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Flow cytometric analysis of neutrophil myeloperoxidase expression in peripheral blood for ruling out myelodysplastic syndromes: a diagnostic accuracy study

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ABSTRACT

Suspicion of myelodysplastic syndromes (MDS) is one of the commonest reasons for bone marrow aspirate in elderly patients presenting with persistent peripheral blood (PB) cytopenia of unclear etiology. A PB assay that accurately rules out MDS would have major benefits. The diagnostic accuracy of the intra-individual robust coefficient of variation (RCV) for neutrophil myeloperoxidase (MPO) expression measured by flow cytometric analysis in PB was evaluated in a retrospective derivation study (44 MDS cases and 44 controls) and a prospective validation study (68 consecutive patients with suspected MDS). Compared with controls, MDS cases had higher median RCV values for neutrophil MPO expression (40.2% *vs.* 30.9%; $P<0.001$). The area under the receiver operating characteristic curve estimates were 0.94 [95% confidence interval (CI): 0.86-0.97] and 0.87 (95%CI: 0.76-0.94) in the derivation and validation studies, respectively. A RCV lower than 30% ruled out MDS with 100% sensitivity (95%CI: 78-100%) and 100% negative predictive value (95%CI: 83-100%) in the prospective validation study. Neutrophil MPO expression measured by flow cytometric analysis in PB might obviate the need for invasive bone marrow aspirate and biopsy for up to 29% of patients with suspected MDS.

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Introduction

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal bone marrow (BM) neoplasms that predominate in the elderly.[1,2] The diagnosis of MDS is based on peripheral blood (PB) cytopenia and morphological dysplasia for one or more hematopoietic cell lineages.[1,3,4] Cytopenia is evidenced with hemogram while dysplasia requires BM aspirate, which is an invasive procedure.[1,2,5]

Because of the limited prevalence of disease among subjects referred for suspected MDS,[6] many patients are exposed to unnecessary BM aspiration with the associated discomfort and risk. Therefore, an objective assay based on a PB sample that accurately discriminates MDS from other cytopenia etiologies is highly desirable. In this context, a few studies have investigated the value of flow cytometric analysis for detecting aberrant phenotypic expression of PB leukocytes in the diagnostic work-up of MDS.[7-9] Although promising, these studies lacked replication of their

results, used a case control design, which was prone to spectrum bias,[10] or yielded imprecise diagnostic accuracy estimates due to relatively limited sample sizes.

Degranulation of mature granulocytes is a classical dysplastic feature of MDS,[11-13] and this can be analyzed using various methods, including hemogram automaton, cytomorphological evaluation, and flow cytometry (side scatter). Degranulation is associated with myeloperoxidase (MPO) cytoplasmic expression, an enzyme synthesized during myeloid differentiation that constitutes the major component of neutrophil azurophilic granules.[14] MPO expression may be studied by immuno-cytochemical staining,[11,15] although this approach is limited by the moderate sensitivity and subjective nature of cytomorphological evaluation of PB in routine practice.

Flow cytometric analysis of MPO expression in BM neutrophil granulocytes has been occasionally used to identify MDS patients and to discriminate between low- *versus* higher-risk patients with MDS.[16] However, the accuracy of flow cytometric analysis of neutrophil MPO expression in PB for the diagnosis of MDS has not been studied.

The present study aimed to assess the performance of flow cytometric analysis of MPO expression in peripheral blood mature granulocytes to rule out a diagnosis of MDS and/or chronic myelomonocytic leukemia (CMML).

Methods

Study design

Using a retrospective case control study design,[17] we assessed the diagnostic accuracy of various parameters of neutrophil MPO expression in PB measured by flow cytometric analysis and defined a threshold that identified patients who were unlikely to have MDS or CMML. We then assessed the diagnostic accuracy of this threshold in a prospective validation cohort of consecutive patients referred for suspicion of MDS. The protocol for this study was approved by the Comité de Protection des Personnes Sud Méditerranée I, Marseille, France.

Study sites

The flow cytometric analysis protocol was jointly developed and pre-tested at three university-affiliated hospitals in France: Clermont-Ferrand, Saint-Etienne, and Grenoble. Participants in the retrospective case control and prospective validation studies were enrolled at two study sites: Clermont-Ferrand and Grenoble. The index test and reference standard were performed at the site of enrollment.

Participants

In the retrospective case control study, cases were adults with established diagnosis of MDS or CMML, as defined by current guidelines.[1,2,4,5,18] They were retrospectively identified by screening the electronic laboratory record using the MDS and CMML diagnosis codes. Controls were individuals referred to the hematology laboratory with normal values for the routine blood cell count. Exclusion criteria for both cases and controls were acute leukemia and admission to the intensive care unit. Cases and controls were matched on gender. The study sample was restricted to controls aged 50 years or older because all cases were over this age.

The prospective validation cohort consisted of consecutive adults who were referred for suspected MDS. Suspicion of MDS was based on medical history and PB cytopenia. All patients enrolled in the validation cohort study were prospectively evaluated for the reference standard and index test.

Index test

Peripheral blood samples were stored at 4°C overnight and processed within 24 hours (h) of collection. We used material remaining after a routine blood cell count with the Sysmex XE-5000 and Sysmex XN-10 automated hematology analyzers (Kobe, Japan).

The blood sample was stained according to the manufacturer's recommendations with a panel of antibodies conjugated to fluorochromes. CD64 FITC (clone 10.1), CD15-PerCPCy55 (clone HI98), CD11b-APC (clone D12), CD16-APCH7 (clone 3G8), CD14-V450 (clone MφP9), and CD45-V500 (clone HI30) antibodies were added. Aliquots were stained for 15 minutes (min) at room temperature. The fixation and permeabilization phases were performed using the BD IntraSure™ Kit (BD Biosciences, San Jose, CA, USA) and MPO-PE was added (clone 5B8) during the permeabilization phase. All antibodies, BD FACS™ Lysing Solution and BD IntraSure™ Kit were obtained from BD Biosciences (San Jose, CA, USA).

At least 10,000 neutrophils were acquired on a 3-laser, 8-color BD FACSCanto-II™ flow cytometer (BD Biosciences, San José, CA, USA) and analyzed using BD FACSDiva Software at each study site. The gating strategy is presented in Figure 1.

Myeloperoxidase expression in the PB neutrophil population within an individual subject was expressed as median, mean, and robust coefficient of variation (RCV).[19] The RCV was calculated as the robust standard deviation divided by the median. The robust standard deviation is a function of the deviation of individual data points to the median of the study population.[20] The RCV was expressed as a percentage and reflected the variability in MPO expression in the PB neutrophil population within an individual subject (Figure 2).

The FranceFlow standard operating procedure was used to standardize instrument settings. Rainbow calibration particles (BD SpheroTM, BD Biosciences, San Jose, CA, USA) were analyzed daily and photomultiplier tubes were adjusted if needed (*Online Supplementary Table S4*).

In the retrospective case control study, flow cytometric analysis was performed within six months of MDS diagnosis and could not be blinded to patient status for logistical reasons. In contrast, flow cytometric analysis was performed within 24 h of BM aspirate and was blinded to the reference standard in the prospective validation cohort.

Reference standard

The reference diagnosis of MDS was established according to current guidelines,[1,2,4,5] based on clinical data, peripheral blood cytopenia, cytomorphology of PB and BM aspirate, and cytogenetic analysis. Peripheral blood cytopenia was defined using standard laboratory values: hemoglobin concentration <12 g/dL (females) and <13 g/dL (males), platelet count $<150x10^9$/L, and/or absolute neutrophil count $<1.8x10^9$/L.[18]

Bone marrow cytomorphology was evaluated prospectively by experienced hematopathologists who were blinded to the index test results. The criteria for MDS

diagnosis were: 1) the presence of ≥10% dysplastic cells in any hematopoietic lineage; 2) the exclusion of acute myeloid leukemia (defined by the presence of ≥20% PB or BM blasts); and 3) the exclusion of reactive etiologies of cytopenia and dysplasia.

Consistent with the World Health Organization (WHO) classification,[1] MDS subcategorization was based on the degree of dysplasia (unilineage *vs.* multilineage), blast percentages, presence of ring sideroblasts, and cytogenetic analysis (del(5q)). The criteria for CMML diagnosis were: 1) the presence of persistent PB monocytosis ≥1x10^9/L; and 2) monocytes accounting for more than 10% of the white blood cell differential count.[1] Idiopathic cytopenia of uncertain significance (ICUS) was defined by unexplained mild persistent cytopenia for 4-6 months and the failure to establish the diagnosis of MDS according to the guidelines.[5,21-23]

In the retrospective case control study, the reference standard was available for MDS cases only and no control subject received cytomorphological evaluations. In contrast, the reference standard was available for all patients enrolled in the prospective validation cohort study.

Sample size

Assuming an area under the receiver operating characteristic (ROC) curve point estimate of 0.95, we estimated that a sample size of 88 participants (comprising 44 MDS patients and 44 controls) would provide a precision of ±0.05 [95% confidence interval (CI) ranging from 0.90 to 1.00].[24]

Precision and reproducibility assessment

We evaluated intra- and inter-assay precision, reproducibility between study sites, and specimen stability for RCV measurements of MPO expression in the PB neutrophil population according to current guidelines.[25-27]

Statistical analysis

We assessed the independent associations of MDS with RCV for neutrophil MPO expression measured by flow cytometric analysis in PB, using multivariable logistic regression. Odds ratio estimates were adjusted for age and baseline characteristics that were significantly associated with MDS in univariable analysis [C-reactive protein (P<0.001) and creatinine (P=0.03) concentrations]. Because hemoglobin concentration, platelet count, and absolute neutrophil count were part of the MDS definition, they were not entered as co-variates in the multivariable model. Twenty-one observations were imputed because of missing values for C-reactive protein and/or creatinine concentrations. Additional variables entered in the imputation model included age, gender, RCV, and MDS diagno-

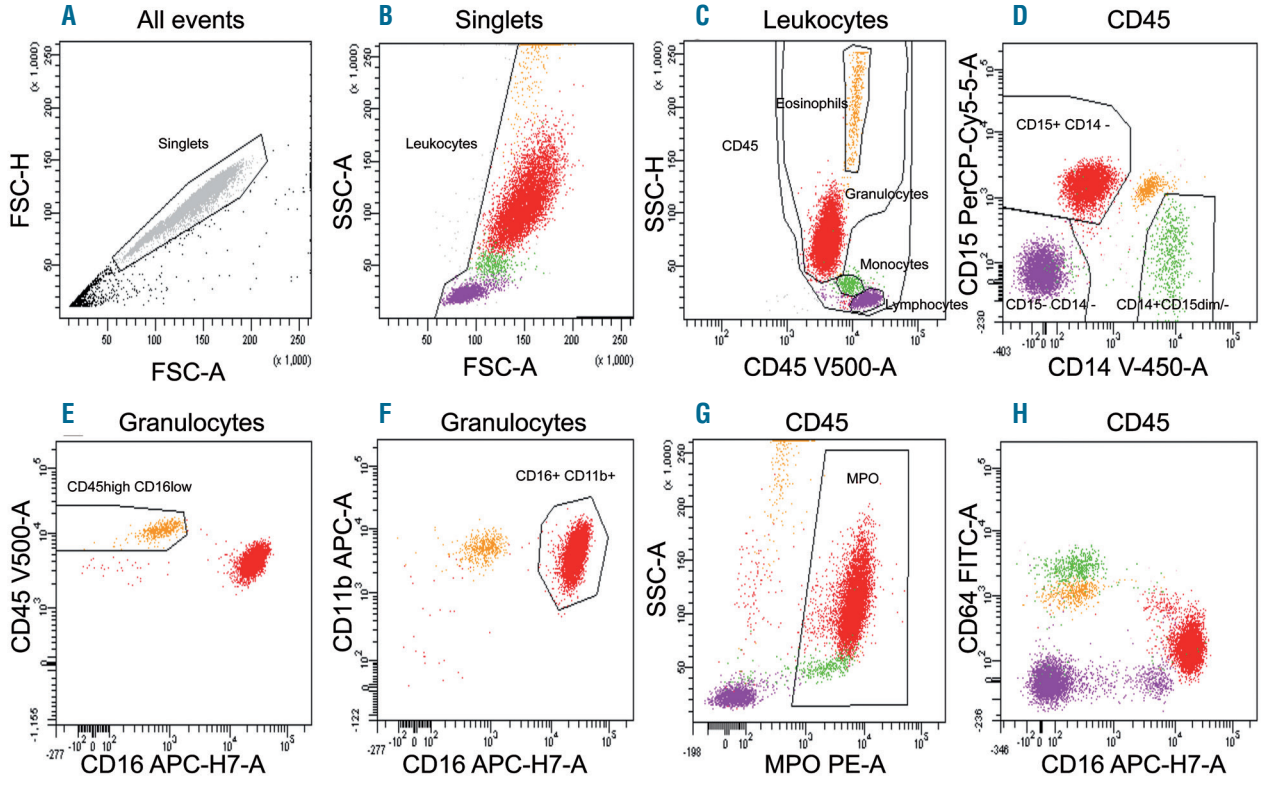

Figure 1. Gating strategy for quantifying peripheral blood neutrophil myeloperoxidase (MPO) expression. CD45$^+$ viable cells were first individualized by crossing the singlet gate (A), FSC-SSC leukocytes (B), and CD45$^+$ gate (C). Three populations including granulocytes (CD15$^+$ CD14$^-$), monocytes (CD14$^+$ CD15low/$^-$), and lymphocytes (CD15$^-$ CD14$^-$) were identified (D). Eosinophils were individualized by CD45high CD16 low (E). Mature neutrophils were individualized by Boolean intersection: [CD15$^+$ CD14$^-$] (D) AND NOT [CD45high CD16 low] (E) AND NOT [CD14$^+$ CD15low/$^-$] (D) AND NOT [CD15$^-$ CD14$^-$] (D) AND [CD16$^+$ CD11b$^+$] (F). Robust coefficient of variation (RCV) MPO was evaluated on the resulting population (G). The CD16 CD64 dot plot (H) was used to verify that the mature neutrophils were correctly selected: they appeared as CD16high and CD64low cluster. The populations identified were lymphocytes (purple), monocytes (green), eosinophils (orange), MPO mature neutrophils (red). CD: cluster of differentiation; FSC-H: forward scatter height; FSC-A: forward scatter area; SSC-H: side scatter height.

sis. Fifty imputed data sets were created with a total run length of 50,000 iterations and imputations made every 1,000 iterations.

We quantified the accuracy of each neutrophil MPO expression parameter in discriminating MDS and non-MDS patients by estimating the area under the ROC curve. We compared the area under the ROC curve for each parameter with that for the RCV. The significance probability was adjusted for multiple comparisons using the Bonferroni method.

The specificity, positive and negative predictive values, and likelihood ratios of the test results were estimated across a range of RCV values that achieved sensitivity of from 100% to 90% in the retrospective case control study. Since neutrophil MPO expression in PB would be mainly used to rule out MDS, we selected a threshold with a likelihood ratio for a negative test result point estimate that was lower than 0.10.[28]

Two-tailed $P<0.05$ was considered statistically significant. Analyses were performed using Stata Special Edition version 14.0 (Stata Corporation, College Station, TX, USA).

Results

Retrospective case control study

Forty-four MDS patients and 44 controls were included in the study. The mean age for all patients was 73.3 years (standard deviation, 10.4), and 38 (43%) were female (Table 1). MDS with excess blasts, MDS with multilineage dysplasia, and CMML accounted for 55% (24 of 44), 20% (9 of 44), and 11% (5 of 44) of all MDS patients, respectively (Table 2). MDS cases had lower median hemoglobin concentration, platelet counts, and absolute neutrophil counts than controls (Table 1).

Compared with controls, MDS cases yielded comparable median and mean values, but a higher RCV for neutrophil MPO expression measured by flow cytometric analysis in peripheral blood (Table 1). Odds ratios of MDS associated with a 1% increase in RCV were 1.80 (95%CI: 1.39-2.33) in univariable analysis and 2.22 (95%CI: 1.31-3.76) in multivariable analysis adjusting for age, C-reactive protein, and creatinine concentrations. RCV values for neutrophil MPO expression in PB were elevated across all WHO classification MDS types, ranging from 28.3% (in a patient with MDS with multilineage dysplasia) to 99.3% (in a patient with MDS with isolated del(5q)) (Table 2). Median RCV values for MPO expression of circulating neutrophils were 41.1% [interquartile range (IQR): 38.6-47.2] and 38.6% (IQR: 36.6-46.0) for 25 low- and 19 high-risk MDS patients, compared with 30.9% (IQR: 29.7-31.9) for 44 controls (*Online Supplementary Table S1*).

The area under the ROC curve (0.94, 95%CI: 0.86-0.97) for the RCV was higher than that for median and mean (Figure 3). These findings were unchanged after excluding CMML cases (*Online Supplementary Table S2*). Sensitivity point estimates ranged from 100% to 91% for RCV thresholds varying between 28% and 32% (Table 3). A RCV value <30% yielded a negative predictive value of 93% and a likelihood ratio of a negative test result of 0.07 (Table 3). All cases but one with established MDS diagnosis had RCV values >30%. The exception was a 72-year old female case with multilineage dysplasia, for whom isolated peripheral thrombocytopenia ($94x10^9$/L) and a 28.3% RCV value for MPO expression in the PB neutrophil population were found. RCV value <28.0%, therefore, excluded MDS with both sensitivity and negative predictive value estimates of 100%, but occurred in a small proportion of patients (3.4%, 3 of 88).

Prospective validation study

Sixty-eight consecutive patients referred for suspected MDS were included in the validation cohort study. The mean age for all patients was 74.7 years (standard deviation, 9.2), and 29 (43%) were female (Table 4). The preva-

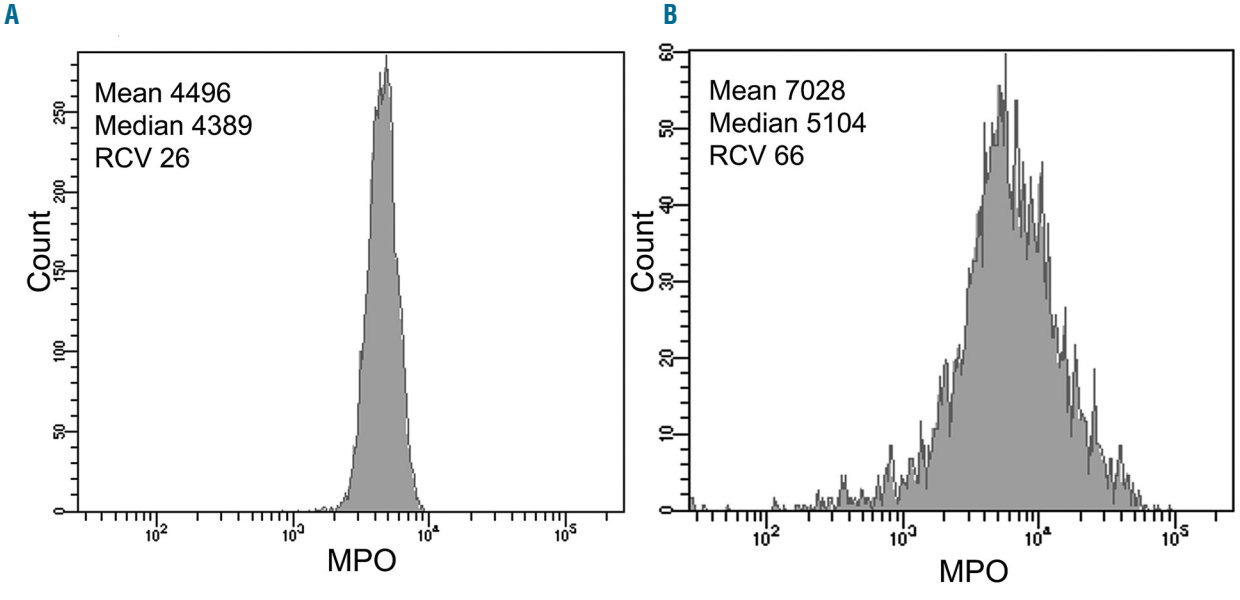

Figure 2. Monoparametric histograms of peripheral blood neutrophil myeloperoxidase (MPO) expression. Values are: mean, fluorescence intensity (FI); median, FI; and robust coefficient of variation (RCV), %. (A) Control subject. (B) Myelodysplastic syndrome case.

lence of MDS and ICUS was 22% and 12%, respectively. The median RCV values for MPO expression in PB were 38.1% (range: 31.3-99.2), 37.2% (range: 32.5-50.2), and 30.6% (range: 26.1-34.1), for patients with MDS, ICUS, and no MDS, respectively (P<0.001) (*Online Supplementary Table S3*). The odds ratios of MDS associated with a 1% increase in RCV were 1.28 (95%CI: 1.10-1.50) in univariable analysis and 1.34 (95%CI: 1.08-1.21) in multivariable analysis adjusting for age, C-reactive protein, and creatinine concentrations. The median RCV values for MPO expression of circulating neutrophils were 37.5% (IQR: 32.7-45.8) and 65.9% for 14 low- and one high-risk MDS cases, compared with 31.0% (IQR: 28.9-32.5) for 53 consecutive patients with unconfirmed suspected MDS (*Online Supplementary Table S4*).

The area under the ROC curve (0.87, 95%CI: 0.76-0.94) for the RCV was higher than that for the median and mean in discriminating patients with *versus* without MDS (Figure 3). A RCV value <30.0% excluded MDS for 29% (20 of 68) of consecutive patients referred for suspected disease, with both sensitivity and negative predictive value point estimates of 100% (Table 3).

Precision and reproducibility assessment

Coefficient of variation point estimates for intra-assay precision ranged from 0.4% to 0.5% for five healthy individuals and from 0.0% to 0.9% for five MDS cases (*Online Supplementary Table S5*). The coefficient of variation point estimate for inter-assay precision was 3.6% in five independent analytical runs at the same laboratory (*Online Supplementary Table S6*).

Compared with baseline values, the mean changes in RCV were -1.8 percentage points (95%CI: -2.4 to -1.3, relative change, -7%) at 24 h and 0.6 percentage points (95%CI: -0.4 to 1.7, relative change, 2%) at 72 h for 10 samples stored at 4°C (*Online Supplementary Table S7*). After post-processing (stained, lysed, fixed), no significant change was observed in mean RCV (-0.1 percentage points, 95%CI: -0.6 to 0.4, relative change, -0.4%) between baseline and 6-h measurements for five samples stored at 4°C (*Online Supplementary Table S8*).

The mean coefficient of variation point estimates across instrument setup procedures were 0.3% (range: 0-0.5) and 0.8% (range: 0.3-1.2) in one laboratory and 2.5% (range: 1.0-3.0) and 1.7% (range: 0.8-3.0) in the other laboratory

Table 1. Baseline patients' characteristics and neutrophil myeloperoxidase expression parameters measured by flow cytometric analysis in peripheral blood for myelodysplastic syndrome cases and controls.

Characteristics	MDS cases* (N=44)		Controls† (N=44)		*P*
Female gender, n (%)	19	(43)	19	(43)	–‡
Age, mean (SD), y	73.2	(10.0)	73.4	(11.0)	0.94
Hemoglobin, median (IQR), g/dL	10.7	(9.0–12.7)	13.8	(13.0–14.9)	<0.001
Platelet, median (IQR), $\times 10^9$/L	142	(75–190)	246	(206–283)	<0.001
Absolute neutrophil count, median (IQR), $\times 10^9$/L	1.9	(1.3–3.0)	3.8	(3.1–4.6)	<.001
Creatinine, median (IQR), µmol/L	87	(67–110)	73	(64–82)	0.03
C-reactive protein ≥ 3 mg/L, n (%)	19	(63)	5	(13)	<0.001
Neutrophil MPO expression in peripheral blood, median (IQR)					
Mean, FI	6083	(3905–9904)	6515	(4230–9749)	0.95
Median, FI	5527	(3777–9482)	6355	(4110–9520)	0.71
Robust coefficient of variation, %	40.2	(37.8–46.9)	30.9	(29.7–31.9)	<0.001

N/n: number; FI: fluorescence intensity; IQR: interquartile range (25–75th percentiles); MDS: myelodysplastic syndrome; MPO: myeloperoxidase; SD: standard deviation. *Values were missing for hemoglobin concentration (n=1), platelet count (n=1), absolute neutrophil count (n=2), C-reactive protein (n=14), and creatinine (n=9) concentrations among myelodysplastic syndrome cases. †Values were missing for C-reactive protein (n=5) and creatinine (n=6) concentrations among controls. ‡Myelodysplastic syndrome cases and controls were matched for gender (See Methods).

Table 2. Flow cytometric robust coefficient of variation estimates for neutrophil myeloperoxidase expression in peripheral blood according to myelodysplastic syndrome type.

	MDS cases			Consecutive patients with confirmed suspicion of MDS		
WHO MDS type	N	Median	(Range)	N	Median	(Range)
MDS with single lineage dysplasia	1	38.6	(–)	1	36.4	(–)
MDS with ring sideroblasts	2	–	(33.3–49.5)	2	–	(31.3–31.5)
MDS with multilineage dysplasia	9	42.1	(28.3–66.3)	3	40.5	(38.1–50.2)
MDS with excess blast 1	7	39.2	(30.3–53.5)	3	32.7	(32.3–61.0)
MDS with excess blast 2	17	38.6	(30.6–73.2)	1	65.9	(–)
MDS with isolated del(5q)	3	40.2	(39.4–99.3)	1	99.2	(–)
Chronic myelomonocytic leukemia	5	45.3	(32.3–66.1)	3	42.5	(35.1–45.8)
Unclassifiable MDS	0	–	(–)	1	36.9	(–)
All	44	40.2	(28.3–99.3)	15	38.1	(31.3–99.2)

N: number; MDS: myelodysplastic syndrome; WHO: World Health Organization.

for healthy individuals and MDS cases, respectively (*Online Supplementary Table S9*). The mean inter-laboratory coefficient of variation point estimates ranged from 4.1% to 5.3% for healthy individuals and from 3.3% to 3.5% for MDS patients, depending on the setup procedures (*Online Supplementary Table S9*).

Discussion

To our knowledge, this is the first study to report on the diagnostic accuracy of neutrophil MPO expression measured by flow cytometric analysis in PB to rule out MDS. Accordingly, a RCV value <30% identified patients at low risk of MDS in whom invasive BM aspirate could potentially be avoided. Because the 95%CI for both sensitivity (78-100%) and negative predictive value (83-100%) estimates were relatively imprecise, these findings warrant replication in a larger and more diverse cohort of patients.

Importantly, all ICUS patients had RCV values >30% and would be recommended for BM aspirate or biopsy, a strategy that complies with published guidelines.[22,23] Although BM aspirate may help establish an alternate diagnosis for patients without MDS, it was not contributive for any of 45 patients with unconfirmed suspicion of MDS in our prospective validation study. This observation may not be consistent with clinical practice and deserves confirmation in an independent sample.

In contrast, flow cytometric analysis of neutrophil MPO expression in PB had limited diagnostic value for ruling in MDS.[28] Indeed, the specificity point estimates for a RCV value >30% ranged from 32% to 38% depending on the study sample, with positive predictive values varying between 31% and 59%. RCV values >38% achieved 100% specificity but at a cost of a 30% false-negative rate. Hence, the RCV of neutrophil MPO expression in PB would not add relevant information to cytomorphological evaluation of BM aspirate.

A thorough understanding of the changes in the RCV of neutrophil MPO expression in MDS patients was not within the scope of this study and requires further investigation. However, we found that RCV values were elevated across all MDS types. This observation might be explained by previous observations of hypogranulation in various MDS types[12,13] and higher variability of neutrophil cell granularity in MDS clone[29,30] as well as in extraclonal cells.[31]

Few studies have reported on the accuracy of flow cytometric analysis of alternate neutrophil antigen expression in PB for the diagnosis of MDS. Rashidi *et al.* reported decreased mean levels of CD10 expression in PB for high-grade MDS compared with cytopenic controls [2.2 (0.7) *vs.* 3.7 (0.7); $P<0.001$).[9] However, this study failed to show a significant difference in levels of CD10 expression

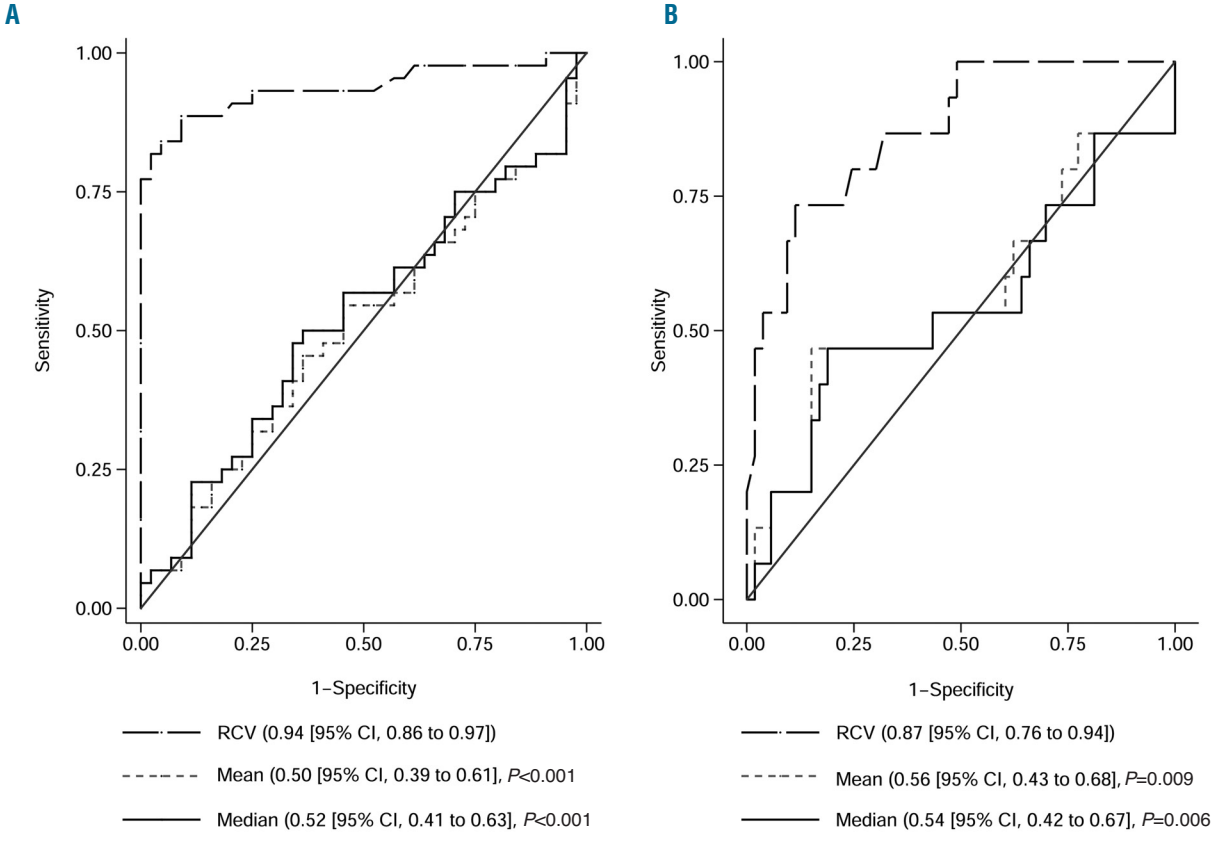

Figure 3. Area under the receiver operating characteristic curve for flow cytometric parameters of peripheral blood neutrophil myeloperoxidase expression in discriminating myelodysplastic syndromes (MDS). (A) Retrospective case control study. (B) Consecutive patients with suspected MDS. The area under the receiver operating characteristic curve for each parameter was compared with that for the robust coefficient of variation (RCV). *P*-values were adjusted for multiple comparisons using the Bonferroni method. CI: Confidence Interval.

Table 3. Accuracy point estimates (95% confidence interval) for predefined thresholds of robust coefficient of variation for peripheral blood neutrophil myeloperoxidase expression in discriminating myelodysplastic syndromes.

MPO RCV, %	N True positive	False negative	False positive	True negative	Sensitivity, %	Specificity, %	PPV, %	NPV, %	LR+*	LR–*
Myelodysplastic syndrome cases *versus* controls†										
28.0	44	0	41	3	100 (92–100)	6.8 (1.4–19)	52 (41–63)	100 (29–100)	1.07 (0.98–1.17)	0.14 (0.01–2.69)
29.0	43	1	38	6	98 (88–100)	14 (5.2–27)	53 (42–64)	86 (42–100)	1.13 (1.00–1.28)	0.17 (0.02–1.33)
30.0	43	1	30	14	98 (88–100)	32 (19–48)	59 (47–70)	93 (68–100)	1.43 (1.17–1.76)	0.07 (0.01–0.52)
31.0	41	3	20	24	93 (81–99)	55 (39–70)	67 (54–79)	89 (71–98)	2.05 (1.47–2.86)	0.13 (0.04–0.38)
32.0	40	4	11	33	91 (78–98)	75 (60–87)	78 (65–89)	89 (75–97)	3.64 (2.16–6.12)	0.12 (0.05–0.31)
Consecutive patients with suspected myelodysplastic syndromes‡										
28.0	15	0	45	8	100 (78–100)	15 (6.8–28)	25 (15–38)	100 (63–100)	1.15 (1.00–1.33)	0.20 (0.01–3.26)
29.0	15	0	38	15	100 (78–100)	28 (17–42)	28 (17–42)	100 (78–100)	1.36 (1.12–1.64)	0.11 (0.01–1.72)
30.0	15	0	33	20	100 (78–100)	38 (25–52)	31 (19–46)	100 (83–100)	1.56 (1.25–1.96)	0.08 (0.01–1.29)
31.0	15	0	27	26	100 (78–100)	49 (35–63)	36 (22–52)	100 (87–100)	1.90 (1.51–2.56)	0.06 (0.01–0.99)
32.0	13	2	20	33	87 (60–98)	62 (48–75)	39 (23–58)	94 (81–99)	2.30 (1.54–3.42)	0.21 (0.06–0.79)

N: number; LR+: likelihood ratio of a positive result; LR–: likelihood ratio of a negative result; MPO: myeloperoxidase; NPV: negative predictive value; PPV: positive predictive value; RCV: robust coefficient of variation. *0.5 was added to all cell frequencies before calculation of likelihood ratios for robust coefficient of variation thresholds with numbers of false-negative cases equal to zero. † The analytical sample consisted of 88 subjects, including 44 myelodysplastic syndrome cases and 44 controls. ‡ The analytical sample consisted of 68 consecutive patients, including 15 and 53 patients with and without myelodysplastic syndrome, respectively.

between low-grade MDS and cytopenic controls [3.7 (0.9) *vs.* 3.7 (0.7)]. The authors also did not report area under the ROC curve estimates for the diagnosis of MDS.[9]

Cherian *et al.* derived and prospectively validated a PB MDS scoring system based on flow cytometry analysis of neutrophils.[7,8] This prediction score combined data on side scatter and four neutrophil immunophenotypic variables (CD11a, CD66, CD10, and CD116 antigen expression). Using published individual participant data,[7] we found that the area under the ROC curve estimate for the PB MDS score was 0.87 (95%CI: 0.70-0.96) compared with 0.94 (95%CI: 0.86-0.97) and 0.87 (95%CI: 0.76-0.94) for the RCV of neutrophil MPO expression in our retrospective case control and prospective validation studies, respectively. Yet a head-to-head comparison of area under the ROC curves between the PB MDS score and the RCV of neutrophil MPO expression on the same sample of patients is currently lacking.

Flow cytometric analysis of neutrophil MPO expression in PB has potential advantages over cytochemical evaluation. While cytochemical evaluation shows moderate reliability and yields normal results in up to 75% of MDS cases,[11] flow cytometric analysis is amenable to standardization across laboratories.[32] Additionally, our study found high intra- and inter-assay precision, satisfactory inter-laboratory reproducibility, and robustness to instrument settings. Because RCV of neutrophil MPO expression in PB is stable with storage at 4°C for up to 24-96 h, blood samples can be shipped to a central facility, without compromising reliability. Interestingly, the results are available within 90 min.

The suspicion of MDS is one of the commonest reasons for BM examination in elderly patients presenting with persistent PB cytopenia of unclear etiology.[33] BM biopsy and aspiration are painful procedures for the majority of patients,[34,35] with 20% of them reporting a moderate level of pain seven days after the procedure.[36] Although infrequent, procedure-related complications (hemorrhage and infection) may be associated with significant morbidity or may even be life-threatening.[37]

The use of flow cytometric analysis of neutrophil MPO expression in PB might be suitable to reduce the unnecessary exposure of patients without MDS to BM aspirate-related discomfort and risk and its associated costs. However, this hypothesis remains speculative because a diagnostic accuracy study cannot provide direct evidence on the clinical benefits and safety of such a strategy.[17] Prospective management studies or randomized controlled trials are needed to evaluate processes of care, short- and long-term patient outcomes, as well as the use of resources associated with the implementation of flow cytometric analysis of neutrophil MPO expression in PB for patients with suspected MDS in routine practice.[17]

Our study has limitations that deserve mention. 1) The retrospective case control study design is prone to spectrum bias,[10] with the potential of providing diagnostic accuracy estimates that are too optimistic. Reassuringly, our prospective validation study replicated the findings in

Table 4. Baseline characteristics for 68 consecutive patients with suspected myelodysplastic syndromes enrolled in the prospective validation study.

Characteristics*	All patients (N=68)		Confirmed MDS				P
			No (N=53)		Yes (N=15)		
Female gender, n (%)	29	(43)	22	(42)	7	(47)	0.72
Age, mean (SD), y	74.7	(9.2)	73.6	(9.2)	78.4	(8.4)	0.07
Hemoglobin, median (IQR), g/dL	10.4	(9.6–12.6)	10.3	(9.6–12.4)	10.7	(9.6–14.1)	0.56
Platelet, median (IQR), $\times 10^9$/L	119	(80–198)	124	(72–205)	104	(80–148)	0.77
ANC, median (IQR), $\times 10^9$/L	3.4	(2.1–4.9)	3.2	(2.3–4.9)	3.8	(1.8–5.3)	0.69
Creatinine, median (IQR), µmol/L	92	(73–114)	93	(76–116)	83	(69–99)	0.22
C-reactive protein ≥ 3 mg/L, n (%)	29/39	(74)	24/33	(73)	5/6	(83)	0.99
ICUS, n (%)	8	(12)	8	(15)	–	(–)	–
Confirmed myelodysplastic syndrome, n (%)	15	(22)	–	(–)	15	(100)	–
Neutrophil MPO expression in peripheral blood, median (IQR)							
Mean, FI	4040	(2828–5739)	3981	(2816–5292)	4296	(2840–6362)	0.46
Median, FI	3883	(2730–5500)	3816	(2732–5184)	4175	(2701–6167)	0.61
Robust coefficient of variation, %	31.9	(29.5–34.6)	31.0	(28.9–32.5)	38.1	(32.7–50.2)	<0.001

N/n: number; ANC: absolute neutrophil count; FI: fluorescence intensity; ICUS: idiopathic cytopenia of undetermined significance; IQR: interquartile range (25–75th percentiles); MDS: myelodysplastic syndrome; MPO: myeloperoxidase; N/n: number; SD: standard deviation. *Values were missing for platelet count (n=2), C-reactive protein (n=29), and creatinine (n=25) concentrations.

68 consecutive patients routinely referred for suspected MDS. 2) Control subjects included in the retrospective study did not undergo BM aspirate or biopsy, with the potential for verification bias.[38] Although overt MDS could not be formally excluded in these subjects, none of the controls had evidence of PB cytopenia, making this hypothesis very unlikely. 3) Peripheral cytopenia was defined based on standard laboratory values, as recommended by others.[18,23] To assess the robustness of our findings, we repeated the analysis after restricting the study sample to patients with evidence of cytopenia according to WHO categorization, and the diagnostic accuracy estimates were similar although less precise (*Online Supplementary Table S10*). 4) Neutrophils of MDS patients can exhibit varying levels of CD14, CD64, or CD16 expression compared with healthy controls. However, we did not have any difficulty separating neutrophils from monocytes because of increased CD14 expression. CD64 was not used in the gating strategy and any modulation of its expression would not alter the results. We rarely observed downmodulation of CD16 in this series and these cells were infrequent among the granulocyte population. Importantly, the RCV for MPO expression of circulating neutrophils remained unchanged depending on whether or not these cells were taken into account. 5) The diagnosis of MDS can be delicate with subtle cytological signs of myelodysplasia. There is some evidence that cytomorphology examination lacks reproducibility, even for experienced hematopathologists. Furthermore, the cytological dysplasia criterion threshold of 10% abnormal cells limited to one lineage is a subject of debate. 6) Our diagnostic accuracy study was carried out in two university-affiliated hospitals in France. For this reason, our findings may lack external validity and may not apply to other regions or healthcare settings.

In conclusion, flow cytometric analysis of neutrophil MPO expression in PB might increase the diagnostic yield of BM aspirate in patients referred for suspected MDS. A RCV value <30.0% accurately rules out MDS, with both sensitivity and negative predictive value estimates of 100%. This strategy might obviate the need for invasive BM aspirate for up to 29% of patients with suspected MDS in real-life practice. Although promising, these preliminary results require replication in a large multicenter prospective diagnostic accuracy study.

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