM patterns in the ERY RP but no predominant pattern was observed. Three of the nine patients had MDS del(5q), four had MDS-EB1 and two MDS-MLD. The MDS-EB1 and the MDS-MLD patients showed a variety of karyotypes (two with normal, two with complex, one with del(20q) and one not available), not allowing for any conclusions about possible correlations. NGS data were available only for one of these nine cases.

We had in the cohort seven other MDS cases without diagnostic morphological changes (<10% dysplasia) in any lineage, no blast excess and a low Ogata score (1 or 2) in the routine FCM analysis. Six cases were diagnosed as MDS based on cytogenetic and/or molecular genetic findings and the clinical picture, and one was a therapy-related MDS. Of these, four cases showed aberrant FCM patterns in erythropoiesis. The cytogenetic and NGS findings in these four patients were as follows: trisomy 11 (NGS N/A), del(20q) (NGS N/A), del(7q) (NGS N/A), and a normal karyotype with TET2 mutation.
Hence, among the total 20 MDS-patients with only borderline morphological erythroid dysplasia, 13 (65%) displayed aberrant FCM patterns of erythropoiesis. We also considered the correlation between low Ogata score (1 or 2) and aberrant erythroid patterns by FCM. In our cohort, 24 (35%) MDS patients had a low Ogata score (1 or 2), without blast increase (<2%). Of these, 21 (88%) had aberrant patterns in the erythroid lineage. Thus, for the “low Ogata score” group (n = 24), the specificity and sensitivity of the combination of Ogata score with aberrant erythropoiesis were 89% and 68%, respectively, whereas the specificity and sensitivity of the Ogata score alone were 83% and 52%, respectively.

Of the 68 MDS patients, seven (10%) had no FCM abnormalities in the myeloid/monocytic lineage analysis (data not shown). Of these, four patients (6% of MDS) showed aberrant FCM patterns in the erythroid lineage. Regarding morphology, two of the cases were MDS-RS-MLD and two were MDS-MLD with significant dysplasia in at least two lineages. Cytogenetics (but not molecular data) were available for three of these seven cases: one with normal karyotype, one with trisomy 21, and one with deletion of 11q.

3.7 Association of the aberrant flow cytometric patterns with clinical parameters in MDS patients

We subsequently investigated whether the three aberrant radar plot patterns and the presence of a CD105-CD117+ population as a
fourth aberrant feature would correlate with any specific clinical characteristics of the MDS patients: MDS entity according to the WHO classification, low- and high-risk subgroups, cytogenetic risk groups (as defined by the Comprehensive Cytogenetic scoring system, CCSS), presence of ring sideroblasts, presence of megaloblastic RBCs (defined as MCV > 100 fL), and with the Ogata score. None of the aberrant FCM patterns showed a statistically proven association with the above parameters (data not shown). The relatively small number of MDS patients (28/68) with available molecular genetic data and the heterogeneity of the various mutations did not allow for a statistical comparison of sufficient power in the current study.

3.8 Effect of treatment on the erythropoietic maturation pattern

Fifteen (22%) of the MDS patients had follow-up specimens under treatment with azacytidine or chemotherapy and six underwent allogenic stem cell transplantation. Although the number of the specimens is too small to lead to definitive conclusions, the preliminary observations are that indeed under therapy, the pattern of maturation changes (Figure S7a). As expected, the pattern normalizes after stem cell transplantation (Figure S7b).

4 DISCUSSION

We have validated a protocol (the ERY tube) for the evaluation of the erythroid lineage differentiation without any substantial loss of erythroid precursors. Our results illustrate that lysis significantly affects the proportion of erythropoiesis in FCM analyses and are consistent with previous observations that highlighted the effect of lysis on the morphology and scatter characteristics of erythroblasts (Mathis et al., 2013; Wangen et al., 2014). Since the early CD117+ erythroid precursors were not affected in the panels using lysis, one can speculate that the decreased fraction of erythropoiesis in lysed BM samples is mainly due to the destruction of the mature erythroblasts. This would be in agreement with the fact that ammonium chloride lysis largely depends on the action of the chloride-bicarbonate exchanger AE1/band 3, which is continually synthesized during maturation of the erythrocyte (mRNA levels peak during the polychromatophilic stage and synthesis of the protein continues until the orthochromatic stage).

Focusing on known erythroid markers, we established the normal maturation pattern of erythropoiesis and continued with the detection of deviations associated with MDS and discriminating MDS patients from patients with cytopenias of nonclonal causes.

We were able to confirm that the previously described aberrant expression of CD36 is significantly associated with MDS (Brodersen et al., 2015; Mathis et al., 2013; Westers et al., 2017). Interestingly, by using the DRAQ5 dye in whole, nonlysed BM samples and collecting all cells including erythrocytes and reticulocytes we could show that a diminished expression of CD36 in erythroblasts in MDS patients paralleled that of the reticulocytes. In addition, aberrant expression of CD36 was observed in five HC, four of which had B12 deficiency or toxic effect of methotrexate treatment. We can assume therefore that it may reflect the phenomenon of asynchronous maturation or, in morphological terms, of megaloblastic changes (Figure S6). This means that, at least on terms of FCM analyses, it can be difficult to distinguish MDS from megaloblastic anemia (although clinically, this differential diagnosis is rarely a challenge).

Regarding CD71 expression, we could not confirm that this marker on its own is a reliable discriminator between MDS and nonclonal cytopenias. However, a combination of low CD36 and CD71 expression resulted in an easily discernible aberrant population seen in radar plots. CD71 has been highlighted by other studies as useful and has been included in the RED score (Mathis et al., 2013; Westers et al., 2017; Xu et al., 2012). The difference between ours and previous studies could be, at least partially, explained by the effect of lysis on CD71 expression.

An additional distinct aberrant pattern that we observed in MDS patients that significantly differentiated them from HC was a diminished expression of CD105 in the earliest CD117+ erythroid precursors. The CV of CD105 expression in all CD105+ erythroid precursors was also proven to be of significance in the distinction between MDS and nonclonal cytopenias. CD105 expression, especially its increase, has also been highlighted by others (Brodersen et al., 2015; Della Porta et al., 2006; Xu et al., 2012) as an important marker for the differential diagnosis between MDS and non-clonal cytopenias. Similar results were presented by the large multicentric study by IMDSFlow working group (Westers et al., 2017). However, CD105 was not included in the proposed score, because data were available only from a limited number of centers. The observation that there was no difference in the CV of CD105 when we compared lysed and nonlysed samples suggests that this marker is stable and not affected by lysis. Our results provide therefore further support for the use of CD105 CV in the differential diagnosis of cytopenias.

Regarding the proportion of the early CD117+ erythroid compartment, we did see MDS cases with both increased and decreased population but did not observe any statistically significant difference between whole MDS and HC cohorts. Still, abnormal percentages of cells in this subpopulation (both increase and decrease) have been proposed (Cremers et al., 2017; Westers et al., 2017) as distinguishing between MDS and nonclonal cytopenia. Our results indicate that the CD117+ early erythroid precursors are not affected by lysis (Figure S4). This may partially explain the observation of increased early erythroid precursors in MDS patients in FCM studies using lysed BM samples. Interestingly, a recent study (Raimbault et al., 2019) that followed the no-lysis method suggested by Mathis et al. (Mathis et al., 2013) has associated the increase of this compartment with better response to erythropoiesis-stimulating agents. This indicates that more studies adopting the no-lysis approach are needed in order to define markers with diagnostic and even prognostic value in the FCM work-up of cytopenia.

We have previously characterized blast region in normal bone marrow and in MDS patients using multivariate radar plots (Jafari
et al., 2018). In the present study, we illustrate the normal erythroid maturation on conventional bivariate dot plots and display the normal maturation pattern on RP. Both methods can be used to assess distribution of different subsets of the erythroid population, considering optimal acquisition of erythroid lineage using a no-lyse method. RP is a graphical display making it easier to evaluate of the FCM aberrancies (Jafari et al., 2018). We recognized three different abnormal erythropoietic patterns by using RP. The left shift or “tail-heavy” represents an increase of early CD117+ erythroid progenitors, the right shift or “heads-heavy” represents a decrease of early erythroid progenitors and the “aberrant subpopulation,” refers to the cluster of CD36+/dim ± CD71−/dim erythropoietic cells. Only the “aberrant subpopulation” pattern showed a statistically significant association with MDS compared to HC, reflecting the most reliable distinguishing marker, that is, aberrant CD36 expression, which often coincides with lower CD71 expression. Even though the two other patterns were not specific for MDS, they may contribute to the diagnosis when integrated with other findings. We believe that RP provide a comprehensive display of erythroid lineage distribution and maturation, which can be used for diagnosis and treatment follow-up.

An additional advantage of the ERY tube and the no-lysis approach is more accurate appreciation of the percentage of CD117+ myeloid blasts in borderline cases between MDS-EB2 and AML. This may have practical consequences while taking both results of cytological assessment and FCM into account before the final diagnosis.

Importantly, FCM evaluation of dyserythropoiesis seems to give additional information in some cases when morphology does not fulfill the WHO criteria for significant dysplasia. Among 20 MDS cases in our cohort without significant dysplasia by morphology, 13 (65%) could be interpreted as aberrant by virtue of their FCM patterns. Thus, FCM may be valuable for additional assessment of dyserythropoiesis when morphological assessment alone is not diagnostic. Interestingly, all three MDS del(5q) cases showed FCM abnormalities, but only slight morphological abnormalities in the erythroid lineage consistent with the diagnosis (Swerdlow et al., 2017). It is tempting to speculate that FCM could provide evidence of dyserythropoiesis in this MDS category. Furthermore, in cases with a low Ogata score (1 or 2, without blast increase), adding an aberrant FCM panel could increase both the specificity and sensitivity of assessment concerning myelodysplasia-related abnormalities.

In summary, we have established the FCM protocol that preserves the total erythroid compartment and allows the collection of robust data for the immunophenotypic assessment of MDS. This study provides evidence that the no-lysis approach is more reliable for evaluation of erythropoiesis and of erythroid dysplasia than protocols with lysis. In addition, it reinforces the value of erythroid markers, mainly of CD36 and of CD105, for the assessment of dyserythropoiesis, supporting the integration of FCM erythroid panels in the standard work-up of cytopenia and MDS, complementary to the established myeloid panels. Incorporation of radar plots facilitates quick visualization of the maturation pattern and detection of aberrant patterns in erythropoiesis, which can be used both for diagnostic purposes and in the follow-up of patients treated for MDS. The major drawback of our study is the lack of correlation with mutational analysis, but this was not possible due to low number of cases with available data. We are now following a new cohort where this kind of correlation will be possible.

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