

# Exploring blast composition in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms: CD45RA and CD371 improve diagnostic value of flow cytometry through assessment of myeloblast heterogeneity and stem cell aberrancy

Afshin Shameli | Poonam Dharmani-Khan | Joanne Luider | Iwona Auer | Meer-Taher Shabani-Rad

Division of Hematology, Alberta Precision Laboratories, South Zone, Department of Pathology & Laboratory Medicine, University of Calgary, Calgary, Alberta, Canada

## Correspondence

Afshin Shameli, Foothills Medical Centre, McCaig Tower, 1403 29 Street NW, Calgary, AB, Canada T2N 2T9.  
Email: afshin.shameli@albertaprecisionlabs.ca

[Correction added on 13 February, 2021, after first online publication: The copyright line was changed.]

## Abstract

**Background:** Flow cytometry immunophenotyping (FCIP) can improve diagnosis of myelodysplastic syndromes (MDS) and myelodysplastic/myeloproliferative neoplasms (MDS/MPN), although its application is challenging due to difficulties in standardization, complexity of antibody panels and subjective interpretation of data. Since blasts are invariably affected in these disorders, we developed a FCIP approach for detailed and objective analysis of the blast population.

**Methods:** FCIP using a one-tube 10-color (13-marker) antibody panel was performed on bone marrow samples from 23 MDS and 8 MDS/MPN patients, 21 cytopenic patients non-diagnostic for MDS (Non-MDS), and 16 Control samples.

**Results:** MDS and MDS/MPN cases demonstrated one to several immunophenotypic abnormalities including: increased myeloblasts, decreased stage-1 hematogones, aberrant stem cells, abnormal myeloblast heterogeneity/divergence from normal, increased or decreased CD45 intensity, increased CD117 or CD123 intensity, decreased CD38 intensity, and aberrant expression of lineage markers (CD5, CD19, CD56). A Blast score was developed that showed sensitivity of 80.6% and specificity of 90.5% for immunophenotypic diagnosis of MDS and MDS/MPN. Expression levels of CD45RA and CD371 were used to evaluate abnormal myeloblast heterogeneity and stem cell aberrancy. Both these features were, for the first time, incorporated into a scoring system and resulted in 19% increase in the sensitivity of the assay for lower-risk MDS.

**Conclusion:** Deep immunophenotypic analysis of the blast population is valuable for diagnosis of MDS and MDS/MPN and can potentially provide sensitivity and specificity figures comparable to those previously described using more comprehensive panels that assess maturing myelomonocytic and erythroid elements in addition to progenitor cells.

## KEYWORDS

blast heterogeneity, Blast score, flow cytometry, myelodysplastic syndromes, stem cells

## 1 | INTRODUCTION

Myelodysplastic syndromes are a group of clonal stem cell disorders characterized by peripheral blood (PB) cytopenia(s) secondary to inefficient hematopoiesis, morphologic dysplasia, and propensity to progress to acute myeloid leukemia (AML) (Swerdlow et al., 2017). Presence of at least 10% dysplastic cells in one or more of erythroid, myeloid, and megakaryocytic lineages is required for morphologic diagnosis of MDS. While these morphologic features are well characterized (Swerdlow et al., 2017; Weinberg & Hasserjian, 2019), diagnosis can be challenging due to borderline changes leading to subjective interpretation and interobserver variability (Font et al., 2015). In fact, without objective evidence of dysplasia such as increased blasts, ring sideroblasts or karyotypic abnormalities, diagnosis sometimes requires follow up of blood counts, repeat bone marrow examinations, and careful exclusion of non-neoplastic causes of cytopenia and dysplasia such as nutritional deficiencies, alcohol toxicity, viral infections, autoimmune conditions, and medications, among others.

Myelodysplastic syndromes are heterogeneous disorders resulting from various combinations of somatic mutations. Recent advances in next generation sequencing (NGS) analysis of such mutations have improved our understanding of pathogenesis, and allowed for better prognostication and prediction of response to therapy (Lindsley, 2017; Swerdlow et al., 2017; Weinberg & Hasserjian, 2019). While NGS study can be diagnostically helpful in some instances (such as SF3B1 mutation in MDS with ring sideroblasts), its application as a diagnostic tool has limitations such as long turn-around time, higher cost, and mutation overlap with pre-malignant conditions such as clonal cytopenia of undetermined significance (CCUS) and clonal hematopoiesis of indeterminate potential (Lindsley, 2017).

FCIP has been extensively studied as a valuable tool to assist in diagnosis of MDS and MDS/MPN. Various groups have shown that numerical and immunophenotypic aberrancies of blasts, maturing myelomonocytic and erythroid elements are helpful in differentiating myelodysplasia from non-neoplastic cytopenia (Shi et al., 2017). However, no single parameter has perfect sensitivity and specificity for such distinction and therefore, different scoring systems have been developed to combine these abnormalities (Della Porta et al., 2012; Kern et al., 2010; Stachurski et al., 2008; van de Loosdrecht et al., 2008). The latest guidelines from International/European LeukemiaNet Working Group for Flow cytometry in MDS (IMDSFlow) recommended application of either a mini-panel (so-called "Ogata score"), or a more comprehensive panel of antibodies that allows for assessment of several parameters on progenitor myeloid and B cells, neutrophils, monocytes and erythroid elements (Porwit et al., 2014). It was noted that Ogata score has limitations in sensitivity and specificity that can be overcome by application of the comprehensive panel (Porwit et al., 2014). The presence of at least three aberrant findings in at least two

compartments was associated with MDS or MDS/MPN. This panel, however, requires various combinations of a total of 18 markers and its implementation can be outside the budgetary limitations of some institutions. Moreover, interpretation of the results requires considerable knowledge of normal and pathological maturation patterns, and such degree of expertise is not readily available in many institutions. That is why, despite its unequivocal value, flow cytometry is not universally used in diagnostic work-up of MDS and MDS/MPN.

FCIP analysis of blasts is an ideal target for diagnosis of myelodysplasia for a number of reasons: (1) MDS and MDS/MPN are clonal stem cell disorders and blasts are expected to be affected in almost all cases; (2) Both frequency and immunophenotypic aberrancies of blasts can be evaluated by flow cytometry; (3) Blasts immunophenotype is likely more stable than maturing myelomonocytic elements (of which immunophenotype and granularity index can be affected by storage time, fixation, marrow regeneration, granulocyte colony stimulating factor [G-CSF] therapy or hemodilution [Alhan et al., 2016; Chen et al., 2019a; Shi et al., 2017; Stachurski et al., 2008]); (4) Blast and stem cell aberrancies, as compared to aberrancies of maturing myelomonocytic elements, are expected to have higher specificity for diagnosis of myelodysplasia (Behbehani et al., 2020; Feng et al., 2018; Kern et al., 2010; Stachurski et al., 2008). Aberrancies of the blast fraction include increased frequency of myeloblasts (CD34<sup>++</sup> CD117<sup>+</sup> precursors), decreased frequency of stage-1 hematogones (CD34<sup>+</sup> CD19<sup>+</sup> CD10<sup>+</sup> precursors), abnormal intensity of markers normally expressed on myeloblasts (CD45, CD117, CD13, CD33, CD38, and HLA-DR), asynchronous expression of markers associated with maturity (CD11b and CD15), and expression of lineage infidelity markers (CD5, CD7, CD19 and CD56) (Chen et al., 2019a, 2020; De Smet et al., 2012; Della Porta et al., 2012; Feng et al., 2018; Goardon et al., 2009; Kern et al., 2010; Kussick et al., 2005; Maftoun-Banankhah et al., 2008; Matarraz et al., 2008; Ogata et al., 2006; Porwit et al., 2014; Schenkel et al., 2019; Shi et al., 2017; Stachurski et al., 2008; van de Loosdrecht et al., 2008).

CD34-positive blast fraction is heterogeneous. This is due to presence of a spectrum of maturation stages and differentiation into distinct lineages including B-lymphoid, erythroid/megakaryocytic and myelomonocytic precursors. Previous studies have shown that such heterogeneity is reduced in myeloid neoplasms, most notably MDS (Jevremovic et al., 2014; Schenkel et al., 2019). For example, combination of CD13 and HLA-DR was used for qualitative (Jevremovic et al., 2014) or quantitative (Schenkel et al., 2019) approaches to define blast heterogeneity. Loss of blast heterogeneity in these studies was likely secondary to reduced stage-1 hematogones and changes in relative composition of different myeloid progenitors. Skewed distribution of progenitor cells in myelodysplastic syndromes has been demonstrated in other studies (Ostendorf et al., 2018; Will et al., 2012).

Normal hematopoietic stem cells (HSC), as defined by lineage (Lin)<sup>-</sup> CD34<sup>++</sup> CD38<sup>-</sup> immunophenotype, are characterized by capacity to self-replicate and differentiate into various myeloid and lymphoid progenitors. While it was long believed that myelodysplastic syndromes are clonal stem cell disorders, this notion has only recently been validated by demonstration of cytogenetics and molecular abnormalities in stem cells (Chen et al., 2019b; Tehrani et al., 2010; Toft-Petersen et al., 2016; Will et al., 2012; Woll et al., 2014). MDS stem cells (MDS-SCs), in contrast to HSCs, have limited replication and skewed differentiation capacity and give rise to dysplastic cells (Will et al., 2012). Moreover, MDS-SCs are resistant to therapies such as lenalidomide or azacitidine and can cause relapsed disease or progression to MDS with excess blasts (MDS-EB) or AML in a subset of patients (Chen et al., 2019b; Tehrani et al., 2010; Will et al., 2012). In AML, leukemic stem cells or AML stem cells (AML-SCs), upon injection into immunodeficient mice, can self-replicate and produce leukemic blasts with the same phenotype as the original disease (Hanekamp et al., 2017). AML-SCs demonstrate resistance to chemotherapy and may escape apoptosis, leading to self-renewal, proliferation and development of relapsed disease after chemotherapy. Stem cells in myeloid neoplasms are heterogeneous and their distinction from normal HSCs requires combination of several markers. Aberrant expression of markers such as C-type lectin-like molecule-1 (CLL-1 or CD371), CD45RA, T-cell immunoglobulin mucin-3 (TIM-3), and lineage markers (CD2, CD5, CD7, CD19, CD22, CD25, CD56, CD11b), as well as bright expression of CD33, CD44 and CD123 can differentiate AML or MDS stem cells from normal HSCs (Chen et al., 2019b; Hanekamp et al., 2017; Kersten et al., 2016; Ostendorf et al., 2018; Toft-Petersen et al., 2016; van Rhenen et al., 2007; Zeijlemaker et al., 2016). Recent studies have suggested that stem cell aberrancies have high sensitivity and specificity for diagnosis of myelodysplastic syndromes (Behbehani et al., 2020), supporting the application of these aberrancies in flow cytometric scoring systems.

Here, we designed a one-tube 10-color (13-marker) flow cytometry assay to simultaneously assess different abnormalities of blast population in bone marrow samples of a series of MDS and MDS/MPN patients. These parameters include myeloblast and stage-1 hematogone frequencies, immunophenotypic aberrancies of myeloblasts, abnormal myeloblast heterogeneity/divergence from normal, and stem cell aberrancies. The combination of CD45RA and CD371 in the panel aided in quantification of the latter two parameters. We also proposed a new scoring system based on deep immunophenotypic analysis of blasts.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and specimens

The study was approved by the University of Calgary Conjoint Health Research Ethics Board. From June 2019 to November 2019, a total of 57 consecutive bone marrow aspirates that were collected from adult patients with cytopenia(s) with or without leukocytosis, with clinical

suspicion of MDS or MDS/MPN were included in the study. Cases with erythrocytosis or thrombocytosis, with or without JAK2 V617F mutation and concern for myeloproliferative neoplasms were not included. Bone marrow diagnosis and subclassification was performed according to 2017 World Health Organization (WHO) classification of tumor of hematopoietic and lymphoid tissue (Swerdlow et al., 2017). Each case was reviewed by at least two hematopathologists with expertise in diagnosis of myelodysplastic syndromes. Twenty-three MDS, 8 MDS/MPN (including 7 chronic myelomonocytic leukemia (CMML) and 1 MDS/MPN-unclassifiable (MDS/MPN-U)), and 21 cases of cytopenia(s) that were not diagnostic for morphologic evidence of MDS (Non-MDS) were included in the study. Conventional cytogenetics using standard G-band karyotyping was performed on all MDS and MDS/MPN cases and 12/21 Non-MDS cases. Karyotypic abnormalities were detected in 13/22 MDS and 3/8 MDS/MPN cases, and cytogenetics study of one MDS case failed to achieve metaphases. All 12 Non-MDS cases that were tested showed normal karyotypes. Two samples were diagnosed with chronic myeloid leukemia (CML), *BCR-ABL1*<sup>+</sup>, and were not included in data analysis. One sample was excluded due to recent G-CSF therapy, and two other samples were excluded due to acquisition of insufficient events by flow cytometry (less than  $1.0 \times 10^5$ ). Sixteen bone marrow aspirates from patients with recent diagnosis of lymphoma (eight follicular lymphoma, two diffuse large B-cell lymphoma, one Burkitt lymphoma, one plasmablastic lymphoma, one classic Hodgkin lymphoma), amyloid deposition disease (one patient), or splenomegaly with concern for lymphoma (two patients) were included as normal reference marrows (Control). All Control bone marrow samples were negative for neoplastic process.

### 2.2 | Flow cytometry analysis

10-color flow cytometry analysis was performed at the Alberta Precision Laboratories, South Zone, Flow Cytometry Laboratory. Aliquots of bone marrow samples were washed two times to remove plasma immunoglobulin, followed by ammonium chloride lysis and washing again. Antibody cocktail was added and incubated for 15 min in dark, followed by washing with PBS and resuspension in 0.1% paraformaldehyde for immediate acquisition on a Navios flow cytometer (Beckman Coulter, Brea, CA). Below is the laser-fluorochrome combinations: (1) 405-nm violet laser with two colors: Brilliant Violet (BV)-421 and BV-510; (2) 488-nm blue laser with five colors: fluorescein isothiocyanate (FITC), phycoerythrin (PE), ECD, PE-cyanine (Cy)-5.5 (PC5.5) and PE-Cy7 (PC7); (3) 638-nm red laser with three colors: allophycocyanin (APC), APC-Alexa Fluor 700 (APC-A700) and APC-A750.

Antibodies against following antigens were used in the cocktail: CD45 (J33)-APC-A700, CD38(LS198.4.3)-APC-A750, CD117(104D2D1)-PC5.5, CD16(3G8)-ECD, CD14(IM2702U)-ECD (Beckman Coulter, Brea, CA); CD371(50C1)-PC7, CD33(WM53)-APC, CD34(581)-FITC, CD5(UCHT2)-PE, CD19(HTB19)-PE, CD56(MEM18)-PE, CD123(6H6)-BV421, and CD45RA(HI100)-BV510 (BioLegend, Inc.). Note that 13 markers were

used in 10 fluorochromes (antibodies to CD16 and CD14 both in ECD and antibodies to lineage markers CD5, CD19 and CD56 all in PE).

An average of 1,473,597 events were acquired with ~90% of cases having more than  $1.0 \times 10^6$  events. High numbers of events were obtained to assess low frequency populations such as stem cells and progenitors, and for reliable assessment of immunophenotypic aberrancies. An average of 21,894 events (range 1137–98,246) in the CD34-positive gate and 3792 events (range 50–52,300) in the CD34<sup>+</sup> CD38<sup>-</sup> stem cell gate were assessed. Analysis was performed using FCS Express 6 software (De Novo Software).

### 2.3 | Statistical analysis

Statistical analysis was performed using Prism 8 (GraphPad software Inc.). Numerical variables were compared using two-tailed Mann-Whitney *U* test. Statistical significance was considered at  $p < 0.05$ . Reference ranges of flow cytometric variables were calculated using mean  $\pm$  2 SD of data derived from 16 Control bone marrow samples. Abnormal values for flow cytometry parameters on CD34<sup>++</sup> CD117<sup>+</sup> myeloblasts were as following: lymphocyte/myeloblast CD45 mean fluorescent intensity ratio  $>9.8$  or  $<5.2$ ; CD117 median fluorescent intensity (MFI)  $>530$ ; CD123 MFI  $>20$ ; CD38 MFI  $<100$ ; percentage of Lin<sup>+</sup> (CD5 + CD19 + CD56)  $> 10\%$ ; percentage of CD371<sup>+</sup>  $< 20\%$  or  $>55\%$ ; and percentage of CD45RA<sup>+</sup>  $< 15\%$  or  $>50\%$ . Upper limit for CD45RA was changed from 45% to 50% to reduce false-positive cases. Abnormal values for myeloblasts percentage ( $\geq 2\%$ ) and stage-1 hematogone frequency ( $<5\%$  of CD34-positive gate) were based on previous studies (Della Porta et al., 2012). Receiver operating characteristic (ROC) curve method was applied to choose the cut-off value for aberrant stem cells with optimal discrimination between MDS and Non-MDS cases (0.02%). Myeloblast divergence index was defined to quantitate the degree of which myeloblasts of each case were different from normal, by calculating the sum of absolute differences in the percentages of each quadrant of CD371 versus CD45RA plot between that specific case and average of Control samples, as following:  $|\text{quadrant 1 (Q1)}|(\text{case-55})| + |\text{Q2 (case-7)}| + |\text{Q3 (case-23)}| + |\text{Q4 (case-15)}|$ . Myelomonocytic aberrancies were assessed by measuring side scatter (SS) ratio and percentage of Lin<sup>+</sup> granulocytes or monocytes. SS ratio was calculated by dividing the mean granulocyte SS value to mean lymphocyte SS value. ROC curve method was applied

to determine the optimal cut-offs for these parameters, with following values considered abnormal: SS ratio  $< 4.4$ , percentage of Lin<sup>+</sup> granulocytes  $>10\%$ , and percentage of Lin<sup>+</sup> monocytes  $>25\%$ .

## 3 | RESULTS

Table 1 summarizes patient demographics, peripheral blood counts and number of patients with cytogenetics abnormalities. Risk stratification was performed according to Revised International Prognostic Scoring System (IPSS-R) for MDS patients (Greenberg et al., 2012), and Mayo prognostic model for CMML patients (Patnaik et al., 2013). Table 2 shows the number of cytopenias and their underlying etiology in Non-MDS group. Table 3 shows WHO diagnoses, IPSS-R/Mayo prognostic risk groups and cytogenetics abnormalities of MDS and MDS/MPN cases. The MDS group included 16 very low- and low-risk, 3 intermediate-risk and 4 high- and very-high risk patients. The MDS/MPN group included seven CMML patients and one MDS/MPN-U patient. The CMML group included one low-risk, three intermediate-risk and three high-risk patients.

Figure 1a summarizes the gating strategy in a Control bone marrow. A usual blast gate was defined in the Dim-CD45/low-SS location. Within the Dim-CD45 gate, myeloblasts, stage-1 hematogones and stem cells were identified by gating on CD34<sup>++</sup> CD117<sup>+</sup>, CD34<sup>+</sup> CD117<sup>-</sup> and CD34<sup>++</sup> CD38<sup>-</sup> populations, respectively. Similar to previous studies of stem cells in AML and MDS (Hanekamp et al., 2017; Ostendorf et al., 2018; Zeijlemaker et al., 2016), we did not exclude lineage positive cells from stem cell gate, due to high frequency of aberrant expression of these markers in myeloid neoplasms. A back-gating strategy was applied to confirm that Dim-CD45 gate is wide enough to include all myeloblast, stage-1 hematogone and stem cell events. As expected, stage-1 hematogones demonstrated lower SS and forward scatter (FS) and dimmer CD34 expression than myeloblasts (Figure 1b), and showed CD38<sup>++</sup> CD45RA<sup>+</sup> Lin (CD19)<sup>+</sup> CD33<sup>-</sup> CD123<sup>-</sup> CD371<sup>-</sup> immunophenotype (Figure 1c, bottom, and data not shown). Normal stem cells showed brighter CD34 and CD45 expression than average myeloblasts and stage-1 hematogones, and SS and FS properties were intermediate between these two populations (Figure 1b). In contrast to stage-1 hematogones (Figure 1c, bottom), myeloblasts showed heterogeneous expression of CD33, CD38, CD123, CD45RA and CD371 (Figure 1c, top). CD34<sup>++</sup> CD117<sup>+</sup>

**TABLE 1** Clinical and hematologic characteristics of the study groups (numbers show mean  $\pm$  SD)

	Non-MDS (n = 21)	MDS (n = 23)	MDS/MPN (n = 8)
Age	63 $\pm$ 11	77 $\pm$ 9	80 $\pm$ 8
Gender (M/F)	11/10	20/3	4/4
Hemoglobin (g/L)	99 $\pm$ 25	100 $\pm$ 20	107 $\pm$ 28
Platelet ( $\times 10^9$ /L)	106 $\pm$ 96	117 $\pm$ 53	94 $\pm$ 59
Neutrophil ( $\times 10^9$ /L)	2.22 $\pm$ 2.89	1.34 $\pm$ 0.90	14.32 $\pm$ 13.38
Morphologic blast count	0.9 $\pm$ 0.9	3.7 $\pm$ 4.3	2.8 $\pm$ 2.0
Abnormal cytogenetics	0/12	13/22	3/8

Abbreviations: MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms.

**TABLE 2** Clinical diagnoses of Non-MDS cohort

Diagnosis/etiology	Number
Anemia	4
Unknown etiology	2
Beta-thalassemia	1
Chronic kidney disease	1
Neutropenia	4
Unknow etiology	1
Cyclin neutropenia	1
Seronegative arthritis	1
Rheumatoid arthritis and history of breast cancer therapy	1
Anemia and thrombocytopenia	2
Unknown etiology	1
Cholestatic liver disease	1
Thrombocytopenia and neutropenia	5
Unknow etiology	2
Cirrhosis	2
Rheumatoid arthritis	1
Pancytopenia	6
Unknow etiology	3
Primary biliary cirrhosis	1
Rheumatoid arthritis	1
Kidney transplant	1

Abbreviation: MDS, myelodysplastic syndromes.

CD38<sup>++</sup> myeloblasts could be divided into common myeloid progenitor (CMP)-like (CD123<sup>+</sup> CD45RA<sup>-</sup>), megakaryocyte-erythroid progenitor (MEP)-like (CD123<sup>-</sup> CD45RA<sup>-</sup>) and granulocyte-monocyte progenitor (GMP)-like (CD123<sup>+</sup> CD45RA<sup>+</sup>) fractions. MEP-like fraction showed the lowest levels of CD33 and CD371 expression, while GMP-like fraction showed the highest expression levels (Figure 1d). There was a positive correlation between expression levels of myeloid markers CD33 and CD371.

This combination of markers allowed for dissection of several other populations within the Dim-CD45 gate. Among myeloid progenitors, expression of CD371 is associated with myelomonocytic differentiation, whereas its absence is seen with erythroid differentiation (Bill et al., 2018; Toft-Petersen et al., 2016; van Rhenen et al., 2007). CD34<sup>-</sup> CD117<sup>+</sup> precursors were differentiated into promyelocytes with bright expression of CD33, moderate CD371 expression and heterogeneous positivity for CD45RA (CD34<sup>-</sup> CD117<sup>+</sup> CD33<sup>++</sup> CD371<sup>++</sup> CD38<sup>++</sup> CD45RA<sup>+/-</sup>), and erythroid precursors showing lack of CD33, CD371 and CD45RA expression (CD34<sup>-</sup> CD117<sup>+</sup> CD33<sup>-</sup> CD371<sup>-</sup> CD38<sup>+</sup> CD45RA<sup>-</sup>) (Figure 2a,b). Later myeloid elements were characterized by moderate CD33 and CD371 expression and loss of CD117, CD38 and CD45RA expression (CD34<sup>-</sup> CD117<sup>-</sup> CD33<sup>+</sup> CD371<sup>++</sup> CD38<sup>-</sup> CD45RA<sup>-</sup>). Monocytic elements were characterized by bright expression of CD33 and CD371 and heterogeneous expression of CD45RA (CD34<sup>-</sup> CD117<sup>-</sup> CD33<sup>++</sup> CD371<sup>+++</sup> CD38<sup>+</sup> CD45RA<sup>+/-</sup>) (Figure 2b). Basophils and plasmacytoid dendritic cells expressed bright CD123 and were separated on the basis of differential CD45RA

and CD33 expression (plasmacytoid dendritic cells: CD34<sup>-</sup> CD117<sup>-</sup> CD33<sup>-</sup> CD371<sup>+</sup> CD38<sup>+</sup> CD123<sup>++</sup> CD45RA<sup>+</sup>; basophils: CD34<sup>-</sup> CD117<sup>-</sup> CD33<sup>+</sup> CD371<sup>+</sup> CD38<sup>+</sup> CD123<sup>++</sup> CD45RA<sup>-</sup>) (Figure 2b, bottom right) (Jegalian et al., 2009). Other populations within the blast gate included plasma cells (bright CD38) and mast cells (bright CD117) (Figure 2b). These populations occupied their expected locations in the Dim-CD45 gate (Figure 2c). Myeloid neoplasms resulted in changes in the composition of different populations within the Dim-CD45 gate. These changes are outside the scope of this article. Some examples are shown in Figure S1.

As expected, MDS cases showed higher percentage of myeloblasts as compared to Non-MDS (Figure 3a). No statistically significant increase in myeloblast percentage was noted in our cohort of MDS/MPN cases. In line with previous studies, we noted significantly decreased frequency of stage-1 hematogones among total CD34-positive blast population in both MDS and MDS/MPN groups, as compared to Non-MDS group (Figure 3b). Moreover, the small MDS/MPN cohort showed lower frequency of stage-1 hematogones, as compared to MDS group (Figure 3b). Various degrees of aberrant expression of CD45, CD117, CD123 and CD38 were detected in myeloblasts of MDS and MDS/MPN cases. Both aberrantly increased or decreased expression of CD45 were detected in a significant number of MDS and MDS/MPN cases (Figure 3c). Myeloblasts of MDS and MDS/MPN cases showed significantly increased expression of CD117 (Figure 3d) and decreased expression of CD38 (Figure 3f), as compared with Non-MDS myeloblasts. MDS/MPN cases showed brighter expression of CD117 than MDS cases (Figure 3d). CD123 expression was significantly increased in myeloblasts of MDS/MPN cases as compared to Non-MDS, and a trend toward higher expression was seen in myeloblasts of MDS cases compared to Non-MDS, although the difference was not statistically significant (Figure 3e). No difference in the frequency of myeloblasts and stage-1 hematogones, and expression levels of CD45, CD117, CD123 and CD38 were observed between Non-MDS and Control groups.

Next, we sought to explore how myeloblast heterogeneity and progenitor cell composition change in MDS and MDS/MPN cases. Among different combinations of markers in our panel, CD45RA versus CD371 plots showed the best separation of sub-populations. CD34<sup>++</sup> CD117<sup>+</sup> myeloblasts were distributed in all four quadrants with highest percentage of cells located in double-negative quadrant, followed by double-positive and single-positive quadrants (Figure 4a). This pattern was highly preserved in myeloblasts of Control bone marrow samples. Myeloblasts from most Non-MDS samples also showed similar distribution patterns (Figure 4b), with only few cases deviating from normal distribution pattern. On the other hand, a significant number of MDS and MDS/MPN cases showed an abnormal distribution pattern (Figure 4c–e, top panels). This abnormal distribution was, to a large extent, secondary to selective expansion of a myeloid progenitor (CMP-like, GMP-like, or MEP-like) and relative decrease in other progenitors (Figure 4c–e, bottom panels). To quantify deviation from normal, myeloblast divergence index was defined (see materials and methods). This index was significantly higher in myeloblasts of MDS and MDS/MPN cases, as compared to Non-MDS cases

**TABLE 3** WHO diagnoses, risk groups and cytogenetics results of individual MDS and MDS/MPN cases

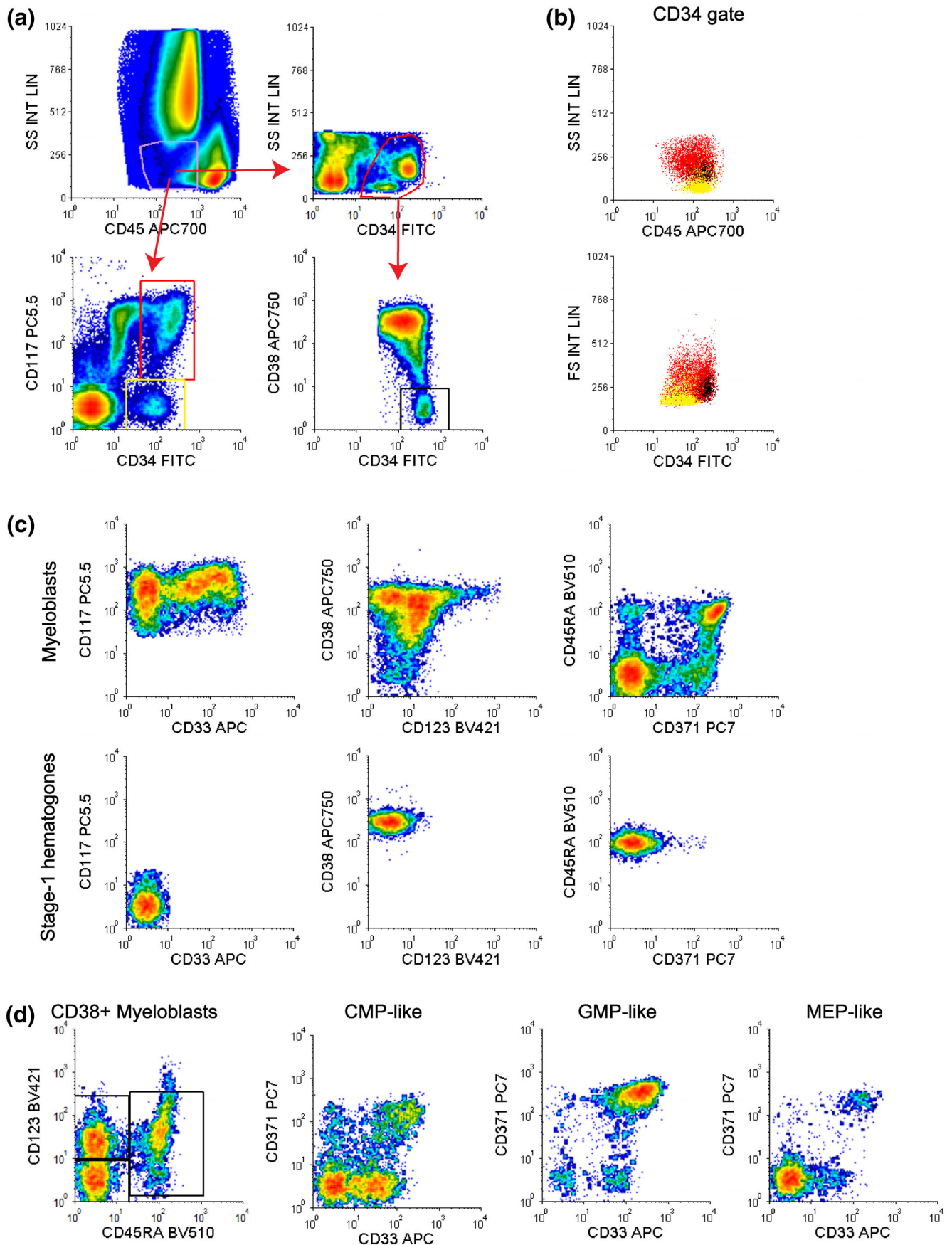
Age/gender	WHO classification	IPSS-R/Mayo	Cytogenetics
86/M	MDS-RS-MLD	Very low	46,XY,del(11)(q13q23)[8]/46,XY[12]
81/M	MDS-MLD	Very low	45,X,-Y[20]
67/M	MDS-MLD	Very low	46,XY[20]
76/M	MDS-RS-SLD	Very low	46,XY[20]
61/M	MDS-SLD	Low	47,XY,+8[3]/46,XY[17]
85/M	MDS-MLD	Low	45,X,-Y[14]/46,XY[6]
86/F	MDS-RS-MLD	Low	46,XX[20]
83/M	MDS-RS-MLD	Low	46,XY,del(20)(q11.2q13)[11]/46,XY[9]
74/M	MDS-MLD	Low	46,XY[20]
66/M	MDS-MLD	Low	46,XY[20]
85/M	MDS-SLD	Low	47,XY,+8[3]/46,XY[17]
64/M	MDS-MLD	Low	47,XY,+8[19]/46,XY[1]
89/M	MDS-MLD	Low	46,XY[20]
69/M	MDS-RS-MLD	Low	44,XY,del(5)(q13q33),-7,-18[5]/46,XY[13]
88/M	MDS-MLD	Low	46,XY[20]
81/M	MDS-MLD	Low	46,XY[20]
68/M	MDS-MLD	Intermediate	47,XY,+8[7]/46,XY[13]
92/M	MDS-EB2	Intermediate	46,XY[20]
81/M	MDS-MLD	Intermediate	46,XY,i(17)(q10)[16]/46,XY[4]
72/M	MDS-EB2	High or Very high	No metaphases
83/F	MDS-EB1	High	46,XX,i(17)(q10)[16]/46,XX[4]
70/F	MDS-EB1	Very high	46,XX,del(5)(q22q35)[2]/54,idem,+1,+6,+8,+8,+9,+11,+14,+19,add(22)(q13)[13]/46,XX[5]
72/M	MDS-EB1	Very high	Complex karyotype
84/M	CMML1	Low	46,XY[20]
81/M	CMML0	Intermediate	46,XY[20]
78/M	CMML0	Intermediate	46,XY[20]
88/F	CMML-0	Intermediate	46,XX[20]
69/F	CMML0	High	46,XX,add(5)(q11.2)[9]/53,idem,+1,+6,+8,+11,+14,+22,+22[10]/46,XX[1]
77/F	CMML0	High	45,X,-X[20]
72/M	CMML1	High	46,XY[15]
94/F	MDS/MPN-U	N/A	47,XX,+8[3]/46,XX[18]

Abbreviations: CMML, chronic myelomonocytic leukemia; MDS/MPN-U, MDS/MPN unclassifiable; MDS-EB, MDS with excess blasts; MDS-MLD, MDS with multilineage dysplasia; MDS-RS-MLD, MDS with ring sideroblasts and multilineage dysplasia; MDS-RS-SLD, MDS with ring sideroblasts and single lineage dysplasia; MDS-SLD, MDS with single lineage dysplasia.

(Figure 4f). Compared to Control cases, Non-MDS myeloblasts showed mild, but statistically significance increase in the myeloblast divergence index (Figure 4f). While myeloblasts from Control and Non-MDS cases showed low-level variation in the frequency of CD45RA and CD371-positive cells, myeloblasts from MDS and MDS/MPN cases demonstrated higher variability in expression levels (Figure 4g,h). Evaluation of CMP-like, GMP-like and MEP-like myeloid progenitors demonstrated wide distribution in MDS myeloblasts, as compared to Control and Non-MDS myeloblasts (Figure S2a–c). Given that this distribution of precursors extended above and below normal distribution, no statistically significant differences were present

between MDS and Non-MDS cases. On the other hand, MDS/MPN myeloblasts showed increased frequency of CMP-like progenitors compared to both Non-MDS and MDS, as well as decreased MEP-like progenitors compared to Non-MDS cases (Figure S2a–c). Compared to Control group, myeloblasts of Non-MDS cases showed decreased frequency of CMP-like progenitors in the expense of smaller (and statistically not significant) increases in GMP-like and MEP-like progenitors.

Last, we studied stem cell aberrancy in MDS and MDS/MPN cases by evaluating the expression of CD371, CD45RA, CD33, CD123, and lineage markers (CD5, CD19 and CD56 in one



**FIGURE 1** Legend on next page.

fluorochrome channel) on CD34<sup>++</sup> CD38<sup>-</sup> stem cells. While some MDS cases showed no significant stem cell aberrancy (Figure 5a), other cases demonstrated stem cell aberrancy characterized by expression of one to several markers (Figure 5b,c). The frequency of aberrant stem cells was significantly higher in MDS cases as compared to Non-MDS cases (Figure 5d). Using a threshold of 0.02% aberrant stem cells, 16 of 23 MDS and 3 of 8 MDS/MPN had increased aberrant stem cells, while only 2 of 21 Non-MDS cases showed stem cell aberrancy.

Figure 6a-top panel summarizes the blast abnormalities evaluated in this study. These parameters include: myeloblasts percentage, percentage of aberrant stem cells, frequency of stage-1 hematogones in CD34-positive gate, abnormal myeloblast heterogeneity/divergence from normal (reflected by increased or decreased percentages of CD45RA or CD371), and immunophenotypic aberrancy (increased CD117 MFI, increased CD123 MFI, decreased CD38 MFI, increased or decreased lymphocyte to myeloblast CD45 ratio, and increased percentage of Lin<sup>+</sup> myeloblasts). A scoring system (Blast score) was developed as following: 2 points were given to increased myeloblasts, one point to decreased hematogones, one point to abnormal heterogeneity (either CD45RA or CD371 aberrancy), and one point to each immunophenotypic aberrancy (CD117, CD123, CD38, CD45 and Lin). Given that stem cell aberrancy was highly correlated with increased myeloblasts, and to avoid duplicate points, no point was given to aberrant stem cells in the presence of more than 2% myeloblasts. However, in cases with less than 2% myeloblasts, one point was given to stem cell aberrancy ( $\geq 0.02\%$ ). MDS and MDS/MPN Blast scores were significantly higher than Non-MDS and Control Blast scores (MDS mean score of 5.1 [range of 1–9], MDS/MPN mean score of 4.9 [range of 2–7], Non-MDS mean score of 0.9 [range of 0–5], and Control mean score of 0.7 [range 0–2]) (Figure 6b). Using a threshold Blast score of 3 for immunophenotypic definition of myelodysplasia, 19 of 23 MDS and 6 of 8 MDS/MPN cases were accurately diagnosed, whereas only 2 of 21 Non-MDS and none of Control cases had scores of 3 or higher. Overall, combining MDS and MDS/MPN cases, the Blast score of at least 3 showed a sensitivity of 80.6% and a specificity of 90.5%. In MDS cases alone, sensitivity was 82.6%, with the same specificity of 90.5%.

Finally, we evaluated myelomonocytic aberrancies (Figure 6a-bottom panel). The parameters that could be evaluated by this panel included granulocyte to lymphocyte SS ratio and percentage of Lin<sup>+</sup> granulocytes or monocytes. Total score was calculated to by adding one point for either of these aberrancies to Blast score (Figure 6c). Again, a total score of 3 or more was used to define dysplasia. Application of Total score did not improve diagnosis of MDS in the current

cohort, nor did it increase the number of false positive cases. However, addition of myelomonocytic parameters improved diagnosis of MDS/MPN cases (Figure 6a,c), supporting their application in the scoring system.

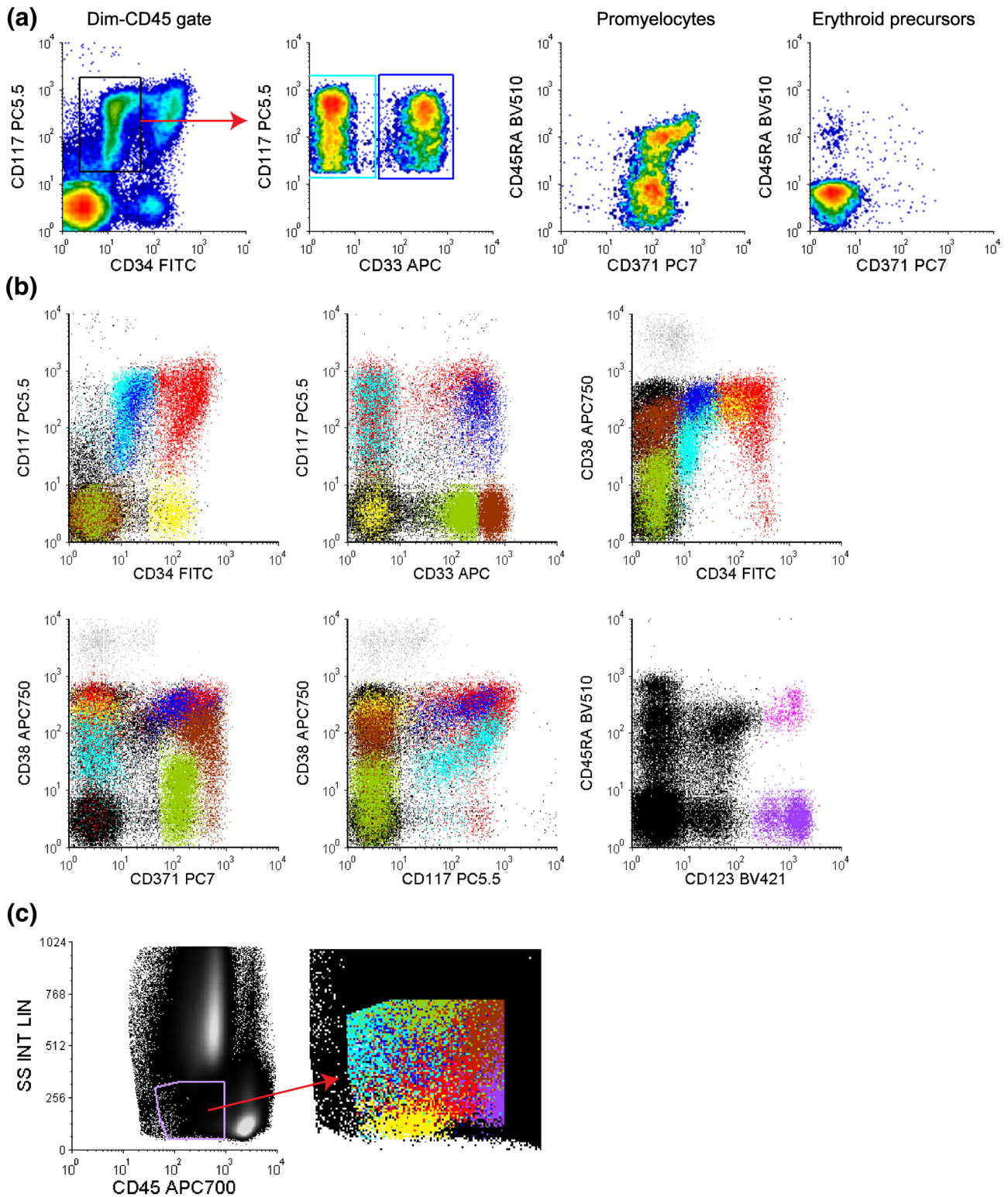
## 4 | DISCUSSION

Using a single-tube 10-color (13-marker) flow cytometry panel we assessed various abnormalities of the blast population in cohorts of MDS and MDS/MPN cases. These abnormalities include myeloblast percentage, frequency of stage-1 hematogones, myeloblast heterogeneity, and stem cell and myeloblast immunophenotypic aberrancies. Changes in frequency of myeloblasts and hematogones and immunophenotypic aberrancies of myeloblasts have been extensively studied in previous publications (Chen et al., 2020; De Smet et al., 2012; Della Porta et al., 2012; Feng et al., 2018; Goardon et al., 2009; Kern et al., 2010; Kussick et al., 2005; Maftoun-Banankhah et al., 2008; Matarraz et al., 2008; Ogata et al., 2006; Porwit et al., 2014; Schenkel et al., 2019; Stachurski et al., 2008; van de Loosdrecht et al., 2008), whereas limited information is available on abnormal blast heterogeneity, changes in relative composition of myeloid progenitors, and frequency of stem cell abnormalities in MDS (Jevremovic et al., 2014; Ostendorf et al., 2018; Schenkel et al., 2019; Toft-Petersen et al., 2016; Will et al., 2012). All parameters measured by this approach are quantitative, eliminating the need for extensive expertise in interpretation. Moreover, the panel mainly focuses on aberrancies of the blast compartment, which (in contrast to maturing myelomonocytic elements) is expected to be more stable, and less commonly affected by storage time, fixation, marrow regeneration, G-CSF therapy and/or hemodilution (Alhan et al., 2016; Chen et al., 2019a; Shi et al., 2017; Stachurski et al., 2008).

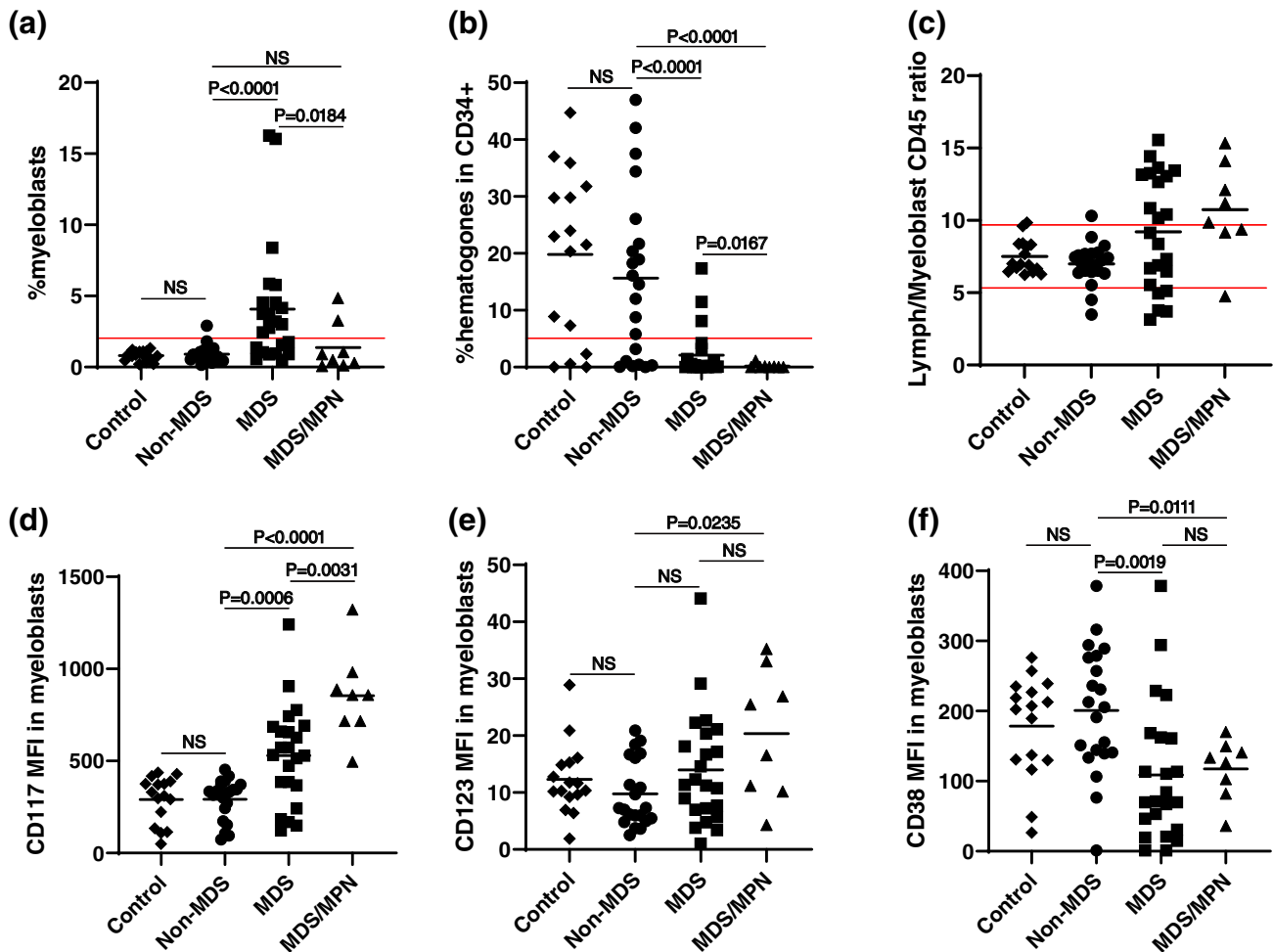
Consistent with previous studies (Chen et al., 2020; De Smet et al., 2012; Della Porta et al., 2012; Goardon et al., 2009; Maftoun-Banankhah et al., 2008; Matarraz et al., 2008; Ogata et al., 2006; Schenkel et al., 2019; van de Loosdrecht et al., 2008), MDS cases showed an increase in the percentage of myeloblasts associated with decreased frequency of stage-1 hematogones. Decreased stage-1 hematogones, despite high sensitivity (20/23 MDS and 8/8 MDS/MPN cases), showed low specificity (7 false positives in 21 Non-MDS cases). Interestingly, in line with a recent study (Chen et al., 2020), 2 of 3 cases with more than 5% stage-1 hematogones were diagnosed as MDS with ring sideroblasts. MDS and MDS/MPN cases showed higher intensity CD117 expression and lower intensity CD38, as compared to Non-MDS cases. Bright CD117 expression on

**FIGURE 1** Summary of the gating strategy in a Control bone marrow. (a) Blast gate was generated in the usual dim-CD45/low-SS location. Within the Dim-CD45 gate, CD34<sup>++</sup> CD117<sup>+</sup> myeloblasts and CD34<sup>+</sup> CD117<sup>-</sup> stage-1 hematogones were defined. A CD34-positive gate was generated from Dim-CD45 gate and was used to define CD34<sup>++</sup> CD38<sup>-</sup> stem cells. (b) Representative SS and FS properties and CD45 and CD34 expression levels of myeloblasts (red), stage-1 hematogones (yellow) and stem cells (black). (c) Representative expression of CD33, CD117, CD38, CD123, CD45RA and CD371 on myeloblasts (top) and stage-1 hematogones (bottom). (d) CD34<sup>++</sup> CD117<sup>+</sup> CD38<sup>+</sup> myeloblasts were divided into CMP-like (CD123<sup>+</sup> CD45RA<sup>-</sup>), GMP-like (CD123<sup>+</sup> CD45RA<sup>+</sup>) and MEP-like (CD123<sup>-</sup> CD45RA<sup>-</sup>) fractions. Representative plots showing CD33 and CD371 expression on these fractions are shown [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]





**FIGURE 2** Immunophenotypic dissection of the blast gate. (a)  $CD34^- CD117^+$  precursors were differentiated into promyelocytes showing CD33 and CD371 expression and erythroid precursors mainly lacking both markers. (b) The following populations could be identified in the blast gate: Myeloblasts (red), promyelocytes (blue), later myeloid elements (light green), monocytic elements (brown), erythroid precursors (turquoise), stage-1 hematogones (yellow), plasma cells (gray), and late hematogones, lymphocytes and residual erythroid elements (black). Basophils (purple), plasmacytoid dendritic cells (pink) are highlighted in the bottom right panel. (c) These populations occupied their expected locations in the blast gate [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

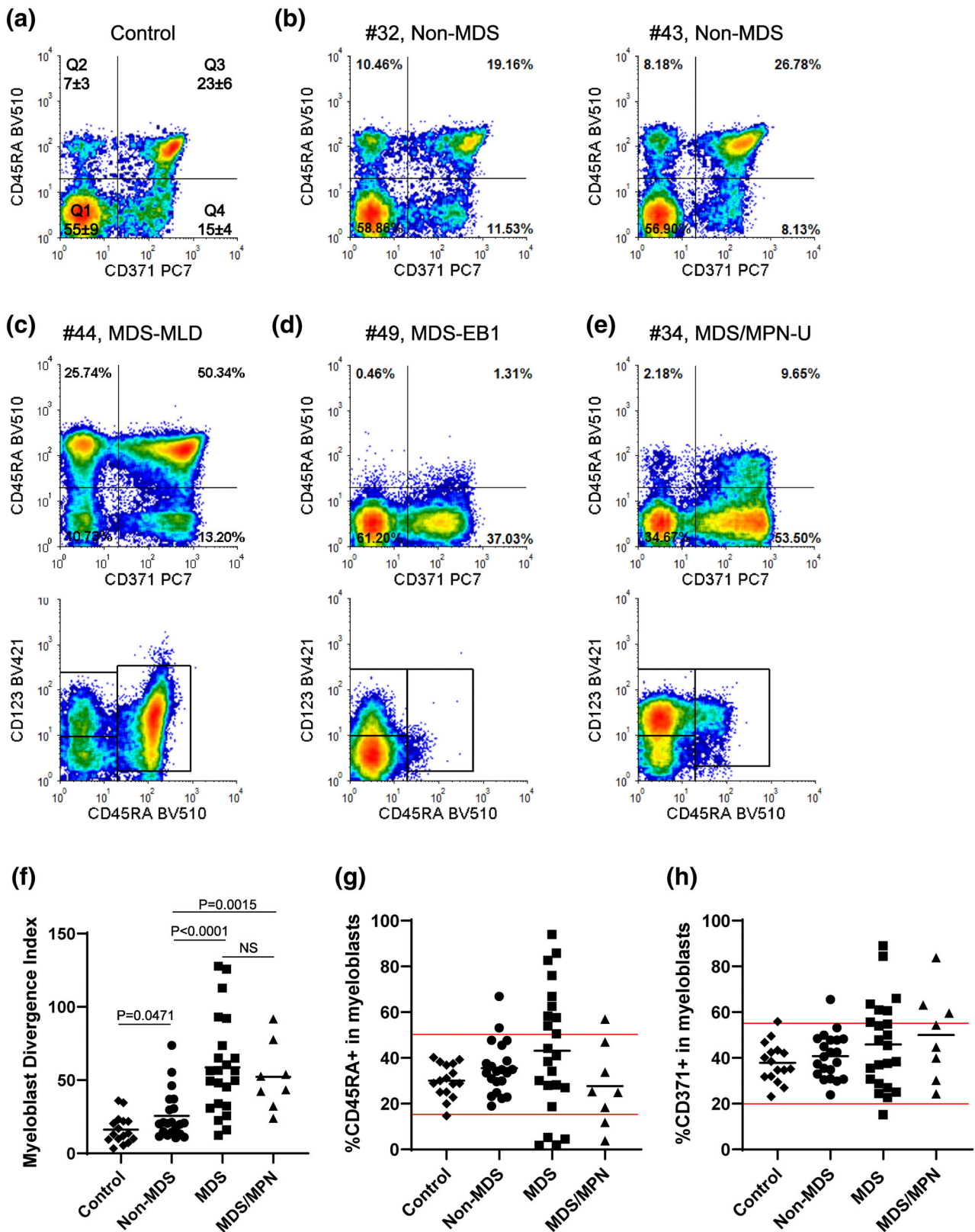


**FIGURE 3** Changes in the frequency of blast fractions and immunophenotypic aberrancies of myeloblasts. (a) Frequency of CD34<sup>++</sup> CD117<sup>+</sup> myeloblasts in Control, Non-MDS, MDS and MDS/MPN cases. Red line shows threshold of 2% myeloblasts. (b) Relative frequency of stage-1 hematogones in total CD34-positive population in Control, Non-MDS, MDS and MDS/MPN cases. Red line shows threshold of 5% stage-1 hematogones. (c) Lymphocyte to myeloblasts CD45 ratio in Control, Non-MDS, MDS and MDS/MPN cases. Red lines show the reference range established using Control samples (5.2–9.8). (d–f) median fluorescent intensity of CD117, CD123 and CD38 expression in myeloblasts of Control, Non-MDS, MDS and MDS/MPN cases. MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

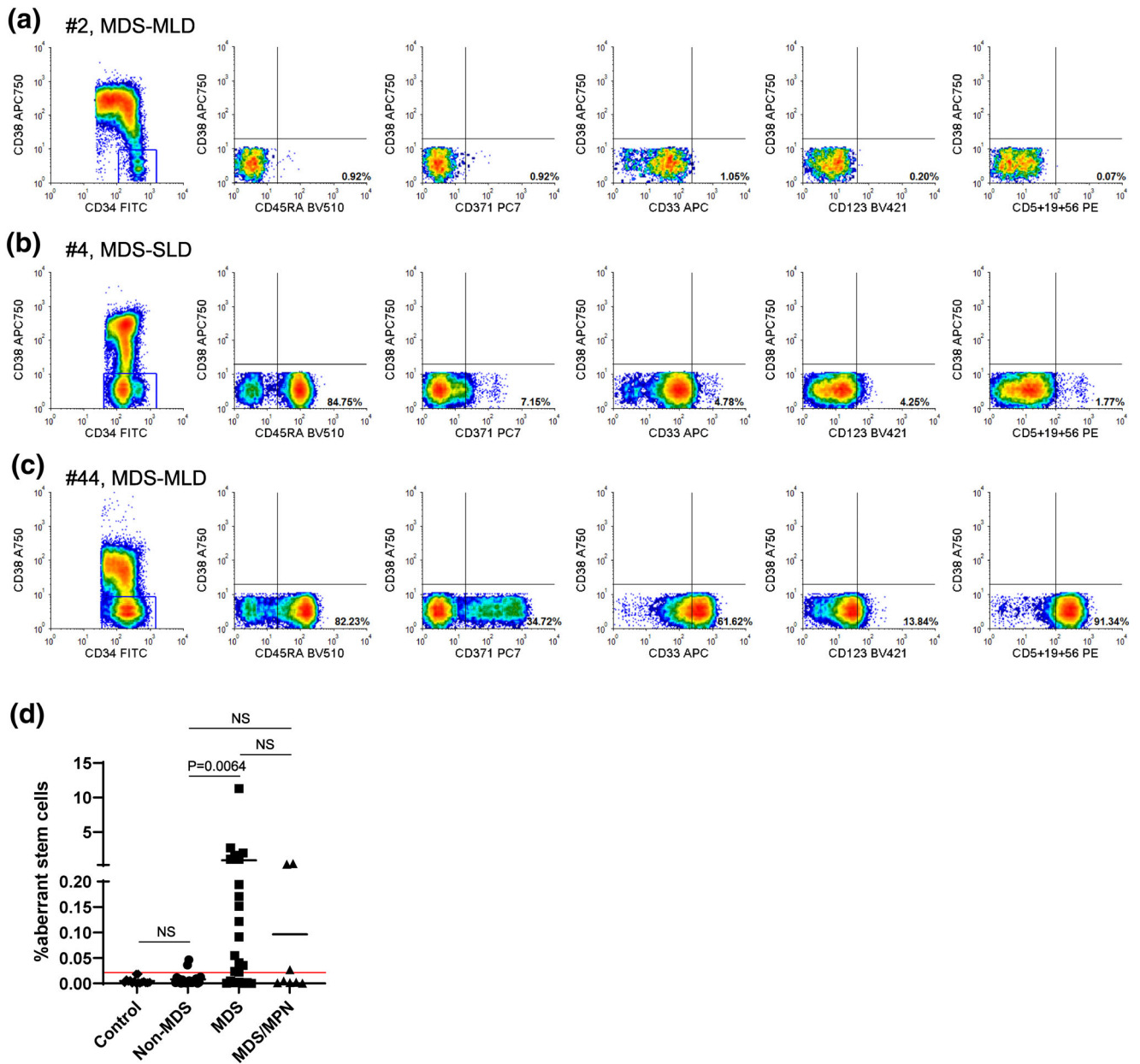
myeloblasts appeared highly specific for myelodysplasia, with positivity in 12/23 MDS, and 7/8 MDS/MPN and none of the 21 Non-MDS cases, in line with a number of previous publications (De Smet et al., 2012; Feng et al., 2018; Matarraz et al., 2008; Ogata et al., 2006; Stachurski et al., 2008). Comparison of lower-risk MDS (very low/low IPSS-R scores) to higher-risk MDS (intermediate/high/very high IPSS-R scores) showed no statistically significant difference in the intensity of CD117 expression (MFI of  $504 \pm 225$  in lower-risk versus  $589 \pm 358$  in higher-risk,  $p = 0.7074$ ), suggesting that this marker is useful irrespective of the risk score. On the other hand, higher-risk MDS group showed significantly lower intensity of CD38 expression, as compared to lower-risk group (MFI of  $138 \pm 103$  in lower-risk versus  $42 \pm 38$  in higher-risk,  $p = 0.0090$ ). This was consistent with previous studies demonstrating decreased CD38 expression in MDS that is more prominent in MDS with excess blasts, at least in part due to expansion of CD34<sup>++</sup> CD38<sup>-</sup> precursors/stem cells in high-risk MDS (Goardon et al., 2009; Monreal et al., 2006; Will

et al., 2012). CD123 intensity on myeloblasts was not significantly different between MDS and Non-MDS cases, although relatively higher number of MDS cases showed intensities above calculated MFI threshold. On the other hand, an increase in CD123 intensity on myeloblasts was seen in MDS/MPN cases as compared to Non-MDS.

Myeloblast population is heterogeneous and composed of immunophenotypically distinct progenitor fractions (CMP, GMP, MEP, dendritic cell progenitors) that give rise to various myeloid lineages. The composition of these myeloid progenitors is altered in MDS (Ostendorf et al., 2018; Will et al., 2012). Two previous studies used a combination of HLA-DR and CD13 to assess heterogeneity of CD34-positive blasts as a potential parameter for MDS diagnosis (Jevremovic et al., 2014; Schenkel et al., 2019). Normal CD34-positive blasts show three distinct populations in the HLA-DR versus CD13 plot representing stage-1 hematogones, stem cells and erythroid/megakaryocytic progenitors, and myelomonocytic progenitors. This heterogeneity is lost in a significant number of myeloid neoplasms,



**FIGURE 4** Myeloblast population is heterogeneous, and this heterogeneity is abrogated in MDS and MDS/MPN. (a) Normal myeloblasts from a Control sample show distinct populations in each quadrant of CD45RA versus CD371 plot. Numbers in each quadrant show mean  $\pm$  SD of myeloblasts from 16 Control samples. (b) CD45RA versus CD371 plots of myeloblasts from two representative Non-MDS cases. (c–e) Top panels show CD45RA versus CD371 plots and bottom panels show CD123 versus CD45RA plots of three representative MDS and MDS/MPN cases. (f) Myeloblast divergence index and (g, h) percentages of CD45RA and CD371 positive myeloblasts in Control, Non-MDS, MDS and MDS/MPN cases. Red lines show the reference ranges established using Control samples. MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

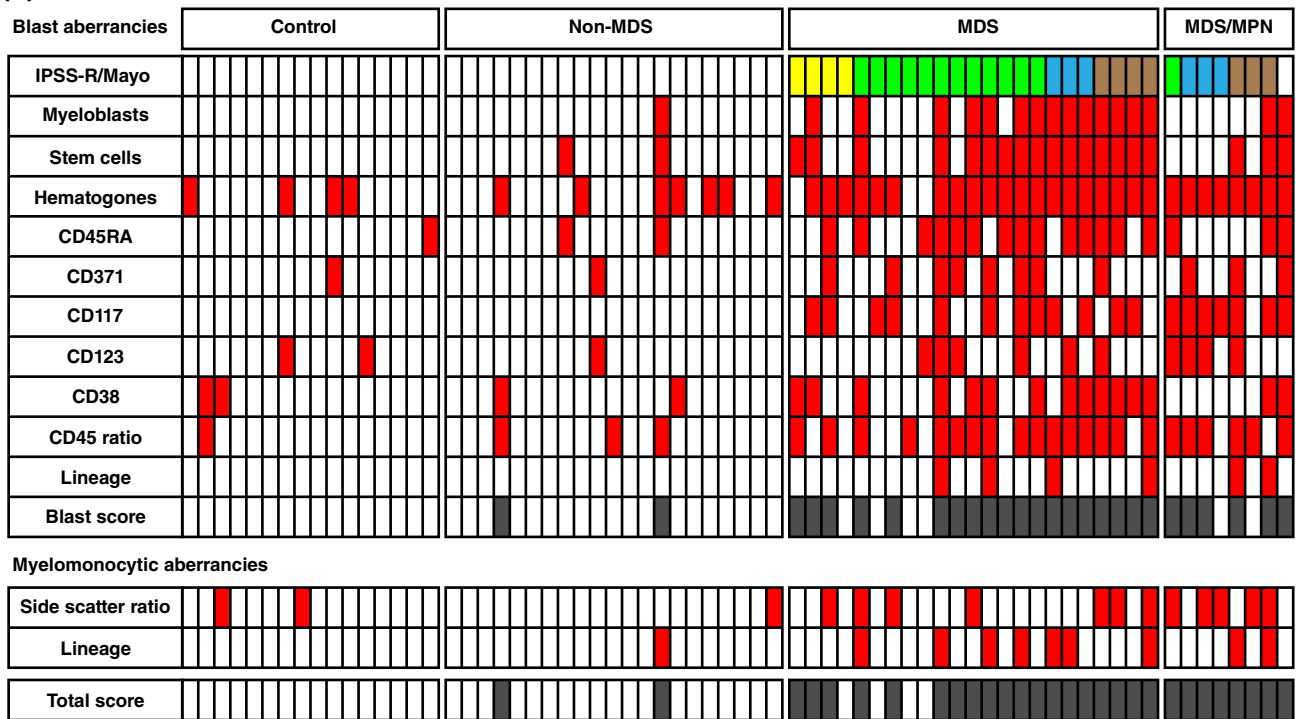


**FIGURE 5** (a–c) Representative plots of aberrant marker expression on CD34<sup>++</sup> CD38<sup>-</sup> stem cells from three MDS cases. (a) A case of MDS-MLD with no significant stem cell aberrancy. (b) Expansion of CD34<sup>++</sup> CD38<sup>-</sup> stem cells associated with large subset aberrant expression of CD45RA in a case of MDS-SLD. (c) Significant stem cell expansion and expression of several markers in a case of MDS-MLD. Note that CD45RA and CD371 often exhibited clear separation of the positive populations, whereas CD33, CD123 showed continuous expression. (d) Percentage of aberrant stem cells in Control, Non-MDS, MDS and MDS/MPN cases. Red line shows threshold of 0.02% aberrant stem cells. MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

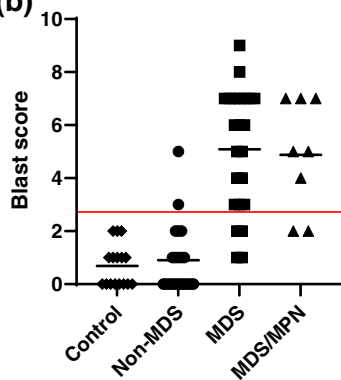
most commonly MDS, with loss of one or two populations. While this concept is appealing, there are a number of considerations with the presented approach: (1) Jevremovic et al. (2014) presented a qualitative approach for assessment of blast heterogeneity which requires expertise in interpretation of normal and pathologic patterns; (2) Total CD34-positive blasts were studied in these two studies and therefore, at least in some cases, this approach may not present an independent value beyond loss of stage-1 hematogones; and (3) In line with the second point, the quantitative measurement of blast heterogeneity

used in Schenkel et al. (2019) does not appear to significantly add to the diagnostic value of decreased stage-1 hematogones. Here we assessed blast heterogeneity and divergence from normal in CD34<sup>++</sup> CD117<sup>+</sup> myeloblast fraction, eliminating the confounding effect of stage-1 hematogone loss. Using CD45RA versus CD371 plot, we calculated Myeloblast Divergence Index and showed that it is significantly increased in MDS and MDS/MPN cases. Abnormal myeloblast heterogeneity resulted in abnormally low or high frequency of CD45RA and/or CD371 positive precursors. Using cut-offs calculated

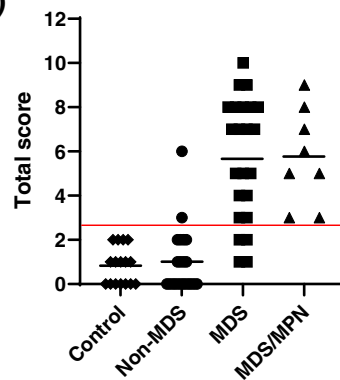
(a)



(b)



(c)



**FIGURE 6** (a) Summary of blast (top) and myelomonocytic (bottom) aberrancies identified in Control, Non-MDS, MDS and MDS/MPN cases. MDS cases are organized according to IPSS-R risk group (Greenberg et al., 2012) (yellow: Very low-risk; green: Low-risk; blue: Intermediate-risk; brown: High- and very high-risk). CMML cases were organized according to Mayo prognostic risk model (Patnaik et al., 2013) (green: Low-risk; blue: Intermediate-risk; brown: High-risk). Gray color shows cases with scores of 3 or more. (b) Blast score and (c) Total score of Control, Non-MDS, MDS and MDS/MPN cases. A score of 3 was used as a threshold for immunophenotypic diagnosis of myelodysplasia. MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

from Control bone marrows, 16/23 MDS and 5/8 MDS/MPN cases showed abnormal myeloblast heterogeneity. On the other hand, only 3/21 Non-MDS cases showed abnormal heterogeneity. Mildly abnormal heterogeneity in these Non-MDS cases may be related to unbalanced regeneration of myeloid progenitors in response to cytopenias.

Myelodysplastic syndromes and acute myeloid leukemias are malignancies that originate from phenotypically defined stem cells (Chen et al., 2019b; Will et al., 2012; Woll et al., 2014). Several markers are used to differentiate AML-SCs from normal HSCs (Hanekamp et al., 2017; Kersten et al., 2016; van Rhenen et al., 2007; Zeijlemaker

et al., 2016), many of which are also expressed by MDS-SCs (Chen et al., 2019b; Ostendorf et al., 2018; Toft-Petersen et al., 2016). Using a combination of markers (CD45RA, CD371, CD33, CD123, CD5, CD19, and CD56), we could efficiently identify aberrant stem cells. Aberrant stem cells were significantly increased in MDS, and their frequency correlated with myeloblast percentage. In fact, all cases with more than 2% myeloblasts showed increased aberrant stem cells, while a small number of cases with less than 2% myeloblasts also demonstrated stem cells aberrancy. To our knowledge, this is the first study to evaluate the diagnostic value of stem cell aberrancy in MDS.

We combined abnormalities of the blast population into a scoring system (Blast score). Scores ranged from 0 to 9. A minimum score of 3 was required for MDS immunophenotypic diagnosis. This system correctly identified 19 of 23 MDS cases and 6 of 8 MDS/MPN cases. Two Non-MDS cases showed abnormal Blast scores of 3 and 5. The first case was a patient with history of kidney transplant on tacrolimus. The blast fraction showed significant expansion of normal CD34<sup>++</sup> CD38<sup>-</sup> stem cells, resulting in lower intensity CD38 expression and higher intensity CD45 expression, leading to abnormal Blast score. Karyotype was normal and a myeloid panel NGS study showed no somatic mutation. The second case was a patient with history of rheumatoid arthritis on methotrexate who presented with pancytopenia. Morphologically, this case showed marginal increase in blasts (3%–4%) with no dysplasia and cytogenetics showed normal karyotype. No specimen was available for NGS analysis and therefore, the possibility of CCUS or evolving MDS cannot be excluded in this case. In fact, a recent study has shown high frequency of flow cytometric abnormalities in patients with CCUS (Dimopoulos et al., 2020).

Comparing MDS and Non-MDS cases, the assay showed a sensitivity of 82.6% and specificity of 90.5%. As expected, the sensitivity was lower in lower-risk MDS (75%) as compared to higher-risk disease (100%). This is comparable to other studies that showed sensitivities of 55%–90% and specificities of 80%–100% in low-risk MDS (Davydova et al., 2020 [Online ahead of print]; Della Porta et al., 2012; Kern et al., 2010; Porwit et al., 2014). Importantly, removing two newly introduced parameter (abnormal myeloblast heterogeneity and stem cell aberrancy) from our scoring system would reduce the sensitivity of the assay from 75% to 56% in lower-risk MDS, while the sensitivity in higher-risk disease remains unchanged at 100%. Evaluation of myelomonocytic aberrancies improved the sensitivity of assay to detect MDS/MPN, although without such improvement in lower-risk MDS cohort.

We understand that this study is limited to relatively low number of cases assessed in a single centre, and therefore, conclusions are preliminary and must be validated in a larger, preferably multi-centre, study. The analysis included bone marrow samples received in our institution with clinical concern for MDS or MDS/MPN over a 6-months period. The study was designed as a proof of concept that deep analysis of the blast population would provide a reasonable sensitivity and specificity for immunophenotypic diagnosis of myelodysplasia. Like many previous studies, the major limitation of this panel is in low-risk MDS where sensitivity approximates 70%–80%. Given the short duration of study, we do not have clinical follow-up and prognostic data. However, higher Blast scores were seen in intermediate/high-risk IPSS-R MDS group ( $4.4 \pm 2.5$  in lower-risk MDS versus  $6.6 \pm 0.8$  in higher-risk MDS,  $p = 0.0467$ ), suggestive of direct correlation between Blast score and adverse outcome. The advantage of our approach is that the scoring system is entirely quantitative and can be automated, bypassing the need for expertise in complex interpretation of maturation patterns of myelomonocytic elements. In addition, for the first time, we introduced quantitative measurement of myeloblast heterogeneity and stem cell aberrancy into a flow cytometry scoring system for diagnosis of myelodysplasia.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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