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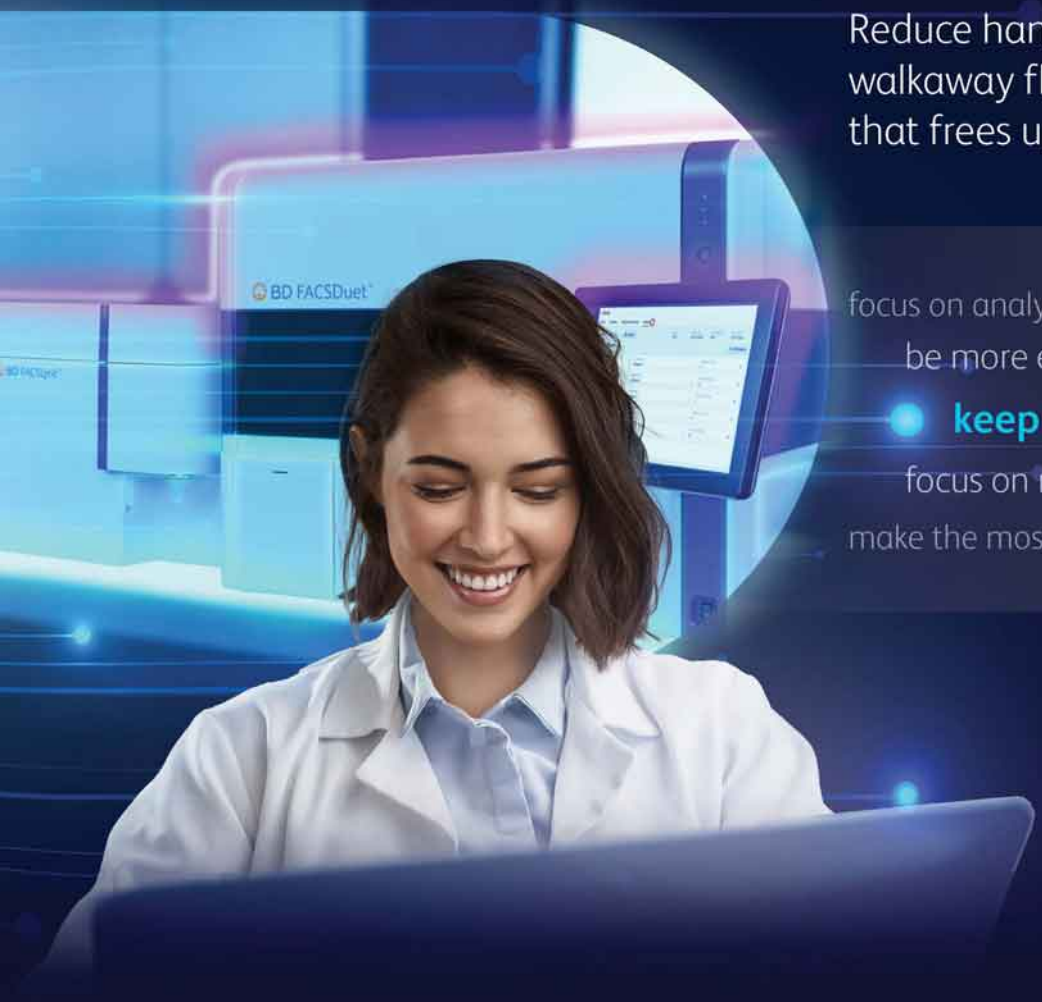

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REVIEW ARTICLE

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Multiparameter flow cytometry in the evaluation of myelodysplasia: Analytical issues

Recommendations from the European LeukemiaNet/International Myelodysplastic Syndrome Flow Cytometry Working Group

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Abstract

Multiparameter flow cytometry (MFC) is one of the essential ancillary methods in bone marrow (BM) investigation of patients with cytopenia and suspected myelodysplastic syndrome (MDS). MFC can also be applied in the follow-up of MDS patients undergoing treatment. This document summarizes recommendations from the International/European Leukemia Net Working Group for Flow Cytometry in Myelodysplastic Syndromes (ELN *i*MDS Flow) on the analytical issues in MFC for the diagnostic work-up of MDS. Recommendations for the analysis of several BM cell subsets such as myeloid precursors, maturing granulocytic and monocytic components and erythropoiesis are given. A core set of 17 markers identified as independently related to a cytomorphologic diagnosis of myelodysplasia is suggested as mandatory for MFC evaluation of BM in a patient with cytopenia. A myeloid precursor cell ($CD34^{+}CD19^{-}$) count >3% should be considered immunophenotypically indicative of myelodysplasia. However, MFC results should always be evaluated as part of an integrated hematopathology work-up. Looking forward, several machine-learning-based analytical tools of interest should be applied in parallel to

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conventional analytical methods to investigate their usefulness in integrated diagnostics, risk stratification, and potentially even in the evaluation of response to therapy, based on MFC data. In addition, compiling large uniform datasets is desirable, as most of the machine-learning-based methods tend to perform better with larger numbers of investigated samples, especially in such a heterogeneous disease as MDS.

KEYWORDS
consensus, ELN, flow cytometry, myelodysplastic syndromes, standardization

1 | INTRODUCTION

The International/European LeukemiaNet (ELN) Working Group for Flow Cytometry in Myelodysplastic Syndromes (ELN *i*MDS Flow) has previously published several papers proposing harmonization of multiparameter flow cytometry (MFC) and integration of this methodology in the diagnostic work-up of patients with suspected myelodysplastic syndrome (MDS) and myelodysplastic-myeloproliferative neoplasms (MDS/MPN) (Porwit et al., 2014; van de Loosdrecht et al., 2009; van de Loosdrecht et al., 2013; Westers et al., 2012; Westers et al., 2017). MFC performed according to *i*MDSFlow has been recommended by ELN guidelines at diagnosis and following therapy of MDS patients (Malcovati et al., 2013) and by the MDS Consensus Group (Valent et al., 2017). Multiple reports from various countries have confirmed the diagnostic value of *i*MDSFlow recommendations (Chauhan et al., 2021; Cremers et al., 2016; Cremers et al., 2017; Davydova et al., 2021; Grille Montauban et al., 2019; Majcherek et al., 2021; Porwit & Rajab, 2015; Takeuchi et al., 2020). However, results of a survey concerning current MFC practice in 229 laboratories around the world showed that although many laboratories used large numbers of markers in MFC workup of MDS (median: 20 ± 4.5), the compliance with *i*MDSflow recommendations was low, and proposed scoring systems were not widely applied (Grille Montauban et al., 2019; Jensen et al., 2019). With the hope of increasing the harmonization of MFC MDS diagnostics, the current paper presents a summary of the progress in this field and an update on consensus *i*MDSFlow guidelines for the assessment of significant anomalies in various bone marrow (BM) cell compartments for MFC features of dysplasia as a part of a special Issue of Clinical Cytometry B, focused on MFC applications in MDS and MDS/MPN (Kern et al., 2022; van de Loosdrecht et al., 2023; van der Velden et al., 2023; Wagner-Ballon et al., 2023; Westers et al., 2021; Westers et al., 2023).

2 | MINIMAL REQUIREMENTS TO ASSESS DYSPLASIA BY MFC

A myelodysplastic syndrome may be suspected in any individual presenting with cytopenia that cannot be attributed to any other known condition. Cytological assessment of BM films remains the first examination in the search for possible hemopoietic dysplasia. MFC will complement the primary morphological evaluation, especially in borderline cases where immunophenotypic aberrations can support or exclude MDS diagnosis (as illustrated in a diagnostic algorithm presented in Figure 1 in van de Loosdrecht et al., 2023). The previous *i*MDSflow recommendation that the four-parameter Ogata score (Ogata et al., 2009) may be used for a preliminary assessment of MFC dysplasia has been confirmed by several publications (Bardet et al., 2015; Dhingra et al., 2020; Grille Montauban et al., 2019; Kárai et al., 2017; Mannelli et al., 2019; Matzen et al., 2018; Muyldermans et al., 2019; Rajab & Porwit, 2015). This score is based on the frequency of myeloid $CD34^+$ myeloid progenitors (MP) (>2%), the fraction of B-cell precursors within $CD34^+$ cells (<5%), abnormal CD45 expression on $CD34^+$ blasts, and low granulocyte scatter (granulocyte/lymphocyte SSC ratio ≤6). In the first publication, the frequency of B-cell precursors was evaluated using gating based on forward vs. side scatter properties (Ogata et al., 2009). However, most laboratories nowadays include CD19 to increase precision (Guo et al., 2020; Matzen et al., 2018; Rajab & Porwit, 2015). The four parameters carry different weights in the evaluation of dysplasia. Increased frequency of $CD34^+$ MP and low side scatter (SSC) of granulocytes are more specific than the decreased fraction of $CD34^+$ B-cells and altered CD45 expression of $CD34^+$ MPs (Bardet et al., 2015; Rajab & Porwit, 2015). The Ogata scores with a cut-off ≥2 have diagnostic and prognostic values (Della Porta et al., 2012; Della Porta et al., 2014). Scores of 2 are observed relatively often in patients with non-clonal cytopenias, while scores 3 or 4 are significantly associated with cytomorphological myelodysplasia and MDS or MDS/MPN diagnosis (Rajab & Porwit, 2015; van Gammeren et al., 2018).

For a detailed evaluation of dysplasia, comprehensive panels including evaluation of MP immunophenotype, myeloid and monocytic maturation and evaluation of erythropoiesis are recommended (Eidenschink Brodersen et al., 2015; Oelschlaegel et al., 2021; Porwit et al., 2014; Selimoglu-Buet et al., 2015; Violidaki et al., 2020; Westers et al., 2012; Westers et al., 2017; Zhu et al., 2019). Alterations of antigen expression should always be evaluated in the context of clinical data, morphological changes, and cytogenetic/molecular findings.

3 | MYELOID PROGENITOR CELLS

The $CD45^{dim}/SSC^{low/int}$ BM cell compartment (a "blast gate" or "MP gate" on a CD45/SSC plot) in normal BM has been characterized as a

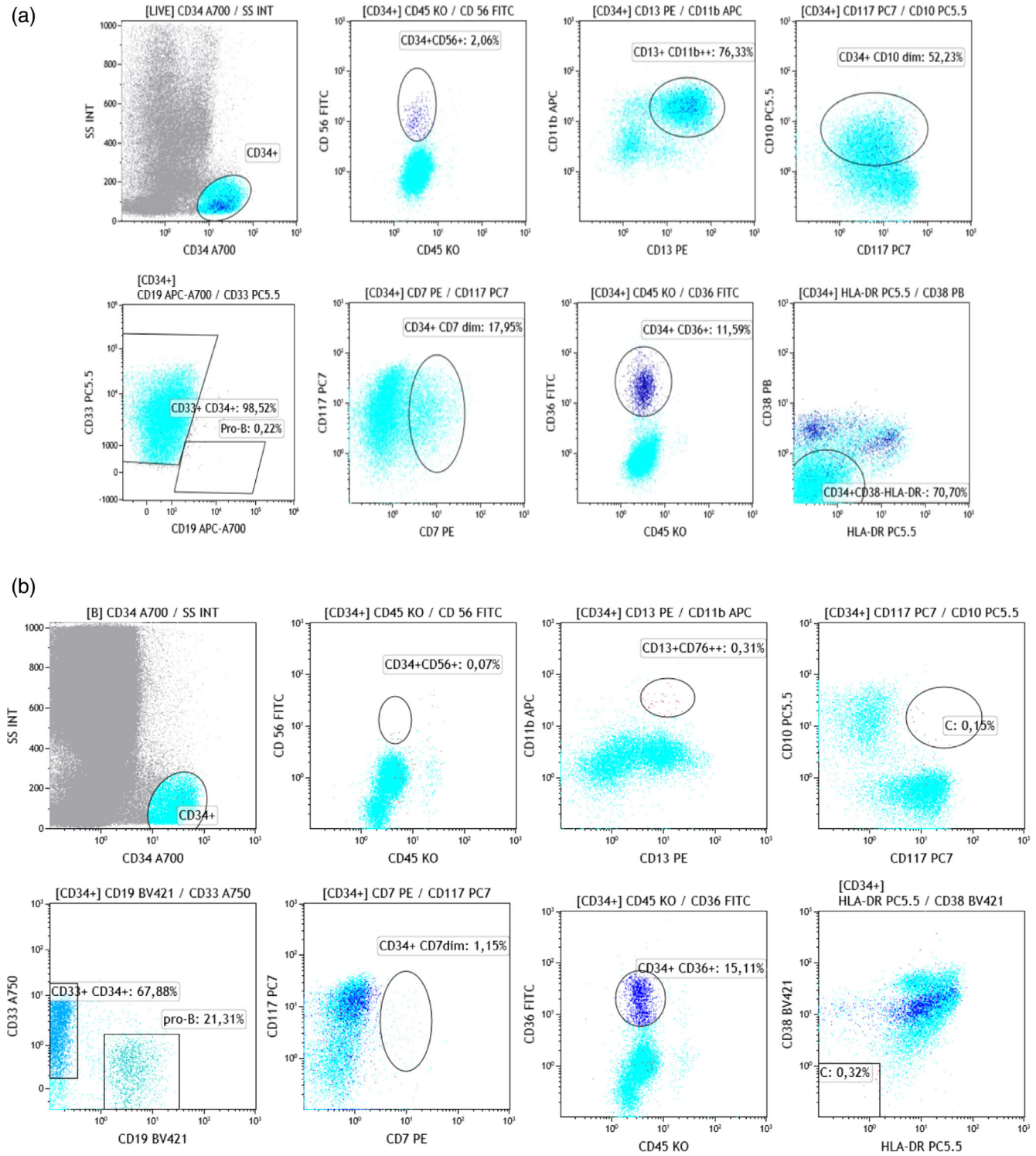

FIGURE 1 Aberrant immunophenotype of myeloid precursors in a case of MDS with excess of blasts in comparison to normal BM pattern. Previously published panels (Violidaki et al., 2020) were applied. Debris was removed and singlets were gated resulting in the "LIVE" gate (not shown). (a) MDS case: $CD34^+$ cells were gated on the SSC/CD34 plot (cyan dots, upper left plot). A small population of $CD56^+$ $CD34^+$ cells (blue dots) was identified (upper, middle-left plot). $CD34^+$ cells were positive for CD11b, CD13, CD117, CD10 (upper middle-right and right plots) and CD33, but negative for CD19 (lower left plot). A small population of $CD7^+$ $CD34^+$ cells was found (lower middle-left plot). Most of $CD34^+$ cells we negative for CD38 and HLA-DR, and a subset of $CD36^+$ $CD34^+$ cells was detected, localized with the $CD38^+$ subset and partly HLA-DR positive (lower right plots). (b) Corresponding plots obtained with the same panel in a normal bone marrow sample showing normal immunophenotypic profile of $CD34^+$ cells. The frequency of cells found in the areas of aberrant immunophenotypes in (a) is indicated

heterogeneous population where progenitor cells of various lineages reside (Arnoulet et al., 2010; Borowitz et al., 1993; Jafari et al., 2018). In MDS, the frequencies of various cell populations in this area change, and MPs predominate while other cell subsets decrease (Jafari et al., 2018; Matarraz et al., 2008). According to Mattarz et al., at least 3×10^3 $CD34^+$ cells should be analyzed for a thorough analysis of the $CD34^+$ compartment (Matarraz et al., 2008). In the ELN *i*MDS Flow experience (van der Velden et al., 2023), a minimum of 100,000 WBCs should be acquired per tube with a minimum of 250 $CD34^+$ cells.

An increase in hematopoietic precursor cells (HPC) and MPs (usually defined as $CD45^{dim}SSC^{low/int}$ $CD34^+CD19^-$) above 2% of total BM nucleated cells is a common finding in MDS that also has prognostic significance (Matarraz et al., 2010; Vido-Marques et al., 2020; Xu et al., 2013). The multicenter study of the ELN *i*MDSFlow WG (Kern et al., 2022) confirms that 3% MPs is a critical cut-off in MFC, above which most cases are MDS or MDS/MPN (whenever acute leukemia is excluded).

Immunophenotypical abnormalities often found in the MP compartment in MDS patients are summarized in Table 1. In some MDS patients, abnormally low CD34 expression in $CD117^+$ precursors can be found, despite increased numbers of cells in the MP region ($CD45^{dim}SSC^{low/int}$). Thus, CD117 expression should also be evaluated both on $CD45^{dim}SSC^{low/int}CD34^+CD19^-$ cells and in the $CD45^{dim}/SSC^{low/int}$ BM compartment (Alhan et al., 2016; Matarraz et al., 2010). An increased CD117 expression is frequently seen in the MP compartment in MDS, and it is one of the MFC variables associated with worse survival (Alhan et al., 2016; Shen et al., 2015). Thus, evaluation of the CD34/CD117 expression ratio in the MP compartment is recommended (Table 1).

Examples of abnormal findings in the MP compartment and corresponding normal BM patterns are illustrated in Figure 1a,b. Overexpression of CD34 and abnormal expression of CD117, CD45 and CD7 were the most frequently encountered findings in the ELN *i*MDSFlow multicenter study (Kern et al., 2022). Although not included by enough participating laboratories in this study, CD38 and CD123 are also of interest since the increased frequency of $CD34^+CD123^+CD38^-$ cells may be associated with increased numbers of cells with leukemia stem-cell characteristics (Al-Mawali et al., 2016; Li et al., 2014). Except for the low frequency of $CD34^+$ B-cell precursors in the MP compartment, CD19 was the least frequent aberrantly expressed marker in the multicenter study.

Aberrant marker expression in the $CD34^+$ compartment is found not only in MDS but also in MDS/MPN, such as chronic myelomonocytic leukemia (CMML). Abnormalities include an increase of $CD34^+$ cells, reduced $CD34^+$ B-cell precursors, increased intensity of expression for CD34, CD13, CD117, and CD123, decreased expression of CD38, aberrant expression of CD2, CD5, CD7, and CD56, and asynchronous expression of CD15 or CD64 (Shen et al., 2015). Of note, aberrancies in the MP compartment may persist after therapy with hypomethylating agents (Shen et al., 2015) and can be occasionally seen in patients after cytostatic therapy or stem-cell transplantation for other malignancies such as B-cell acute lymphoblastic leukemia (Kriegsmann et al., 2018).

TABLE 1 Aberrant multiparameter flow cytometry features in a myeloid progenitor cell population ($SSC^{low}/CD45^{dim}$) in BM samples of patients with myelodysplastic syndrome

Marker/pattern	Aberrant feature	References[a]
CD45	Increased CD45dim population	Stetler-Stevenson et al. (2001); Maynadié et al. (2002); Truong et al. (2009); Della Porta et al. (2011)
CD34	Increased number in BM (>3%)	Malcovati et al. (2005); Ogata et al. (2006); Matarraz et al. (2008); van de Loosdrecht et al. (2008); Kern et al. (2022)
CD34	Increased number of $CD34^{bright}$ cells	Maynadié et al. (2002); Pirruccello et al. (2006)
CD34/CD117 ratio	Aberrant ratio may be caused by lack of $CD34^+$ population or decreased $CD34^+CD117^+$ population or increased $CD34^-/CD117^+$ population	Matarraz et al. (2008); (2010); Truong et al. (2009); Stachurski et al. (2008); Alhan et al. (2016)
$CD34^+$/CD38	Increased frequency of $CD38^{-/dim}$ CD34 cells	Kussick et al. (2005); Monreal et al. (2006); Goardon et al. (2009); Xie et al. (2010); Tang et al. (2012)
$CD34^+$/CD45	Increased number of $CD45^-$ $CD34^+$ cells	Kussick et al. (2005); Scott et al. (2008)
CD34+/CD123+	Increased number of $CD123^+$ $CD34^+$ cells	Tang et al., 2012
$CD34^+$/CD19	Decreased proportion of $CD34^+/CD19^+$ lymphoid progenitors	Malcovati et al. (2005); Matarraz et al. (2010)
$CD34^+$ /HLA-DR	Increased proportion of $HLA\text{-}DR^{neg/dim}$ $CD34^+$ progenitors	Kussick et al. (2005); Scott et al. (2008)
CD13/CD33	Increased numbers of $CD13^+/CD33^-$ or $CD13^-/CD33^+$ progenitors	Scott et al. (2008); Harrington et al. (2010); Chu et al. (2011)
CD2, CD4, CD5, CD7[b], CD56	Aberrant expression on $CD34^+$ and or $CD117^+$ progenitors	Stetler-Stevenson et al. (2001); van de Loosdrecht et al. (2008); Kern et al. (2010)
CD11b[c]	High expression on $CD34^+$ cells	Kern et al. (2010)

[a]Examples of references that reported this feature.
[b]Minimal $CD34^+/CD7^+/$ $CD13^{low}$ populations (<0.1%) can be seen in regenerating bone marrow.
[c]Can be increased in patients treated with G-CSF.

BM immunophenotyping in MDS is usually performed using antibody combinations to detect abnormal patterns in immature (MPs) and mature cell compartments. Multicolor (8–10) color panels are recommended and examples of such panels from the literature are presented in a separate paper from the ELN *i*MDS Flow WG on preanalytical aspects (van der Velden et al., 2023). The absence of aberrant MPs at diagnosis and/or a decrease in the abnormalities during treatment identified Intermediate-2 risk and High-risk (Greenberg et al., 2012) MDS patients who are likely to respond to treatment with azacitidine (Alhan, Westers, van der Helm, et al., 2014; Subirá et al., 2021).

In summary, based on data from the literature and the results of the multicenter study of the ELN *i*MDSFlow WG (Kern et al., 2022), evaluation of MPs for MDS should include their quantification with a cut-off of >3% $CD34^{+}$ cells (of viable BM cells), which is strongly indicative of MDS or MDS/MPN on its own. Moreover, the assessment of CD45, CD117, HLA-DR, CD13, CD5, CD7, and CD56 in the MP compartment is recommended since aberrant expressions of these markers have been demonstrated to be independently related to a diagnosis of MDS.

4 | MATURING GRANULOCYTIC COMPARTMENT

Comprehensive MFC analysis of the maturing granulocytic compartment is an essential part of the MDS diagnostic workup (Table 2), and it is incorporated in several MDS MFC scores (Alhan et al., 2016; Alhan, Westers, Cremers, et al., 2014; Barreau et al., 2020; Chu et al., 2011; Cremers et al., 2016; Ogata et al., 2009; Wells et al., 2003; Xu et al., 2013). Accurate immunophenotypic identification of the maturation pattern of myeloid cells is a prerequisite. This can be accomplished by using a multidimensional gating strategy: (1) *inclusion* of $SSC^{int/high}/CD45^{int}$ cells, (2) *exclusion* of progenitor cells ($CD45^{int}CD34^{hi}$), monopoiesis ($CD45^{hi}$HLA-$DR^{hi}CD33^{hi}$ and /or $CD64^{hi}$ and/or $CD14^{hi}$), eosinophils ($SSC^{hi}CD45^{hi}CD13^{hi}CD16^{neg}$) (Hassani et al., 2020), basophils ($SSC^{lo}CD45^{int}CD123^{hi}$HLA-$DR^{neg}$), plasmacytoid dendritic cells ($SSC^{lo}CD45^{int}CD123^{hi}$HLA-$DR^{hi}$), most immature erythroid cells ($SSC^{lo}CD45^{int}CD71^{hi}CD117^{int}$ or $CD105^{hi}$; optional: $CD33^{neg}CD13^{neg}$), and (3) *purity control* of the gated population of maturing myeloid cells via back-gating with a combination of CD13/CD16, CD13/CD11b or HLA-DR/CD11b (Figure 2). The patterns of these three latter combinations are very stable in normal maturing BM, and their disruption is a significant sign of underlying MDS (Orfao et al., 2019).

Analysis of antigen expression patterns in BM samples can be performed in different ways. The ELN *i*MDS Flow WG recommends establishing laboratory-specific reference ranges until standardized protocols and reference ranges are available for various MFC systems. Published cut-offs and antigen expression patterns should be verified in-house. We recommend the analysis of 10–20 BM samples from patients with non-clonal cytopenias (such as idiopathic thrombocytic purpura) or lymphoma staging BM without any signs of infiltration to establish laboratory-specific reference ranges.

TABLE 2 The most common multiparameter flow cytometry aberrant features in granulopoiesis in myelodysplastic syndrome

Marker/pattern	Aberrant feature	References[a]
Maturing myeloid cells-to-lymphocytes ratio		
SSC	Decreased	Wells et al. (2003); Stetler-Stevenson et al. (2001)
Percentage	Decreased	Cremers et al. (2017); Porwit et al. (2014)
Aberrancies in antigen expression		
CD34 (%)	Asynchronous expression	Matarraz et al. (2008)
CD5; CD7; CD56 (%)	Cross lineage expression	van de Loosdrecht et al. (2008)
CD13/CD16/CD11b	Aberrant pattern	Stetler-Stevenson et al. (2001); Kussick et al. (2005)
CD15/CD10	Aberrant pattern	Huang et al. (2010); Chung et al. (2012)
CD33 (MFI)	Decreased	Wells et al. (2003); Kussick et al. (2005); Kern et al. (2010)

[a]Examples of references that reported this feature.

4.1 | MDS-related abnormalities measured as numerical or phenotypic changes in maturing granulocytic cells in relation to lymphocytes

The number of maturing granulocytic (Gr) cells compared to lymphocytes (Ly) is the first parameter to be analyzed since it reflects myeloid cell differentiation. A decreased Gr/Ly ratio (for many laboratories <1.0) suggests a limited ability of the immature myeloid cells to differentiate into granulocytes and it has been included in MFC scoring systems (Cremers et al., 2017; Wells et al., 2003). However, this ratio is also altered in aplastic anemia due to a reduction in maturing myeloid cells (Wells et al., 2003). Therefore, the ELN *i*MDS Flow WG recommends applying this parameter with caution, particularly if clinicopathological characteristics point toward aplastic anemia or hypoplastic MDS. Caution should also be exerted in pediatric samples (Aalbers et al., 2015; van der Velden et al., 2023).

Neutrophil hypogranularity is a crucial feature of myeloid dysplasia by cytomorphology. MFC allows a more objective assessment of this feature by investigating very large numbers of cells and applying a parameter set in the context of a reference range. The value used here is the SSC ratio of maturing granulocytic cells relative to that of mature lymphocytes as an internal standard. This strictly defined parameter, first introduced by Ogata and colleagues (2009), has since then been incorporated in recent MFC scores and is widely used in daily practice (Cremers et al., 2017; Kern et al., 2010; Matarraz

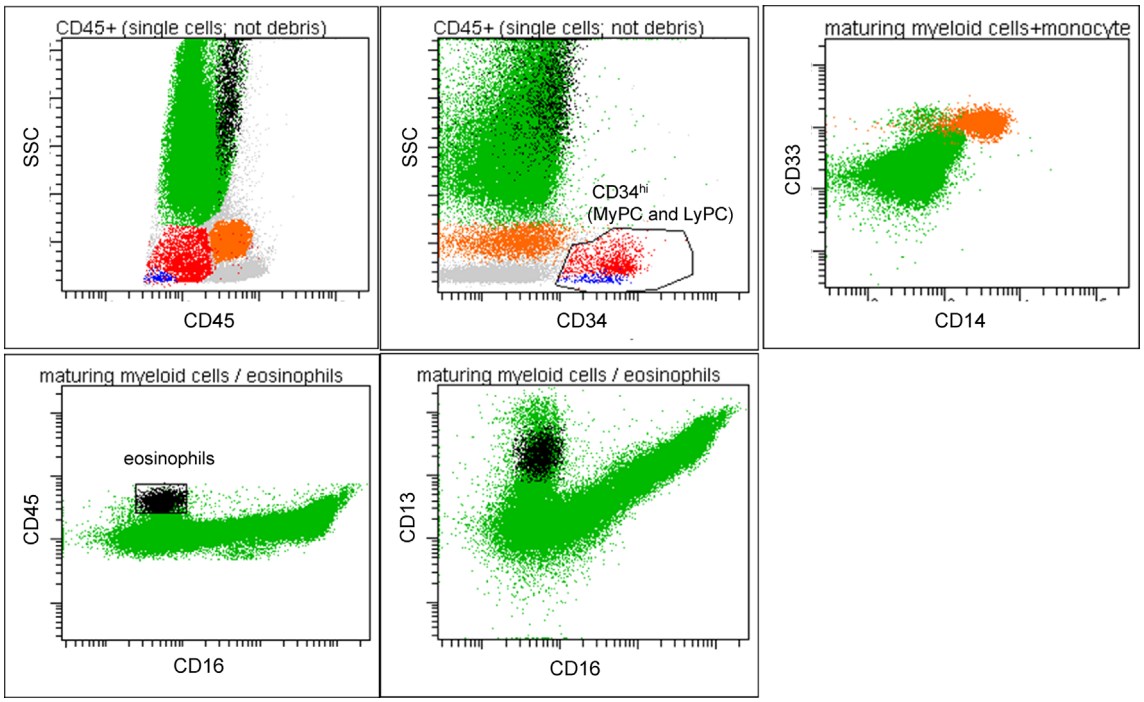

FIGURE 2 Gating procedure of maturing granulocytic cells: Maturing myeloid cells ($SSC^{int/high}/CD45^{int}$) are shown in green. Most important cell populations, which should be excluded from the maturing myeloid compartment are color coded as follows: myeloid progenitor cells (MyPC in red; $SSC^{lo}CD45^{int}CD34^{hi}$), lymphoid progenitor cells (LyPC in blue; $SSC^{verylo}CD45^{lo}CD34^{hi}$), monocytes (in orange; $SSC^{int}/CD45^{hi}CD33^{hi}$ and/or $CD14^{hi}$), eosinophils (in black; $SSC^{hi}CD45^{hi}CD13^{hi}CD16^{neg}$)

et al., 2010; Oelschlaegel et al., 2021; Rajab & Porwit, 2015; Wells et al., 2003). The correct gating procedure (in particular the exclusion of MPs, see Figure 2) may not be easy to accomplish in some cases, however, it should be performed precisely to guarantee a correct SSC analysis (Westers et al., 2012). An abnormal SSC signal has also been reported in a fraction of control cases in the ELN *i*MDSFlow multicenter study (20.1%, Kern et al., 2022) and might be affected by decreased cell viability with sample aging.

Another frequently analyzed parameter is the ratio of the expression of the common leukocyte antigen CD45 on maturing granulocytes relative to lymphocytes. Normal myeloid cells show an intermediate expression intensity (measured as mean fluorescence intensity, MFI) compared to a bright expression on lymphocytes (Figure 2). As with the SSC ratio, a reference range should be established for this parameter (Aalbers et al., 2013). A decrease in CD45 expression on maturing granulopoietic cells has been described in MDS and incorporated in various MFC scoring systems (Barreau et al., 2020; Matarraz et al., 2010).

4.2 | MDS-related abnormalities measured as aberrancies in antigen expression patterns

Multiple changes in the expression of myeloid maturation antigens result in abnormal expression patterns. Abnormalities in the granulopoiesis maturation patterns using CD13/CD16/CD11b markers combination (Figure 3) have been reported in many publications (Chopra et al., 2012; Chung et al., 2012; Kern et al., 2010; Kussick et al., 2005; Kussick & Wood, 2003; Stetler-Stevenson et al., 2001). CD13/CD16 and CD13/CD11b marker combinations displayed aberrant patterns of maturation in granulopoiesis in 29% and 40% in low-risk MDS patients in the ELN *i*MDSFlow multicenter study by comparison to 16% and 11% of hospital controls (Kern et al., 2022).

The exact measurement and determination of "aberrant" versus "within range" patterns may vary between laboratories. Different panels and conjugates may result in slight differences in observed patterns. Most ELN *i*MDS WG laboratories use pre-defined gates or software-generated pathways and measure the proportion of maturing myeloid cells that fall outside these standards. Exact cut-offs, if applied, are validated 'in-house'. A complete or partial loss of CD16 (a glycosyl-phosphatidyl-inositol [GPI] anchored protein, Figure 3) might suggest the presence of a paroxysmal nocturnal hemoglobinuria (PNH) clone which requires further testing for other GPI anchored antigens such as CD14 on mature monocytes and CD24 on mature neutrophils (Sutherland et al., 2018; Westers et al., 2021). Aged BM specimens, eosinophils contaminating the granulocyte gate and genetic polymorphisms may also account for changes in CD16 expression (de Haas et al., 1995; Loken et al., 2009; Stachurski et al., 2008). An altered maturation pattern skewed to more mature myeloid cells suggests hemodilution. The skewed pattern in such a case is not an aberrancy but a sample quality problem. Loken et al. (2009) proposed to consider the fluorescence intensity of CD16 to address this problem with two possible scenarios: (1) only samples with less than 30% of mature myeloid cells ($CD16^{hi}$) are considered adequate or (2) in

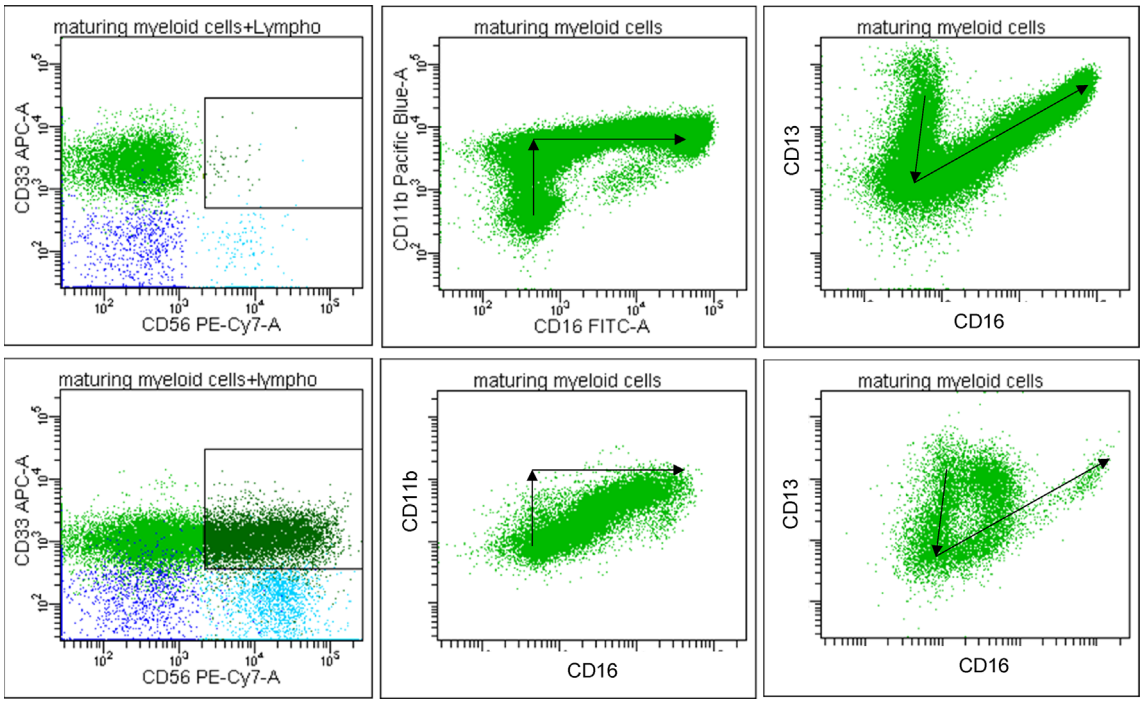

FIGURE 3 Examples of antigen expression in normal bone marrow (upper row) in comparison to immunophenotypic aberrancies in maturing myeloid cells in myelodysplastic syndrome (MDS) and in paroxysmal nocturnal hemoglobinuria (PNH). Left: aberrant CD56 expression (dark green) detected in maturing myeloid cells of MDS (lower row), but was detectable only in a minority of normal bone marrow cells; NK cells (light blue) which regularly express CD56 and T-/B-lymphocytes (dark blue) without CD56 expression are displayed as an internal control. Middle: abnormally shaped CD11b/CD16 expression pattern in MDS (lower row) compared to normal bone marrow as $CD16^{neg}$ $CD11b^{+}$ via $CD16^{+}CD11b^{+}$ to $CD16^{++}CD11b^{+}$. Right: partial CD16 deficiency due to PNH (lower row) compared to normal bone marrow as $CD16^{neg}$ $CD13^{+}$ via $CD16^{neg}$ $CD13^{neg}$ to $CD16^{++}$ $CD13^{+}$. Black arrows mark the normal maturation pattern

samples with higher proportions, the use of a correction formula is needed (Loken et al., 2009). Pont et al. (2018) reported that the ratio of immature granulocytes ($SSC^{int/hi}CD45^{lo}CD10^{neg}CD16^{neg/lo}$) to neutrophils ($SSC^{int/hi}CD45^{lo}CD10^{+}CD16^{hi}$) (IGRA/N ratio) was the best parameter to assess the hemodilution of a BM specimen. Thus, CD16 expression should only be interpreted in the context of other maturation antigens and in the absence of PNH. Additionally, in some non-clonal conditions, such as nutritional deficiencies (e.g., folic acid), the maturation pattern of granulopoiesis can also be altered. In these cases, if abnormal WBC values persist, examination of a post-supplementation BM aspirate is recommended to clarify whether the maturation abnormalities persist after substitution.

Asynchronous antigen expression of progenitor cell-related antigens (CD34, CD117, and HLA-DR) on maturing myeloid cells and asynchronous shift toward an immature immunophenotype (ex. decreased CD15 expression in parallel to a normal CD13/CD16/CD11b expression) are commonly observed MFC features of dysgranulopoiesis (Cremers et al., 2017; Wells et al., 2003; Westers et al., 2012). The relationship between CD15 and CD10 could also be abnormal if CD10 is lost or decreased despite a bright CD15 expression (Barreau et al., 2020; Duetz et al., 2019; van de Loosdrecht et al., 2009). Of note, abnormal strong CD15 expression can also be due to hemodilution, and decreased CD10 expression has been described in autoimmune diseases, hemophagocytic lymphohistiocytosis, and reactive conditions (McCall et al., 2012; Morisaki et al., 1992; Ui Mhaonaugh et al., 2019). A lack of CD33 antigen has also been described on maturing granulopoietic cells and incorporated in different diagnostic MFC scores (Cremers et al., 2017; Kern et al., 2010; Matarraz et al., 2010; Wells et al., 2003). However, caution should be exercised since changes due to polymorphisms have been described for CD33 as well as for CD16 (Pérez-Oliva et al., 2011; Raptis et al., 1998). Thus, in the presence of concomitant decreased CD33 expression on granulocytes, monocytes, and myeloid progenitor cells, a genetic polymorphism should be suspected.

Cross-lineage expression of lymphoid antigens (such as CD5, CD7, CD19, or CD56) in maturing myeloid cells has high importance in the MFC assessment of dysgranulopoiesis (Barreau et al., 2020; Cremers et al., 2017; van de Loosdrecht et al., 2008; Wells et al., 2003). Hence, lymphocytes should be excluded from the granulopoiesis gate for analysis of cross-lineage expression. CD56 expression on maturing myeloid cells and myeloid progenitors and/or monocytes can occur in inflammatory conditions, in diabetes and after chemotherapy treatment (so-called "stressed bone marrow") (Friedrich et al., 2019; Grip et al., 2007, Krasselt et al., 2013). Thus, the ELN *i*MDSFlow WG would not consider CD56 expression alone as significant in assessing post-treatment MDS flow scores.

Furthermore, CD14 or CD64 expression intensity may be altered in maturing myeloid cells (Barreau et al., 2020; Kern et al., 2010; Matarraz et al., 2010). Overexpression of CD14 on maturing granulocytic cells has been described as associated with genetic abnormalities such as del(5q) or chromosome 7 monosomy (Chen et al., 2019; Keerthivasan et al., 2014). A normal, bright expression of these antigens on monocytic cells strengthens the necessity of proper exclusion of monocytes from the granulopoiesis gate, as described above. Of note, an increased CD64 expression on maturing granulocytic cells could be associated with infection (especially in patients with sepsis) (reviewed in Patnaik et al., 2020).

In some MDS cases, a prominent BM eosinophilia may be present without significant eosinophilia in blood. In such cases, specific WHO-defined eosinophilic disorders should be excluded (reviewed in Shomali & Gotlib, 2019). Blood eosinophilia is an adverse prognostic factor (Andersen et al., 2015), and increased eosinophils and basophils have been described as a sign of progression in MDS (Wimazal et al., 2008). Recent studies point out the presence of neutrophils with features of eosinophils, monocytes, and dendritic cells, as well as eosinophil subsets expressing neutrophil markers in various disease states (reviewed in Berdnikovs, 2021). However, to our knowledge, dysplastic MFC features of eosinophils in MDS are not yet defined and currently do not contribute to MDS diagnosis.

In summary, analysis of MDS-related aberrancies in the maturing granulocytic compartment may be challenging due to the large variety of possible aberrancies and because some of these also occur in conditions other than MDS. Therefore, the *i*MDS Flow WG does not recommend using any of the mentioned markers in isolation. The analysis of several antigen expression patterns is necessary for a correct classification between aberrant vs. non-aberrant maturing granulocytic cells. This has already been implemented in some MFC diagnostic scores such as FCSS and *i*FS (Cremers et al., 2017; Wells et al., 2003).

Based on data available and the results of the multicenter study of the ELN *i*MDSFlow WG (Kern et al., 2022), the evaluation of the granulocytic compartment in BM of patients with suspect MDS or MDS/MPN should include the quantification of granulocytes (aberrant percentages) as well as the assessment of a reduced SSC-Gr signal, of an aberrant expression of CD33 in granulocytes and evaluation of CD13/CD16 expression pattern in granulocytes, which all have been demonstrated to be independently related to a diagnosis of MDS or MDS/MPN.

5 | MONOCYTES

5.1 | Monocytic lineage in the bone marrow

Early monocytic commitment among bone marrow CD34$^+$ HPCs is defined by the progressive acquisition of CD64 starting from low intensity and increasing to heterogeneous expression levels (Matarraz et al., 2008; Matarraz et al., 2010; Matarraz et al., 2017; Orfao et al., 2019). In parallel, these early monocytic precursors (morphologically corresponding to monoblasts), downregulate the immature markers CD34 and CD117. Early promonocytes acquire myelomonocytic maturation antigens such as CD11b, CD11c, CD15, cytoplasmic (Cy) lysozyme, CyCD68, and CD36, followed by the acquisition of surface CD14 and CD35 (Dunphy, 2011; Matarraz et al., 2017; van Dongen et al., 2012). The further immunophenotypic transition toward mature monocytic stages is characterized by the acquisition of CD300e and CD312 (Matarraz et al., 2017; Orfao et al., 2019). CD62L expression is preserved throughout monocytic maturation in the BM, while in blood and lymphoid tissues the presence or absence of CD62L defines distinct maturation-associated subsets of monocytes (Damasceno et al., 2019).

The most frequently reported altered monocytic immunophenotypes in myelodysplasia include (Table 3):

1. decreased light scatter characteristics (20%–30% of MDS cases),

TABLE 3 The most common multiparameter flow cytometry aberrant features in monocytes in myelodysplastic syndrome and chronic myelomonocytic leukemia (CMML)

Merker/pattern	Aberrant feature	References[a]
Aberrancies in scatter characteristics	Decreased	Matarraz et al. (2010)
Aberrancies in antigen expression (including CMML)		
CD13, CD14, CD15, CD36, CD64 HLA-DR, CD11b, CD11c	Decreased	Xu et al. (2005); Subirá et al. (2008); Matarraz et al. (2010); Kern et al. (2011); Sojitra et al. (2013); Harrington et al. (2016); Kern et al. (2022)
CD56, CD2	Cross lineage expression	Xu et al. (2005); Subirá et al. (2008); Matarraz et al. (2010); Kern et al. (2011); Kern et al. (2022)
Aberrancies in monocyte subpopulation partition in CMML		
cMo %	Increased ≥94%[b]	Selimoglu-Buet et al. (2015); Selimoglu-Buet et al. (2017) Talati et al. (2017); Patnaik et al. (2017); Hudson et al. (2018); Tarfi et al. (2018); Wagner-Ballon et al. (2023)
slan$^+$ ncMo%	Decreased <1.7%	Tarfi (2019)

[a]A relative accumulation of circulating cMO has been reported in roughly one third of the MDS patients (Selimoglu-Buet et al., 2017; Talati et al., 2017).
[b]Examples of references that reported this feature.

2. downregulation of monocyte-associated differentiation markers such as CD11b, CD13, CD14, CD36, CD64 (16%–34% of MDS cases),
3. abnormally low frequency of CD300e^{+} mature monocytes (18% of MDS cases) (Matarraz et al., 2010).

In addition, cross-lineage marker expression is a relatively frequent finding among monocytic lineage cells in MDS, including aberrant expression of the CD56 and/or CD2 lymphoid-associated antigens, reported in 48% and 32% of the cases respectively (Matarraz et al., 2010; Subirá et al., 2008). Cross lineage expression of CD2, CD7, and CD56 has also been reported in CMML, together with low expression of CD14 and CD11c, down-regulation or lack of CD13 or HLA-DR, and increased numbers of monocytic lineage cells (Kern et al., 2011; Sojitra et al., 2013; Subirá et al., 2008). Thus, the occurrence of persistent monocytosis, associated with ≥2 immunophenotypic alterations on monocytic cells, >20% of CD14^{low} BM monocytes and decreased expression of CD11c, has been described as highly specific for CMML (Sojitra et al., 2013; Xu et al., 2005). However, caution should be exerted while evaluating patients who developed cytopenias during active therapy for other medical conditions since some changes observed in the monocytic compartment may be due to activation or regeneration (Tang et al., 2012).

As expected, the multicenter study of the ELN *i*MDSFlow WG found a higher frequency of aberrant monocyte immunophenotypes in CMML by comparison to MDS (Kern et al., 2022). CD56 expression on monocytes was the most frequent abnormal feature in BM with myelodysplasia. Abnormal expressions of CD13 and HLA-DR were found in a considerable fraction of hospital controls, making these markers less specific for the evaluation of immunophenotypically defined myelodysplasia (Kern et al., 2022).

In summary, based on data available and the multicenter study of the ELN *i*MDSFlow WG (Kern et al., 2022) in particular, the evaluation of monocytes for MDS should include their quantification (aberrant percentages) as well as the assessment of an aberrant expression of CD13 and of CD56, and of an aberrant HLA-DR/CD11b expression pattern which all have been demonstrated to be independently related to a diagnosis of MDS or MDS/MPN.

5.2 | Monocyte subsets in peripheral blood

Overall, blood monocytes can be stratified in three major subsets according to their expression of CD14 and CD16 (Figure 4): that is, CD14^{++}CD16^{-} classical monocytes (cMo), CD14^{++}CD16^{+} intermediate monocytes (iMo) and CD14$^{-/low}$CD16^{+} non-classical (ncMo) (Wong et al., 2011). From an analytical point of view, it is also recommended in the diagnostic work-up of MDS or MDS/MPN to acquire at least 10,000 cMo events to ensure the robustness of cMo identification (Tarfi et al., 2018), which should include an exclusion gating strategy to remove cell populations that may overlap ncMo (e.g., NK cells) (Selimoglu-Buet et al., 2017).

A relative accumulation of cMo (≥94% of total blood monocytes) is associated with a CMML diagnosis with high specificity (94.1%) and sensitivity (92.8%, reaching 100% for CMML type 2) (Selimoglu-Buet et al., 2017). The 94% threshold has been subsequently validated in independent studies (Hudson et al., 2018; Patnaik et al., 2017; Talati et al., 2017) and in the CMML prospective trial conducted by the ELN *i*MDS Flow WG (Wagner-Ballon et al., 2023). The prospective trial has also indicated that the 94% cMo threshold could be valid for BM samples (see Figure 4 in Wagner-Ballon et al., 2023).

However, the monocyte assay can be compromised by the co-occurrence of an inflammatory state in CMML patients, leading to an increase in the iMo subset and a subsequent decrease in the relative cMo percentage that may not reach the diagnostic cut-off of 94%. Although the typical CMML signature may be erased in such cases,

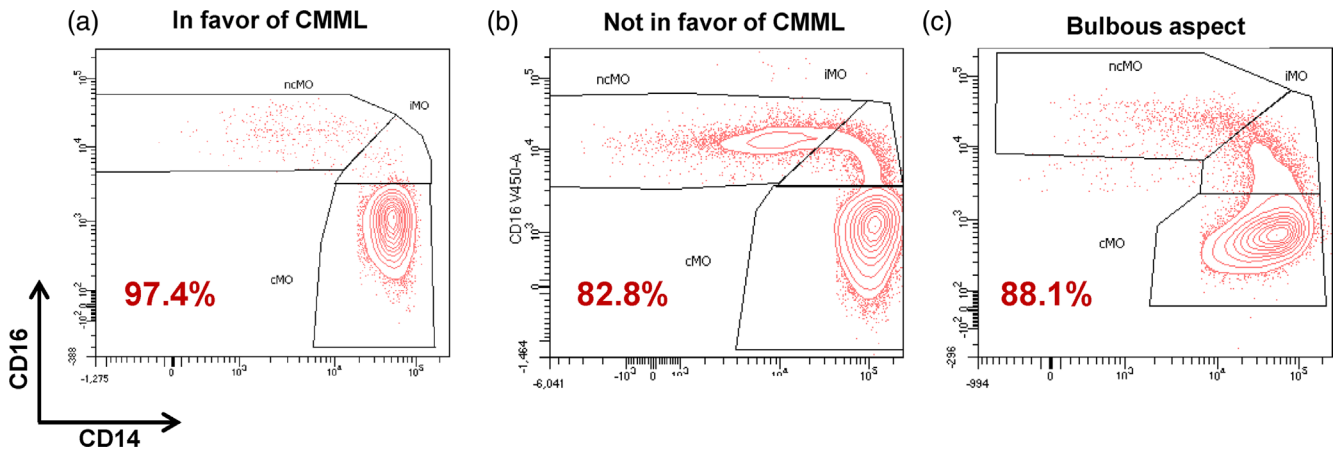

FIGURE 4 Representative examples of blood monocyte subpopulation distribution profiles obtained with the monocyte assay. (a) Immunophenotype in favor of chronic myelomonocytic leukemia (CMML) showing an accumulation of cMo that is, a percentage ≥94. (b) Immunophenotype not in favor of CMML showing no accumulation of cMo that is, a percentage <94. (c) Immunophenotype in favor of CMML with an easily recognized bulbous aspect, due to an increase in iMo fraction combined with the near disappearance of the ncMo subset, leading to a decrease in the cMo percentage below the 94% threshold [Color figure can be viewed at wileyonlinelibrary.com]

there is an easily recognized "bulbous" profile (Figure 4) (Selimoglu-Buet et al., 2017). Identification of this MFC profile can be further refined by incorporating a ncMo specific marker, such as *slan* (M-DC8 6-sulfo LacNAc) to better identify CMML patients with an associated inflammatory state (Tarfi et al., 2019). It should be noted that relative accumulation of circulating cMo ≥94% of total monocytes has been described in roughly one-third of MDS patients at diagnosis (Selimoglu-Buet et al., 2017; Talati et al., 2017), half of them considered likely to evolve into overt CMML (Selimoglu-Buet et al., 2017).

6 | ERYTHROID LINEAGE

CD235a (glycophorin A) and CD71 (transferrin receptor) are well-known markers for studying the erythroid lineage (Loken et al., 1987). In addition, CD117 (stem cell factor receptor), CD105 (endoglin), CD36 (thrombospondin receptor), $CD45^{low/neg}$ and SSC^{low} enable dissection of the erythroid maturation in clear steps (Eidenschink Brodersen et al., 2015; Fajtova et al., 2013; Machherndl-Spandl et al., 2013; Orfao et al., 2019). This knowledge facilitates the analysis of dyserythropoiesis in MDS. MFC analysis of markers for erythroid dysplasia includes aberrant surface marker expression (intensity, homogeneity, and heterogeneity) and abnormal distribution of maturing erythroid cell subsets.

In MDS, an aberrant expression pattern of CD71 versus CD235a is a classic example of dyserythropoiesis (Stetler-Stevenson et al., 2001). In addition, an increase in the coefficient of variation (CV) of CD36 and CD71 expression serves as a specific feature of MDS-associated erythroid dysplasia (Mathis et al., 2013). The multicenter study performed by the ELN *i*MDSFlow WG confirmed these findings and demonstrated that the CV of CD36 and CD71 expression, median expression of CD71 and frequency of $CD117^+$ erythroid progenitors form a combination of parameters that enable differentiation between MDS-associated erythroid dysplasia and non-clonal cytopenic controls (Table 4, Figure 5) (Westers et al., 2017). Nevertheless, various conditions (e.g., reactive changes, medication-induced anomalies, vitamin B12, and folate deficiencies) may induce dysplastic features in the BM erythroid lineage.

The sensitivity of detecting MDS-associated erythroid dysplasia by MFC is relatively low (approximately 30%–40%), but the specificity is high at 90%. The addition of erythroid lineage evaluation to current (mainly myelomonocytic-oriented) MFC scores improved the total diagnostic sensitivity (up to 85%; Cremers et al., 2017; Mathis et al., 2013).

As described in detail in van der Velden et al. (2023), the erythroid compartment is sensitive to lysing procedures used to remove mature erythrocytes, which may cause an altered frequency of erythropoietic precursors, subset distribution or marker expression (Violidaki et al., 2020). Immature $CD117^+$ and $CD105^+$ erythroid progenitors, as well as other erythroid precursors seem to be less affected by ammonium chloride-based lysis than other lysing protocols. It may be in part due to a 10-fold lower expression of carbonic anhydrase in erythroid progenitors than in reticulocytes and mature erythrocytes (Gautier et al., 2016; Wangen et al., 2014).

Nevertheless, CV values of CD36 and CD71 MFI as assessed in both lysed and non-lysed BM were equally informative concerning the analysis of erythroid dysplasia (Mathis et al., 2013; Violidaki et al., 2020; Westers et al., 2017). The ELN *i*MDS Flow WG multicenter study confirmed CD71 as a helpful marker for evaluating erythropoietic dysplasia (Kern et al., 2022). In this study, data on other markers, such as CD117, CD105, and CD235a, was not sufficient to draw definitive conclusions.

CD105 is a potential marker to be included in MFC analysis of the erythroid lineage. In addition to an aberrant frequency of $CD105^+$ progenitors, CD105 overexpression has been reported in

TABLE 4 Recommended erythroid markers to assess myelodysplastic syndrome-associated erythroid dysplasia

Marker/pattern	Aberrant feature	References[a]
Recommended by ELN *i*MDS Flow WG		
CV of CD36	Increased	Mathis et al. (2013); Westers et al. (2017)
CV of CD71	Increased	Mathis et al. (2013); Westers et al. (2017)
CD71 MFI	Decreased	Westers et al. (2017)
Percentage of $CD117^+$ erythroid cells	Decreased or increased	Westers et al. (2017)
Optional		
Relationship CD71-CD235a	Disturbed	Stetler-Stevenson et al. (2001); Eidenschink Brodersen et al. (2015)
Percentage of nucleated erythroid cells	Increased	Eidenschink Brodersen et al. (2015)
Percentage of $CD105^+$ erythroid cells	Decreased or increased	Della Porta et al. (2006); Eidenschink Brodersen et al. (2015); Westers et al. (2017)
CD105 MFI	Decreased or increased	Della Porta et al. (2006); Eidenschink Brodersen et al. (2015); Westers et al. (2017)

[a]Examples of references that reported this feature.

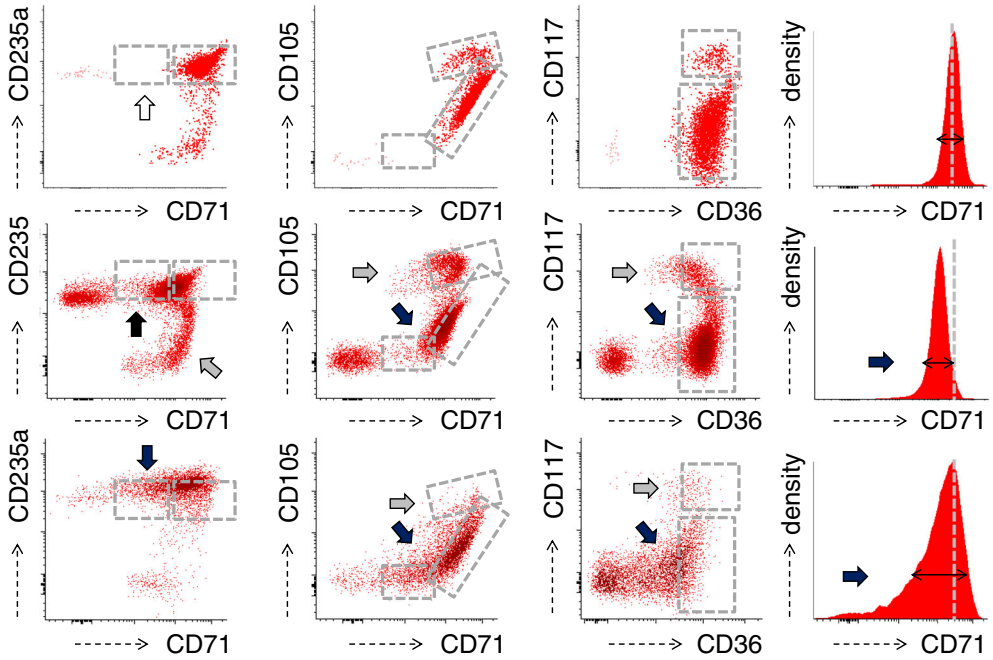

FIGURE 5 MFC assessments of erythroid dysplasia in MDS. The upper row displays patterns of normal erythroid maturation and a normal homogenous CD71 expression (histogram, right). All plots were generated from assessments of ammoniumchloride-based lysed bone marrow aspirates. The gray dashed boxes delineate most of the normal erythroid maturation patterns. The most immature erythroid cells are defined as $CD71^+$ $CD235a^-$, $CD71^+$ $CD105^+$ and $CD36^+$ $CD117^+$, respectively. Upon maturation, expression follows a pattern upwards in the CD71 versus CD235a plot and downwards in the CD71 versus CD105 and CD36 versus CD117 plots. The normal absence of cells in the box between erythroid progenitors and the remaining mature $CD71^-$ $CD235^+$ erythrocytes is marked with an open arrow. The middle row displays an example of dysplastic erythropoiesis in a case of MDS-MLD with increased CV of CD71 (black arrows) and increased frequency of immature erythroid progenitor cells (%CD117 and %CD105, gray arrows). The expression of CD71 is decreased as well. The bottom row demonstrates an example of MDS-RS-MLD where the erythroid cells show heterogeneous and decreased CD36 and CD71 expression as compared to the normal control (indicated by black arrows). The frequency of immature progenitors is relatively low (%CD117 and %CD105, gray arrows); in addition, CD105 expression is decreased. Notably, the displayed results are not typical for either of the WHO classification subtypes, but just an example of possible dysplastic features associated dyserythropoiesis by MFC. CV, coefficient of variation; dim, diminished; LD, multilineage dysplasia; RS, ring sideroblasts [Color figure can be viewed at wileyonlinelibrary.com]

some cases of MDS (Della Porta et al., 2006; Westers et al., 2017; Xu et al., 2012). Remarkably, CD105 expression was shown to be negatively correlated with age in normal controls (Westers et al., 2017). Ongoing studies may elucidate whether CD105 MFI is truly valuable in the analysis of MDS-associated erythroid dysplasia.

Additional markers may support the identification of dysplastic features and hence, the separation between MDS and cytopenic controls. Other markers that have been reported to be informative in assessing MDS-related erythroid dysplasia are CD35, CD44, CD43, CD49d, and the *major coxsackie adenovirus receptor* (CAR) (Bauer et al., 2014; Laranjeira et al., 2015; Machherndl-Spandl et al., 2013; Oliveira et al., 2019). However, these findings have not yet been validated in multiple centers.

In summary, we highly recommend including the following variables validated in a multicenter setting: CV of CD36 and CD71, MFI of CD71 and percentages of $CD117^+$ and $CD105^+$ progenitors, with reference values determined for each standardized work-up, on either non-lysed or lysed BM.

7 | OTHER BM CELL SUBSETS

7.1 | Lymphocyte subsets in MDS

The low frequency of B-cell progenitors in BM $CD34^+$ cells has been included in several MDS MFC diagnostic scores (Ogata et al., 2009; Ribeiro et al., 2006). However, the low frequency of B-cell precursors had a relatively low specificity as an MDS-related feature (Bardet et al., 2015; Rajab & Porwit, 2015). Preserved B-cell progenitors are seen in approximately one-quarter of low-grade MDS (Chen et al., 2020) and a low frequency of $CD34^+CD19^+$ cells can be seen in older adults with no MDS features (Lorand-Metze et al., 2018). Moreover, an independent prognostic value of BM progenitor B-cell frequencies in patients with lower-risk MDS has been suggested (Kahn et al., 2015).

Immune dysregulation plays an essential role in MDS (reviewed in Lambert et al., 2016). In general, activation of myeloid-derived inflammation, signified by NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3), is a characteristic element of MDS pathophysiology

(Sallman & List, 2019). However, adaptive immune responses follow a more stepwise transformation from a protective activated adaptive immunity to a more immunosuppressive cellular response as the disease progresses.

Expansion of pro-inflammatory T cells, including T helper 17 cells (Th17), has been shown in lower-risk MDS (Epling-Burnette, Painter, et al., 2007; Kordasti et al., 2009). At the same time, a predominantly suppressive milieu characterizes higher-risk disease with significant expansion of immunosuppressive cells such as regulatory T cells (Tregs) (Kordasti et al., 2009; Kotsianidis et al., 2009) accompanied by a reduction in the number and function of BM dendritic cells (Saft et al., 2013) and NK cells (Epling-Burnette et al., 2007).

Overall, the adaptive immune system has an essential role in the pathophysiology and risk stratification of MDS (Platzbecker & Kordasti, 2020; Winter et al., 2020). Although there is no consensus on the role of B- and T-cell studies in MDS diagnostics (van de Loosdrecht et al., 2023), it is highly advisable to include T and B cell markers in routineimmunophenotyping for MDS to evaluate the frequency of B-cell precursors and to exclude the possibility of clonal populations suggesting lymphoma. Multicentre studies, using similar MFC panels are needed to clarify the predictive value of T and B cell subpopulations and further design robust diagnostic panels and an "immune scoring system" in the future.

7.2 | Dendritic cells

The role of dendritic cells (DCs) in MDS is largely unknown. Since DCs are key players in the immune system by serving as regulators of immune responses, they likely have an essential function in the pathogenesis of MDS. Recent findings point to an impaired ability of DC subsets to adequately respond to cellular stress and DNA damage in immune escape and progression of MDS toward AML (Carenza et al., 2019; Van Leeuwen-Kerkhoff et al., 2021). The development of human DCs occurs in the BM, where they originate from common precursor cells and differentiate into specialized subsets, respectively conventional myeloid DC (cDC) and plasmacytoid DC (pDC). These DC subsets are decreased in the blood and BM of MDS patients(Carenza et al., 2019; Saft et al., 2013; Van Leeuwen-Kerkhoff et al., 2021). cDC are further separated in cDC1 ($CD141^+$) and cDC2 ($CD1c^+$) DC. Initially, a fourth DC subset, slanDC, was identified based on the expression of M-DC8 (6-sulfo LacNAc or *slan*) and CD16. Further studies showed that these SLAN+ cells are more closely related to monocytes than to DC and they were renamed as slan+ nc monocytes (van Leeuwen-Kerkhoff et al., 2017; 2018). A decrease in the specific slan+ ncMo subset and increased CD1c + cDC (Meyerson et al., 2016) are features of CMML but their use in the diagnosis or prognostication of MDS is unclear (Van Leeuwen-Kerkhoff et al., 2021). Re-appearance of the slan+ ncMo subset is associated with response to hypomethylating drugs in CMML (Tarfi et al., 2019). Conversely, in approximately 20% of CMML patients, an excess of $CD123^+/CD56^-/BDCA2^+/BDCA4^+$ pDC was found to correlate with the presence of somatic *RAS* mutations and an increased risk of transformation (Lucas et al., 2019).

7.3 | Mast cells

Immunophenotyping of BM mast cells by MFC is recommended if a concomitant mast cell disease is to be confirmed or excluded in patients with MDS (Valent et al., 2017). In normal BM, mast cells express CD33, CD44, CD45, and CD117 (KIT) but do not express CD2, CD25, CD30 or CD34 (Morgado et al., 2014; Valent et al., 2010). Depending on their maturation stage, BM mast cells may also have low expression of CD11a, CD123, and CD203c. In MDS patients, mast cells may display CD25, while in MDS with concomitant systemic mastocytosis (SM-MDS) mast cells express aberrant CD2, CD25, and CD30 (Escribano et al., 2001; Sotlar et al., 2011). In these cases, mast cells also display higher levels of CD44, CD123, and CD203c expression than normal BM mast cells (Hauswirth et al., 2008; Pardanani et al., 2015).

7.4 | Basophils

Basophil numbers may increase in MDS with disease progression and basophilia in MDS has an adverse prognostic significance (Matsushima et al., 2003; Wimazal et al., 2008; Wimazal et al., 2010). Normal mature basophils express CD25, CD33, CD44, CD123, CD203c, and FcεRI but do not express substantial amounts of CD30, CD34, or CD117 (Florian et al., 2006). In MDS, basophils express the same markers as normal basophils. The expression of CD117 may be substantial in immature basophils. Although basophil counting is considered helpful in MDS, basophil immunophenotyping is not considered mandatory in these patients.

8 | FLOW CYTOMETRY SCORING SYSTEMS

Several MFC scoring systems for immunophenotypic signs of myelodysplasia have been described and validated (summarized in Table 5). Some of the scoring systems focus on MPs, while others focus on erythropoietic precursors. Some scoring systems require large panels to explore both MPs, granulopoiesis, monopoiesis, and erythropoiesis. Two comparisons of several scoring systems (Davydova et al., 2021; Oelschlaegel et al., 2021) recommend the *i*FS score (Cremers et al., 2017, Table 6) as the one with the best balance between sensitivity and specificity. However, the correct application of this score requires approximately 40 markers in a multicolor setting (Cremers et al., 2017; Kern et al., 2022).

The ELN *i*MDSFlow WG multicenter study confirmed the diagnostic potential of MFC aberrancies and identified 17 markers as the most specific, independently related to MDS diagnosis (Kern et al., 2022). These include the percentage of aberrant MPs, aberrant expression of CD45, CD13, CD117, CD5, CD7, CD56, and HLA-DR on MPs, percentage of aberrant neutrophils, aberrant scatter of neutrophils, aberrant CD33 expression and CD13/CD16 pattern in neutrophils, percentage of aberrant monocytes, HLA-DR/CD11b pattern on monocytes, aberrant CD13 and CD56 expression on monocytes as

TABLE 5 Scoring systems for multiparameter flow cytometry diagnosis of immunophenotypic myelodysplasia

Name	No of parameters	Progenitors	Maturing granulocytes and monocytes	Erythropoiesis	Scores	Interpretation	Reference
FCSS	40	% Abnormal MP	Abnormal patterns Lineage infidelity		0–1 2–3 ≥4	Normal Moderate Severe dysplasia	Wells et al. (2003); Alhan, Westers, Cremers, et al. (2014)
Ogata score	4	% $CD34^+$ cells Fraction of $CD34^+$ B-cells CD45MFI of $CD34^+$ MP cells	Abnormal scatter of granulocytes		0–1 ≥2	Low High	Ogata et al. (2009); Della Porta et al. (2012)
Quantitative FC score	28	% CD34, CD117 CD45 Lineage infidelity	Abnormal patterns Lineage infidelity	Abnormal CD71	≥3	Suggestive of MDS	Chopra et al. (2012)
RED	3			Abnormal CD36 Abnormal CD71 Hb	0–2 ≥3	Suggestive of MDS	Mathis et al. (2013)
MDS immunophenotype	21	CD11b, CD5, CD7, CD56, CD15, and CD64, and HLA-DR on $CD34^+$ MP	SSC signal, abnormal patterns	Abnormal CD71	≥3 aberrantly expressed antigens in ≥2 cell lineages	Suggestive of MDS	Kern et al. (2013); Kern et al. (2015)
Extended Ogata	7	% $CD34^+$ cells Fraction of lyCD34$^+$ cells CD45MFI of $CD34^+$ cells Lineage infidelity	Lineage infidelity on monocytes		0–1 >2	Normal Suggestive of MDS	Bardet et al. (2015)
IFS	44	% MP Fraction of ly $CD34^+$ cells 45 MFI of $CD34^+$ cells Lineage infidelity	Abnormal patterns Lineage infidelity	Abnormal CD36 Abnormal CD71 Abnormal CD117	A B C	No MDS related features Limited MDS features Consistent with MDS	Cremers et al. (2017)
ELN-NEC	3			Abnormal CD36 Abnormal CD71 Abnormal CD117	0–4 ≥5	Low Erythroid dysplasia	Westers et al. (2017)
DIFF score	10		the sum of absolute values of SD outside the threshold of all markers ($n = 10$) at all stages of		17.2 SD	Consistent with MDS	Matarraz et al. (2010); Barreau et al. (2020)

(Continues)

TABLE 5 (Continued)

Name	No of parameters	Progenitors	Maturing granulocytes and monocytes	Erythropoiesis	Scores	Interpretation	Reference
			granulocytic and monocytic maturation. The sum of absolute values of SD outside the threshold of all markers ($n = 10$) at all stages of granulocytic and monocytic maturation. The sum of absolute values of SD outside the threshold of all markers ($n = 10$) at all stages of granulocytic and monocytic maturation. Total number of SDs outside ±2SD of the reference database				

well as aberrant CD71 expression on erythropoietic precursors. The multicenter study did confirm that finding three aberrant markers was of diagnostic significance but did not confirm the necessity of aberrant immunophenotypic changes in two hematopoietic compartments. The set of markers that were identified as independently related to a diagnosis of MDS/CMML (Kern et al., 2022), and MP count >3% of BM cells in the appropriate clinical setting, should be considered indicative of MDS or MDS/MPN. Again, MFC results should always be evaluated as a part of a multimodal, integrated hematopathology work-up.

9 | COMPUTATIONAL ANALYSIS/ FUTURE DIRECTIONS

The increasing number of markers that can be measured simultaneously, both by flow and mass cytometry (Bachas et al., 2022), has accelerated the development of novel analysis tools. Such tools as Phenograph or FlowSOM apply clustering methods to group cells with similar MFC properties (Levine et al., 2015; Quintelier et al., 2021; Van Gassen et al., 2015). There are several advantages to using these tools as compared with manual analysis. As these methods rely on unsupervised analysis, the multidimensionality of MFC data is considered and a predetermined gating strategy is not necessary (Pedreira et al., 2013; Saeys et al., 2016). This results in reproducible and objective identification of relevant cell populations and may also reveal novel relevant cellular properties due to the largely unbiased approach of unsupervised analysis. As aberrancies in MDS are subtle and require particularly precise gating in supervised strategies, implementation of such tools may be advantageous in MDS work-up. Moreover, identifying novel relevant cellular properties could be relevant for diagnosis, risk stratification and prognostication in MDS.

Several successful efforts have been made to implement such tools for clinical applications in hematological malignancies, including MDS (Duetz et al., 2019; Duetz et al., 2020; Duetz et al., 2021). A combination of FlowSOM and Kaluza® has been developed and published (Béné et al., 2020; 2021; Lacombe, Dupont, et al., 2019; Lacombe, Lechevalier, et al., 2019), which allows for rapid and complete characterization of subsets generated by the unsupervised analysis of classical MFC list-modes. In a first attempt, this has been applied to delineate normal BM subsets with four 10-color antibody panels used on merged normal BM samples (Lacombe, Dupont, et al., 2019). Then, the combination of diagnosis and follow-up samples from patients with hematological malignancies (acute leukemia or MDS) together with this reference normal BM has been developed for direct comparison and identification of subsets of interest (Lacombe, Lechevalier, et al., 2019). This approach is easy to apply but requires some critical pre-analytical features. The same panel must be used for all samples on a single instrument or several harmonized instruments. Compensations must be carefully controlled, and the files should be normalized for proper comparison after merging. The tools necessary for these preliminary operations have

TABLE 6 Integrated MDS-MFC score (iFS) (modified from Cremers et al., 2017)

Diagnostic score	<2 abnormalities								≥2 abnormalities							
Aberrant myeloid progenitors	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+
Aberrant neutrophils (SSC or ≥2 other aberrancies) Aberrant monocytes (CD56/≥2 aberrancies)	−	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+
Aberrant erythroid (≥2 aberrancies)	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
iFS[a]	A	B	B	C	B	C	C	C	A/B	C	C	C	C	C	C	C

Note: The four-parameter diagnostic score is as described by Della Porta et al., 2006. Abnormal features in progenitors, granulocytes, monocytes, and erythropoiesis as depicted in Tables 1–4.

[a]*i*FS Category A: no MDS-related features; B: limited number of changes associated with MDS, or C: features consistent with MDS. Choice for A or B and B or C depends on the kind and number of aberrancies that are encountered. Note that patients with ≥2 points in the four-parameter score can still be concluded as not consistent with MDS by the *i*FS when there are no other abnormalities.

been developed. In another study, FlowSOM was used in a fully automated diagnostic workflow, combined with preprocessing of raw data by Flow-artificial intelligence (FlowAI) and a random forest machine learning algorithm (Duetz et al., 2021). This workflow outperformed currently used manually analyzed scores such as the iFS and the Ogata score regarding accuracy (sensitivity 85%–97% and specificity 93%–97%), analysis time (<3 min), and the number of antibodies needed. In addition, this method confirmed the discriminative value of CD36 and CD71 MFI CVs for the erythroid lineage. It has also identified an increased SSC of more mature erythroid cells as a highly informative feature of MDS-associated dysplasia (Duetz et al., 2021; Westers et al., 2023). This finding was independently confirmed by others using traditional analysis (Johansson et al., 2021). Likewise, Barreau et al. (2020) developed a semi-automated approach for a MFC diagnosis of MDS. BM-derived monocytes and neutrophils were analyzed using the maturation tool in Infinicyt™. This approach resulted in increased diagnostic accuracy, especially with increased sensitivity, and proved valuable in prognostication.

Other studies focused primarily on risk stratification, such as a large study of Ko et al. (2018) who applied Gaussian Mixture Models on MFC data from healthy donors and MDS or AML patients, before and after treatment. These models allowed for the prediction of progression-free and overall survival in AML and MDS patients (van Spronsen et al., 2019).

These studies are promising and may make MDS MFC accessible to more laboratories, by reducing time investment for analysis and increasing the accuracy of diagnosis and risk stratification by identifying novel parameters. Nonetheless, several steps and studies are desirable to facilitate widespread (clinical) implementation. Essential aspects to consider are uniformity and quality standards for panels, sample preparation, flow cytometers and optimization of raw MFC data. Although this is important for (computational) MFC in general, it may be particularly important in MDS, as MDS aberrancies are more complex and subtle than those of acute leukemia or multiple myeloma. Therefore, rigorous quality measures may be necessary for MDS to reach a sufficiently high signal-to-noise ratio, especially regarding studies and clinical applications for MFC data from different centers acquired over a long time. Several suggestions for optimization of pre-analytical and analytical steps for high dimensional MFC data and computational analysis are made in the paper by Brummelman et al. (2019). If retrospective data are used from multiple centers or flow cytometers, without standardized instrument settings and protocols several methods must be developed to normalize raw MFC data (Azad et al., 2016; Finak et al., 2014; Subirá et al., 2021). Some of these normalization methods use control samples as a reference, and therefore, require a different study design (Hahne et al., 2010; Van Gassen et al., 2020). It seems that complete standardization of protocols may be critical for inter-laboratory comparison of results unless every laboratory undertakes its own machine-learning process on controls.

The development of novel computational systems for detecting cell (sub)populations is still ongoing (Finak et al., 2016; Flores-Montero et al., 2017; Pedreira et al., 2019). It is of interest that several of these tools will be applied in parallel to classical analyses to investigate which are most suited for MDS integrated diagnosis, risk stratification, and potential response to therapy based on MFC data. In addition, compilations of large uniform datasets are desirable, as most of these machine-learning-based methods tend to perform better with larger sample numbers, especially in such a heterogeneous disease as MDS.

AUTHOR CONTRIBUTIONS

All persons listed as co-authors contributed to pre-conference and post-conference discussions and actively participated in the Standardization Conferences. All co-authors contributed equally by discussing criteria, standards, and recommendations at the Working Conferences: 14th/2nd virtual annual ELN MDS Flow Working Conference April 30, 2021; 13th/1st virtual meeting Nov 6, 2020; 12th ELN MDS Flow Working Conference Oct 31-Nov 2, 2019, Nijmegen, Netherlands; 11th Annual meeting Nov 1–3, 2018, Munich, Germany; 10th Annual meeting, Nov 2–4, 2017, Lund, Sweden; 9th Annual meeting, Oct 27–29, 2016, Paris, France; 8th Annual meeting Oct 29–31, 2015, Athens, Greece. In addition, all persons listed as co-authors provided essential input by drafting parts of the manuscript and by approving the final version of the document.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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