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

Running title: Myeloperoxidase in myelodysplastic syndromes

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ABSTRACT (205 words)

Suspicion of myelodysplastic syndromes is one of the commonest reasons for bone marrow aspirate in elderly patients presenting with persistent peripheral blood cytopenia of unclear etiology. A peripheral blood assay that accurately rules out myelodysplastic syndromes would have major benefits. The diagnostic accuracy of the intraindividual robust coefficient of variation for neutrophil myeloperoxidase expression measured by flow cytometric analysis in peripheral blood was evaluated in a retrospective derivation study (44 myelodysplastic syndrome cases and 44 controls) and a prospective validation study (68 consecutive patients with suspected myelodysplastic syndromes). Compared with controls, myelodysplastic syndrome cases had higher median robust coefficient of variation values for neutrophil myeloperoxidase expression (40.2% versus 30.9%, $P<.001$). 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 robust coefficient of variation lower than 30% ruled out myelodysplastic syndromes with 100% sensitivity (95% CI, 78%–100%) and 100% negative predictive value (95% CI, 83%–100%) in the prospective validation study. Neutrophil myeloperoxidase expression measured by flow cytometric analysis in peripheral blood might obviate the need for invasive bone marrow aspirate and biopsy for up to 29% of patients with suspected myelodysplastic syndromes.

INTRODUCTION

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal bone marrow neoplasms that predominate in the elderly.[1,2] The diagnosis of MDS is based on peripheral blood cytopenia and morphologic dysplasia for one or more hematopoietic cell lineages.[1,3,4] Cytopenia is evidenced with hemogram while dysplasia requires bone marrow 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 bone marrow aspiration-related discomfort and harm. Therefore, an objective assay based on a peripheral blood sample that accurately discriminates MDS from other cytopenia etiologies is highly desirable. In this context, few studies have investigated the value of flow cytometric analysis for detecting aberrant phenotypic expression of peripheral blood 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] which can be analyzed using various approaches, including hemogram automaton, cytomorphologic evaluation, and flow cytometry (side scatter). Degranulation is associated with myeloperoxidase (MPO) cytoplasmic expression, an enzyme synthetized 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 cytomorphologic evaluation of peripheral blood in routine practice.

Flow cytometric analysis of MPO expression in bone marrow neutrophil granulocytes has been occasionally used for identifying MDS patients and discriminating between low-

versus higher-risk patients with MDS.[16] Yet, a study reporting on the accuracy of flow cytometric analysis of neutrophil MPO expression in peripheral blood for the diagnosis of MDS is still lacking.

The present study aimed to assess the performance of flow cytometric analysis of MPO expression in peripheral blood mature granulocytes for ruling out the 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 for various parameters of neutrophil MPO expression in peripheral blood 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 pretested 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 above 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 peripheral blood 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 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 min at room temperature. The fixation and permeabilization phases were performed using the BD IntraSure^{TM} Kit (BD Biosciences, San Jose, CA, USA) and MPO-PE was added (clone 5B8) during the permeabilization phase. All antibodies, BD FACS TM Lysing Solution (BD Biosciences, San Jose, CA, USA) and BD IntraSure^{TM} Kit were obtained from BD Biosciences (San Jose, CA, USA).

At least 10,000 neutrophils were acquired on a three-laser, eight-color BD FACSCanto-II ^{TM} 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.

MPO expression in the peripheral blood 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 peripheral blood neutrophil population within an individual subject (Figure 2).

The FranceFlow standard operating procedure was used to standardize instrument settings. Rainbow calibration particles (BD Sphero^{TM}, BD Biosciences, San Jose, CA, USA) were analyzed daily and photomultiplier tubes were adjusted if needed.

In the retrospective case–control study, flow cytometric analysis was performed within 6 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 bone marrow 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 peripheral blood and bone marrow 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 $<150\times10^9$/L, and/or absolute neutrophil count $<1.8\times10^9$/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% peripheral blood or bone marrow blasts), and 3) the exclusion of reactive etiologies of cytopenia and dysplasia.

Consistent with the WHO classification,[1] MDS subcategorization was based on the degree of dysplasia (unilineage versus multilineage), blast percentages, presence of ring sideroblasts, and cytogenetic analysis (del(5q)). The criteria for CMML diagnosis were 1) the presence of persistent peripheral blood monocytosis $\geq 1\times10^9$/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 cytomorphologic 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 peripheral blood 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 peripheral blood, 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<.001] and creatinine [P=.03] concentrations). Because hemoglobin concentration, platelet count, and absolute neutrophil count were part of the MDS definition, they were not entered as covariates 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 diagnosis. 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 ranging from 100% to 90% in the retrospective case–control study. Since neutrophil MPO expression in peripheral blood 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-values less than 0.05 were 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% (24/44), 20% (9/44), and 11% (5/44) 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 peripheral blood 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 (Supplemental Table 1).

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 rather unchanged after excluding CMML cases (Supplemental Table 2). Sensitivity point estimates ranged from 100% to 91% for RCV thresholds varying between 28% and 32% (Table 3). A RCV value lower than 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 higher than

30%. The exception was a 72-year-old female case with multilineage dysplasia, for whom isolated peripheral thrombocytopenia (94×10^9/L) and a 28.3% RCV value for MPO expression in the peripheral blood neutrophil population were found. A RCV value lower than 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% [3/88]).

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 prevalence of MDS and ICUS was 22% and 12%, respectively. The median RCV values for MPO expression in peripheral blood 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 ($P<.001$) (Supplemental Table 3). 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 (Supplemental Table 1).

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 lower than 30.0% excluded MDS for 29% (20/68) 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 (Supplemental Table 5). The coefficient of variation point estimate for inter-assay precision was 3.6% in five independent analytical runs at the same laboratory (Supplemental Table 6).

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 (Supplemental Table 7). 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 (Supplemental Table 8).

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 for healthy individuals and MDS cases, respectively (Supplemental Table 9). 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 (Supplemental Table 9).

DISCUSSION

To our knowledge, this is the first study reporting on the diagnostic accuracy of neutrophil MPO expression measured by flow cytometric analysis in peripheral blood for ruling out MDS. Accordingly, a RCV value lower than 30% identified patients at low risk of MDS in whom invasive bone marrow 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 higher than 30% and would be recommended bone marrow aspirate or biopsy, a strategy that complies with published guidelines.[22,23] Although bone marrow 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 peripheral blood had limited diagnostic value for ruling in MDS.[28] Indeed, the specificity point estimates for a RCV value higher than 30% ranged from 32% to 38% depending on the study sample, with positive predictive values varying between 31% and 59%. RCV values higher than 38% achieved 100% specificity but at a cost of a 30% false-negative rate. Hence, the RCV of neutrophil MPO expression in peripheral blood would not add relevant information to cytomorphologic evaluation of bone marrow 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 peripheral blood for the diagnosis of MDS. Rashidi et al. reported decreased mean levels of CD10 expression in peripheral blood for high-grade MDS compared with cytopenic controls (2.2 [0.7] versus 3.7 [0.7], $P<.001$).[9] Yet, this study failed to show a significant difference in levels of CD10 expression between low-grade MDS and cytopenic controls (3.7 [0.9] versus 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 peripheral blood 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 peripheral blood 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 peripheral blood 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 peripheral blood 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 peripheral

blood 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 bone marrow examination in elderly patients presenting with persistent peripheral blood cytopenia of unclear etiology.[33] Bone marrow biopsy and aspiration are painful procedures for the majority of patients,[34,35] with 20% of them reporting a moderate level of pain 7 days after the procedure.[36] Although infrequent, procedure-related complications (hemorrhage and infection) may be associated with significant morbidity or even be life-threatening.[37]

The use of flow cytometric analysis of neutrophil MPO expression in peripheral blood might be suitable to reduce the unnecessary exposure of patients without MDS to bone marrow aspirate-related discomfort and harm 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 resource use associated with the implementation of flow cytometric analysis of neutrophil MPO expression in peripheral blood for patients with suspected MDS in routine practice.[17]

Our study has limitations that deserve mention. First, the retrospective case–control study design is prone to spectrum bias,[10] with the potential for optimistic diagnostic accuracy estimates. Reassuringly, our prospective validation study replicated the findings in 68 consecutive patients routinely referred for suspected MDS.

Second, control subjects included in the retrospective study did not undergo bone marrow 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 peripheral blood cytopenia, making this hypothesis very unlikely.

Third, 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 (Supplemental Table 10).

Fourth, neutrophils of MDS patients can exhibit varying levels of CD14, CD64, or CD16 expression, compared with healthy controls. Yet, 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 down-modulation 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.

Fifth, the diagnosis of MDS can be delicate with subtle cytologic signs of myelodysplasia. Evidence exists that cytomorphology examination lacks reproducibility, even for experienced hematopathologists. Furthermore, the cytologic dysplasia criterion threshold of 10% abnormal cells limited to one lineage is debated.

Sixth, our diagnostic accuracy study was carried out in two university-affiliated hospitals in France. For this reason, our findings may lack external validity and not apply to other regions or healthcare settings.

In conclusion, flow cytometric analysis of neutrophil MPO expression in peripheral blood might increase the diagnostic yield of bone marrow aspirate in patients referred for suspected

MDS. A RCV value lower than 30.0% accurately rules out MDS, with both sensitivity and negative predictive value estimates of 100%. This strategy might obviate the need for invasive bone marrow 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.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest relevant to this study.

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AUTHOR CONTRIBUTIONS

RV-M had full access to the data in the study and takes responsibility for data integrity and accuracy of data analysis.

TR, M-CJ, SP, JK and RV-M contributed to the study design, data acquisition, interpretation of the results, and manuscript preparation.

CMA and LC contributed to the study design, interpretation of the results, and manuscript preparation.

JL and BP contributed to data management, statistical analysis, interpretation of the results, and manuscript preparation.

J-YC and MB provided project leadership, contributed to the interpretation of the results, and manuscript preparation.

All authors approved the final version of the manuscript.

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Table 1. Baseline patient 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)	.94
Hemoglobin, median (IQR), *g/dL*	10.7	(9.0–12.7)	13.8	(13.0–14.9)	<.001
Platelet, median (IQR), $\times 10^9/L$	142	(75–190)	246	(206–283)	<.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)	.03
C-reactive protein ≥ 3 mg/L, *n (%)*	19	(63)	5	(13)	<.001
Neutrophil MPO expression in peripheral blood, median (IQR)					
Mean, *FI*	6083	(3905–9904)	6515	(4230–9749)	.95
Median, *FI*	5527	(3777–9482)	6355	(4110–9520)	.71
Robust coefficient of variation, %	40.2	(37.8–46.9)	30.9	(29.7–31.9)	<.001

Abbreviations: 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)

Abbreviations: MDS, myelodysplastic syndrome; WHO, World Health Organization.

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*				Sensitivity, %	Specificity, %	PPV, %	NPV, %	LR+*	LR−*
	True positive	False negative	False positive	True negative						
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 (.98–1.17)	.14 (.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)	.17 (.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)	.07 (.01–.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)	.13 (.04–.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)	.12 (.05–.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)	.20 (.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)	.11 (.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)	.08 (.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)	.06 (.01–.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)	.21 (.06–.79)

Abbreviations: 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.

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)	.72
Age, mean (SD), *y*	74.7	(9.2)	73.6	(9.2)	78.4	(8.4)	.07
Hemoglobin, median (IQR), *g/dL*	10.4	(9.6–12.6)	10.3	(9.6–12.4)	10.7	(9.6–14.1)	.56
Platelet, median (IQR), $\times 10^9/L$	119	(80–198)	124	(72–205)	104	(80–148)	.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)	.69
Creatinine, median (IQR), *µmol/L*	92	(73–114)	93	(76–116)	83	(69–99)	.22
C-reactive protein ≥ 3 mg/L, *n (%)*	29/39	(74)	24/33	(73)	5/6	(83)	.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)	.46
Median, *FI*	3883	(2730–5500)	3816	(2732–5184)	4175	(2701–6167)	.61
Robust coefficient of variation, %	31.9	(29.5–34.6)	31.0	(28.9–32.5)	38.1	(32.7–50.2)	<.001

Abbreviations: ANC, absolute neutrophil count; FI, fluorescence intensity; ICUS, idiopathic cytopenia of undetermined significance; IQR, interquartile range (25–75th percentiles); MDS, myelodysplastic syndrome; MPO, myeloperoxidase; SD, standard deviation.

* Values were missing for platelet count (*n*=2), C-reactive protein (*n*=29), and creatinine (*n*=25) concentrations.

Figure 1. Gating strategy for quantifying peripheral blood neutrophil myeloperoxidase expression.

CD45+ viable cells were first individualized by crossing the singlet gate (A), FSC-SSC leukocytes (B), and CD45-positive 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). 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).

Abbreviations: CD, cluster of differentiation; FSC-H, forward scatter height; FSC-A, forward scatter area; SSC-H, side scatter height; MPO, myeloperoxidase; RCV, robust coefficient of variation.

Figure 2. Monoparametric histograms of peripheral blood neutrophil myeloperoxidase expression.

Values are mean, *FI*; median, *FI*; and RCV, *%*.

Panel A. Control subject.

Panel B. Myelodysplastic syndrome case.

Abbreviations: FI, fluorescence intensity; MPO, myeloperoxidase; RCV, robust coefficient of variation.

Figure 3. Area under the receiver operating characteristic curve for flow cytometric parameters of peripheral blood neutrophil myeloperoxidase expression in discriminating myelodysplastic syndromes.

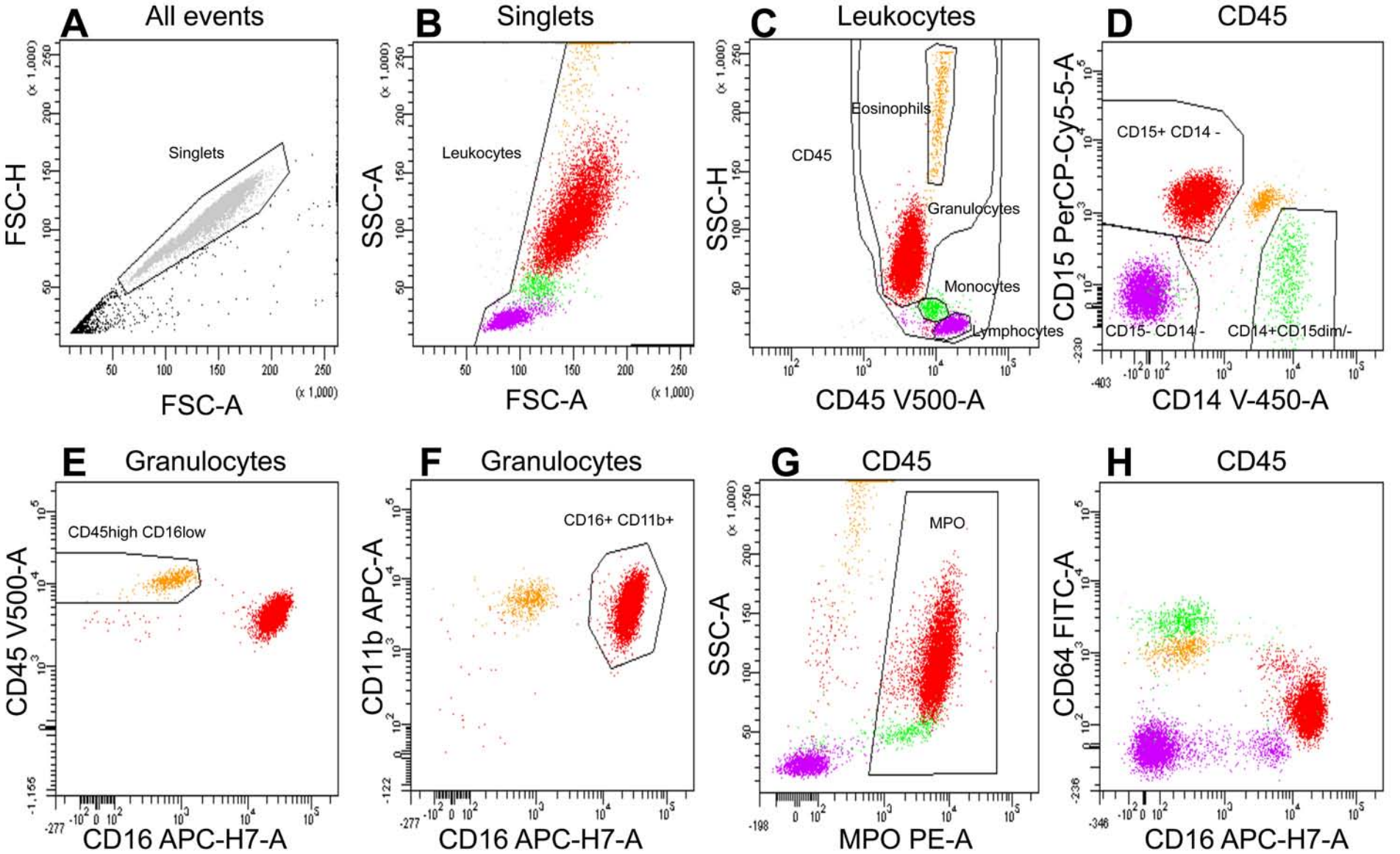
Panel A. Retrospective case–control study.

Panel B. Consecutive patients with suspected myelodysplastic syndromes.

The area under the receiver operating characteristic curve for each parameter was compared with that for the robust coefficient of variation. *P*-values were adjusted for multiple comparisons using the Bonferroni method.

Abbreviations: CI, confidence interval; RCV, robust coefficient of variation.

Figure 1


A
All events
Singlets
FSC-H
FSC-A
B
Singlets
Leukocytes
SSC-A
FSC-A
C
Leukocytes
Eosinophils
CD45
Granulocytes
Monocytes
Lymphocytes
SSC-H
CD45 V500-A
D
CD45
CD15+ CD14 -
CD15- CD14 -
CD14+CD15dim/-
CD15 PerCP-Cy5-5-A
CD14 V-450-A
E
Granulocytes
CD45high CD16low
CD45 V500-A
CD16 APC-H7-A
F
Granulocytes
CD16+ CD11b+
CD11b APC-A
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CD64 FITC-A
CD16 APC-H7-A

Figure 2

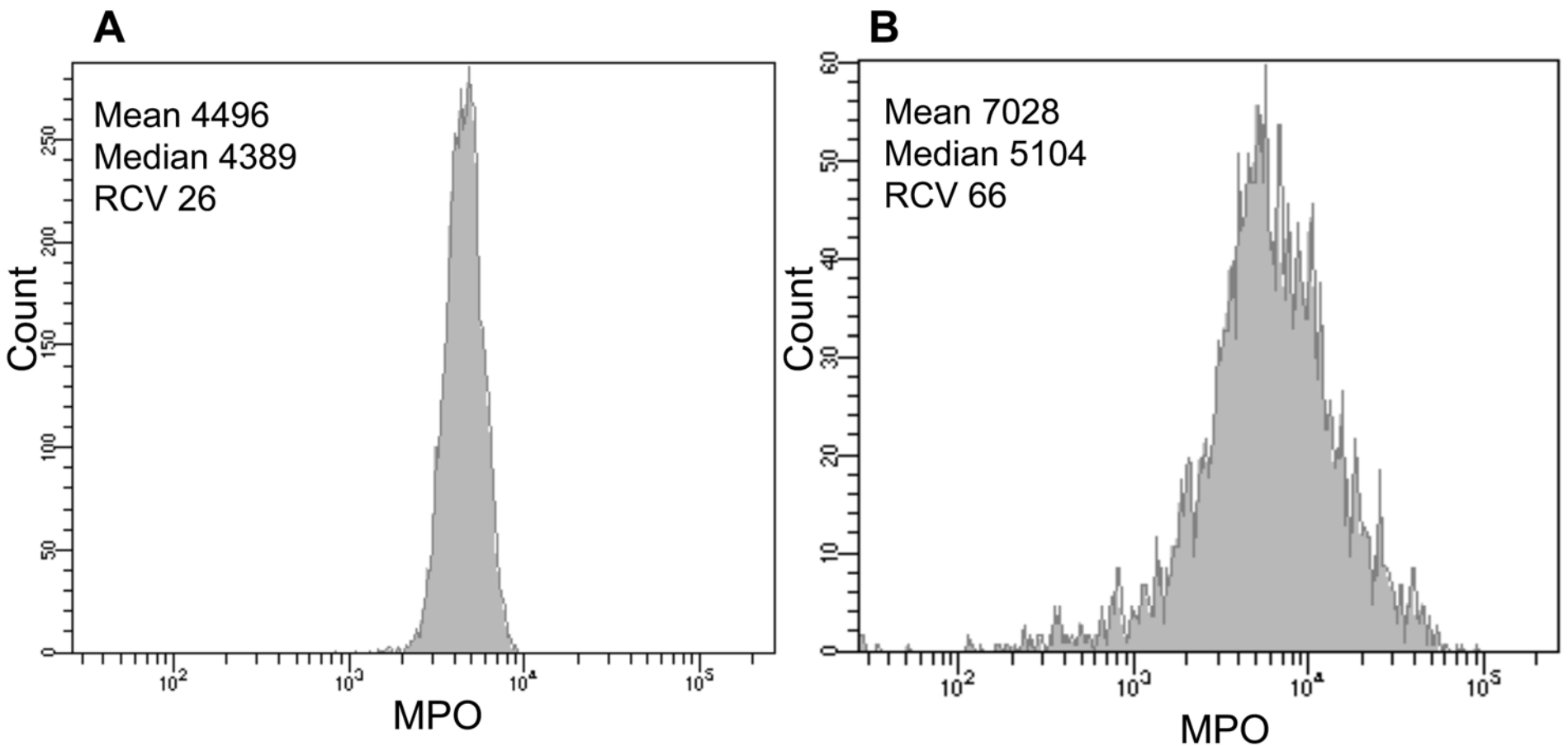
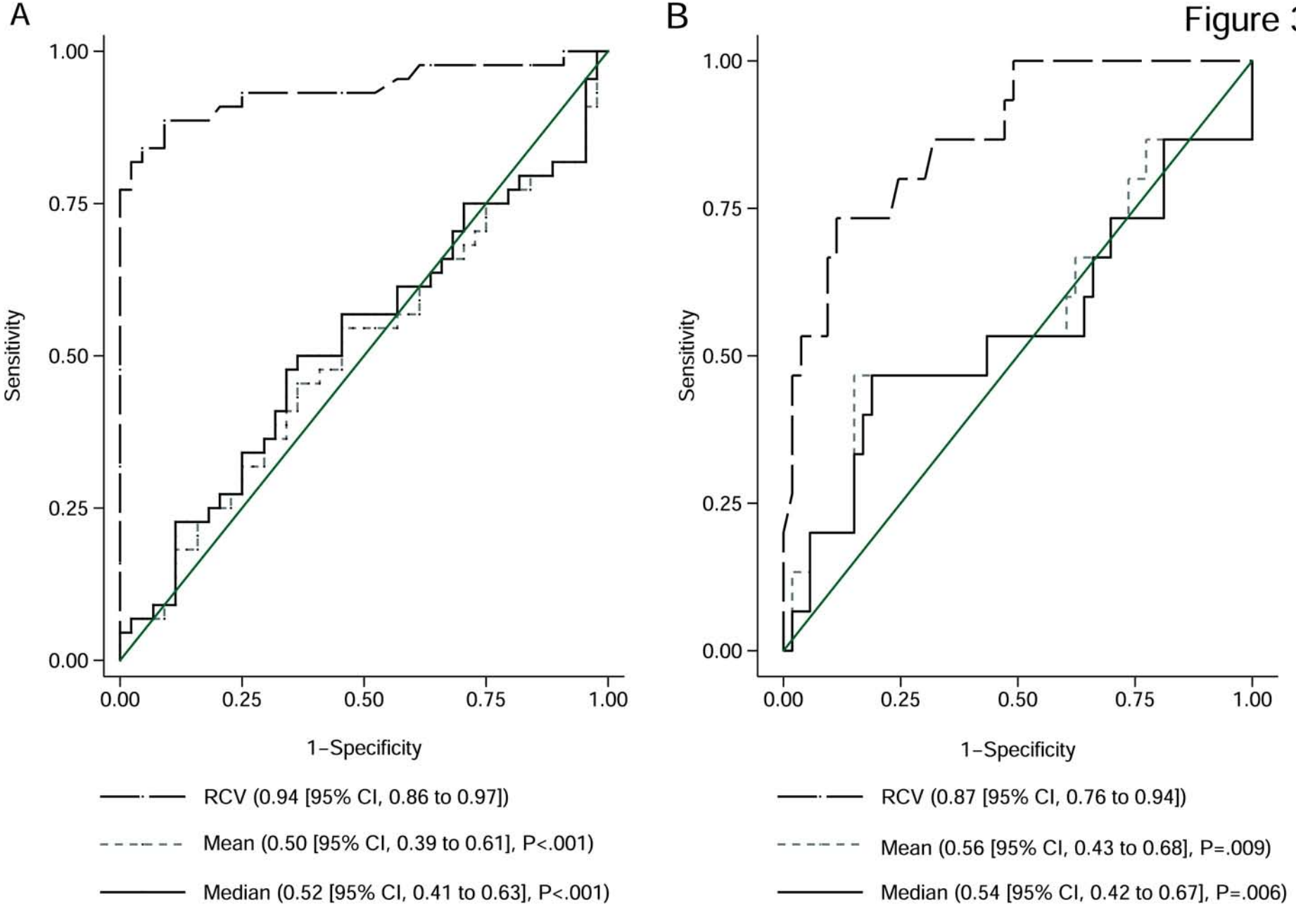

A
Mean 4496
Median 4389
RCV 26
Count
MPO
B
Mean 7028
Median 5104
RCV 66
Count
MPO

Figure 3


A
1.00
0.75
0.50
0.25
0.00
Sensitivity
0.00
0.25
0.50
0.75
1.00
1–Specificity
RCV (0.94 [95% CI, 0.86 to 0.97])
Mean (0.50 [95% CI, 0.39 to 0.61], P<.001)
Median (0.52 [95% CI, 0.41 to 0.63], P<.001)
B
1.00
0.75
0.50
0.25
0.00
Sensitivity
0.00
0.25
0.50
0.75
1.00
1–Specificity
RCV (0.87 [95% CI, 0.76 to 0.94])
Mean (0.56 [95% CI, 0.43 to 0.68], P=.009)
Median (0.54 [95% CI, 0.42 to 0.67], P=.006)

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

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SUPPLEMENTAL METHODS

Study design

Using a retrospective case–control study design,[1] we assessed the diagnostic accuracy for various parameters of neutrophil MPO expression in peripheral blood 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 present article complies with the updated Standards for Reporting Diagnostic Accuracy Studies statement.[2]

Study sites

The flow cytometric analysis protocol was jointly developed and pretested 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 an established diagnosis of MDS or CMML, as defined by current guidelines.[3-7] 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 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 above 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 peripheral blood 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 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 manufacturers' recommendations with a panel of antibodies conjugated to fluorochromes. CD64-FITC (clone 10.1), CD15-PerCP-Cy5.5 (clone HI98), CD11b-APC (clone D12), CD16-APC-H7 (clone 3G8), CD14-V450 (clone MΦP9), and CD45-V500 (clone HI30) antibodies were added. Aliquots were stained for 15 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, the BD FACS™ Lysing

Solution, and the BD IntraSure™ Kit were obtained from BD Biosciences (San Jose, CA, USA).

At least 10,000 neutrophils were acquired on a three-laser, eight-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.

MPO expression in the circulating neutrophil population was expressed as median, mean, and robust coefficient of variation (RCV).[8] The median and mean fluorescence intensity (MFI) reflected the central location of MPO expression in the circulating neutrophil population within an individual subject. 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.[9] The RCV was expressed as a percentage and reflected the variability in MPO expression in the circulating neutrophil population within an individual subject (Figure 2).

The FranceFlow standard operating procedure was used to standardize instrument settings. The voltage for each photomultiplier tube was set to reach the target MFI of the FranceFlow-validated lot of Rainbow beads (target MFI ± 2%) (Supplemental Table 4). Fluorescence compensation was calculated using CompBeads (BD Biosciences, San Jose, CA, USA) with Diva v6 or Diva v8 software (BD Biosciences, San Jose, CA, USA). Rainbow calibration particles (BD Sphero™, BD Biosciences, San Jose, CA, USA) were analyzed daily and photomultiplier tubes were adjusted if needed (target MFI ± 15%).[10]

In the retrospective case–control study, flow cytometric analysis was performed within 6 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 MDS diagnosis 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,[3-6] based on clinical data, peripheral blood cytopenia, cytomorphology of peripheral blood and bone marrow 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 $<150\times10^9$/L, and/or absolute neutrophil count $<1.8\times10^9$/L).[7]

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% peripheral blood or bone marrow blasts), and 3) the exclusion of reactive etiologies of dysplasia.

Consistent with the WHO classification,[3] MDS subcategorization was based on the degree of dysplasia (unilineage versus multilineage), blast percentages, presence of ring sideroblasts, and cytogenetic analysis (del(5q)). The criteria for CMML diagnosis were 1) the presence of persistent peripheral blood monocytosis $\geq1\times10^9$/L and 2) monocytes accounting for more than 10% of the white blood cell differential count.[3] 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.[5,11-13]

In the retrospective case–control study, the reference standard was available for MDS cases only and no control subjects received cytomorphologic evaluations. In contrast, the reference standard was available for all patients enrolled in the prospective validation cohort study. Additionally, we categorized MDS patients as “low risk” (low- and intermediate−1-risk categories) and “high risk” (intermediate−2- and high-risk categories), using the International Prognostic Scoring System.[14]

Sample size

We estimated that a sample size of 88 participants (comprising 44 MDS patients and 44 controls) would provide a precision of ±0.05 for an area under the receiver operating characteristic (ROC) curve point estimate of 0.95 (95% confidence interval [CI] ranging from 0.90 to 1.00).[15]

Precision and reproducibility assessment

Using the bootstrap method with 1,000 replications, 2.5% and 97.5% percentile point estimates for RCV for neutrophil MPO expression in 44 healthy controls were 25.4 (95% CI, 25.2–28.3) and 36.9 (95% CI, 33.4–37.3), respectively. We evaluated intra- and inter-assay precision, reproducibility between study sites, and specimen stability for RCV measurements of MPO expression in the peripheral blood neutrophil population according to current guidelines.[16-18] For this purpose, we calculated the coefficient of variation for RCV measurements as the standard deviation multiplied by 100 and divided by the mean.

To assess intra-assay precision, blood samples were collected from five healthy individuals and five SMD patients, respectively.[18] Each sample was assayed in triplicate in a single analytical run by the same operator.[16,18] To assess inter-assay precision, a single blood sample from a healthy individual was assayed by five different operators, in five independent analytical runs at the same laboratory and on the same day.

To assess specimen stability, blood samples from 10 healthy individuals were assayed at five different time points (at baseline, 24 h, 48 h, 72 h, and 96 h).[18] To assess the stability of the processed (stained, lysed, fixed) specimens, five samples held at 4°C were tested at baseline (within 1 h of staining) and 6 h.[18]

To assess inter-laboratory reproducibility, blood samples from five healthy individuals and five MDS cases were split, stored at 4°C, and assayed simultaneously at two laboratories,

24 h after collection. Additionally, we examined reproducibility using three alternate setup procedures (manufacturer's recommendations [cytometer setup and tracking research beads], FranceFlow and EuroFlow instrument setups) within each laboratory.

Statistical analysis

Patient characteristics were reported as percentages for categorical variables and mean and standard deviation or median and interquartile range (IQR, 25th and 75th percentiles) or range for continuous variables. Patient characteristics and neutrophil MPO expression in peripheral blood were compared between study groups using the χ^2 test, replaced by the Fisher exact test where appropriate, for categorical variables, and the Student *t*-test, or the nonparametric Wilcoxon test where appropriate, for continuous variables.

We assessed the independent associations of MDS with RCV for neutrophil MPO expression measured by flow cytometric analysis in peripheral blood, 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<.001] and creatinine [P=.03] concentrations). Because hemoglobin concentration, platelet count, and absolute neutrophil count were part of the MDS definition, they were not entered as covariates 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 diagnosis. 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 the area 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 ranging from 100% to 90% in the retrospective case–control study. Since neutrophil MPO expression in peripheral blood 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.[19]

Two-tailed *P*-values less than 0.05 were considered statistically significant. Analyses were performed using Stata Special Edition version 14.0 (Stata Corporation, College Station, TX, USA).

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Supplemental Table 1. Flow cytometric robust coefficient of variation estimates for neutrophil myeloperoxidase expression in peripheral blood according to International Prognostic Scoring System.

				MDS*					
	Non-MDS			Low-risk			High-risk		
Study sample	*N*	Median RCV (IQR)		*N*	Median RCV (IQR)		*N*	Median RCV (IQR)	*P*
Retrospective case–control study	44	30.9	(29.7–31.9)	25	41.1	(38.6–47.2)	19	38.6 (36.6–46.0)	<.001
Consecutive patients with suspected MDS	53	31.0	(28.9–32.5)	14	37.5	(32.7–45.8)	1	65.9 (…)	<.001

Abbreviations: IQR, interquartile range (25–75th percentiles); MDS, myelodysplastic syndrome; RCV, robust coefficient of variation.

* MDS patients were categorized as low-risk (low and intermediate−1-risk categories) versus high-risk (intermediate−2- and high-risk categories), using the International Prognostic Scoring System (See Supplemental Methods).

Supplemental Table 2. Flow cytometric robust coefficient of variation estimates for neutrophil myeloperoxidase expression in peripheral blood stratified by myelodysplastic syndromes versus chronic myelomonocytic leukemia.

	N	Median RCV (range)		Area under the ROC curve (95% CI)	
Retrospective case–control study					
Controls	44	30.9	(25.2–37.3)	…	(…)
MDS cases	39	39.9	(28.3–99.3)	0.93	(0.86–0.98)
CMML cases	5	45.3	(32.3–66.1)	0.96	(0.86–0.99)
Consecutive patients with suspected MDS					
Unconfirmed suspicions of MDS	53	31.0	(26.1–50.2)	…	(…)
Confirmed suspicions of MDS	12	37.5	(31.3–99.2)	0.85	(0.74–0.92)
Confirmed suspicions of CMML	3	42.5	(35.1–45.8)	0.95	(0.85–0.99)

Abbreviations: CI, confidence interval; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndrome; RCV, robust coefficient of variation; ROC, receiver operating characteristics.

Supplemental Table 3. Flow cytometric robust coefficient of variation estimates for neutrophil myeloperoxidase expression in peripheral blood according to final diagnoses for consecutive patients with unconfirmed suspected myelodysplastic syndrome.

		MPO RCV, %	
Final diagnosis	*N*	Median	(Range)
ICUS	8	37.2	(32.5–50.2)
Drug-induced cytopenia	7	30.8	(26.9–33.6)
Immune thrombocytopenic purpura	6	30.9	(27.2–32.1)
Chronic liver disease	6	29.1	(26.7–31.9)
Chronic kidney disease	4	30.9	(26.1–34.1)
Transient/unconfirmed cytopenia	4	30.6	(28.7–32.5)
Iron, vitamin B_{12}, and/or folate deficiency	4	31.6	(28.3–34.0)
Bone marrow infiltration	3	30.6	(28.2–32.8)
Inflammation	2	…	(27.0–29.7)
Autoimmune disease	2	…	(30.6–33.8)
Post-transplant cytopenia	2	…	(27.5–27.7)
Hairy cell leukemia	1	32.2	(…)
Large granular lymphocytic leukemia	1	31.9	(…)
Other	3	31.7	(28.7–32.4)

Abbreviations: MPO, myeloperoxidase; RCV, robust coefficient of variation.

Supplemental Table 4. Target mean fluorescence intensity values for three different lots of Rainbow beads according to the FranceFlow standard.

Lot no. (year)	23755 (2015)		3319908 (2016)		5173576 (2016)	
Fluorochrome channel	Rainbow peak	MFI	Rainbow peak	MFI	Rainbow peak	MFI
FITC	8th	56163	8th	59689	7th	28171
PE	8th	88401	8th	86002	7th	35489
PerCP-Cy5.5	8th	221025	8th	220558	7th	71801
PE-Cy7	8th	29327	8th	29335	8th	28048
APC	8th	208842	7th	134990	6th	49100
APC-H7	8th	44591	8th	94477	7th	42456
Horizon™ V450	7th	180118	7th	183461	6th	69155
Horizon™ V500	7th	155930	7th	129664	6th	42190

Abbreviations: APC, allophycocyanin; APC-H7, allophycocyanin hilite7; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; PE, phycoerythrin; PE, PE-Cy7, phycoerythrin-cyanin7; PerCP-Cy5.5, peridinin-chlorophyll-protein-cyanin5.5

Supplemental Table 5. Intra-assay precision estimates for robust coefficient of variation of neutrophil myeloperoxidase expression in peripheral blood.

	MPO RCV, %*					
	1	2	3	Mean (SD)		CV, %
Healthy individuals						
HI 1	28.3	28.5	28.3	28.4	(0.1)	0.4
HI 2	30.8	31.0	31.0	30.9	(0.1)	0.4
HI 3	33.3	33.3	33.0	33.2	(0.2)	0.5
HI 4	25.0	25.0	25.2	25.1	(0.1)	0.5
HI 5	27.8	27.6	27.8	27.7	(0.1)	0.4
MDS patients						
MDS 1	30.4	30.5	30.6	30.5	(0.1)	0.3
MDS 2	34.9	34.9	34.9	34.9	(0.0)	0.0
MDS 3	33.4	33.2	33.8	33.5	(0.3)	0.9
MDS 4	41.8	41.9	42.0	41.9	(0.1)	0.2
MDS 5	41.0	41.0	41.0	41.0	(0.0)	0.0

Abbreviations: CV, coefficient of variation; HI, healthy individual; MDS, myelodysplastic syndrome; MPO, myeloperoxidase; RCV, robust coefficient of variation; SD, standard deviation.

* Blood samples were collected from five healthy individuals and five MDS cases. Each sample was assayed in triplicate in a single analytical run by the same operator (see Supplemental Methods).

Supplemental Table 6. Inter-assay precision estimates for robust coefficient of variation of neutrophil myeloperoxidase expression in peripheral blood.

	MPO RCV, %*							
	1	2	3	4	5	Mean (SD)		CV, %
Healthy individual	27.0	24.7	26.0	27.0	26.4	26.2	(0.9)	3.6

Abbreviations: CV, coefficient of variation; MPO, myeloperoxidase; RCV, robust coefficient of variation; SD, standard deviation.

* A single blood sample from a healthy individual was assayed by five different operators, in five independent analytical runs at the same study site and at the same day (see Supplemental Methods).

Supplemental Table 7. Specimen stability estimates for robust coefficient of variation of neutrophil myeloperoxidase expression in peripheral blood according to timing.*

Healthy individual	Baseline	24 h		48 h		72 h		96 h	
1	29.0	26.9	(−7)	27.9	(−4)	28.0	(−3)	28.8	(−1)
2	27.5	27.0	(−2)	27.5	(0)	31.2	(13)	31.3	(14)
3	27.5	24.3	(−12)	24.7	(−10)	27.1	(−1)	28.0	(2)
4	28.4	25.8	(−9)	25.7	(−10)	27.5	(−3)	28.5	(0)
5	27.0	25.6	(−5)	27.5	(2)	27.7	(3)	29.3	(9)
6	26.6	25.3	(−5)	25.5	(−4)	27.8	(5)	28.8	(8)
7	27.1	25.7	(−5)	28.2	(4)	29.1	(7)	29.4	(8)
8	27.1	25.3	(−7)	26.1	(−4)	27.8	(3)	27.7	(2)
9	28.0	26.3	(−6)	25.8	(−8)	27.4	(−2)	27.6	(−1)
10	27.6	25.4	(−8)	26.1	(−5)	28.4	(3)	28.6	(4)
Mean	27.6	25.8	(−7)	26.5	(−4)	28.2	(2)	28.8	(4)
SD	0.7	0.8	(3)	1.2	(5)	1.2	(5)	1.1	(5)

Abbreviations: SD, standard deviation.

* Values are robust coefficients of variation for neutrophil myeloperoxidase expression in peripheral blood (relative change from baseline expressed as a percentage). Blood samples from 10 healthy individuals were assayed at five different time points (i.e., at baseline, 24 h, 48 h, 72 h, and 98 h) at 4°C (See Supplemental Methods).

Supplemental Table 8. Stability of processed samples for robust coefficient of variation of neutrophil myeloperoxidase expression in peripheral blood.

	MPO RCV, %*		(Change from
Healthy individual	Baseline	6 h	baseline, %)
1	28.5	28.2	(−1)
2	31.0	31.4	(1)
3	33.5	32.9	(−2)
4	25.1	24.8	(−1)
5	35.1	35.3	(1)
Mean	30.6	30.5	(−0.4)
SD	4.0	4.1	(1)

Abbreviations: MPO, myeloperoxidase; RCV, robust coefficient of variation; SD, standard deviation.

* Five processed (stained, lysed, fixed) samples held at 4°C were tested at baseline (within 1 h of staining) and 6 h (see supplemental Methods).

Supplemental Table 9. Inter-laboratory and instrument setup procedure comparisons for robust coefficient of variation of neutrophil myeloperoxidase expression in peripheral blood.

	Laboratory 1*				Laboratory 2*				Inter-laboratory CV, %†		
Individuals	MR	FF	EF	CV, %	MR	FF	EF	CV, %	MR	FF	EF
Healthy individuals											
HI 1	31.0	31.3	31.1	0.5	29.9	28.5	28.7	2.6	2.6	6.6	5.7
HI 2	28.2	28.2	28.4	0.4	26.9	28.4	27.3	2.8	3.3	0.5	2.8
HI 3	28.1	28.2	28.2	0.2	28.8	27.2	28.5	3.0	1.7	2.6	0.7
HI 4	31.7	31.8	31.9	0.3	28.6	27.3	28.8	2.9	7.3	10.8	7.2
HI 5	27.0	27.0	27.0	0.0	25.0	24.7	25.2	1.0	5.4	6.3	4.9
Mean (HI 1–5)	29.2	29.2	29.3	0.3	27.8	27.2	27.7	2.5	4.1	5.3	4.3
MDS cases											
MDS 1	31.8	31.2	31.4	1.0	33.3	33.9	34.4	1.6	3.3	5.9	6.4
MDS 2	48.0	47.4	47.1	1.0	45.3	46.0	45.7	0.8	4.1	2.1	2.1
MDS 3	30.6	30.3	30.7	0.7	31.2	32.1	30.7	2.3	1.4	4.1	0.0
MDS 4	34.7	34.1	34.9	1.2	31.3	32.8	31.2	3.0	7.7	2.7	7.9
MDS 5	33.3	33.2	33.1	0.3	33.5	34.1	33.6	1.0	0.4	1.9	1.1
Mean (MDS 1–5)	35.7	35.2	35.4	0.8	34.9	35.8	35.1	1.7	3.4	3.3	3.5

Abbreviations: CV, coefficient of variation; EF, EuroFlow instrument setup; FF, FranceFlow instrument setup; HI, healthy individual; MDS, myelodysplastic syndrome; MR, manufacturer's recommendation (cytometer setup and tracking research beads).

* Blood samples from five healthy individuals and five MDS cases were split and assayed simultaneously with three alternate instrument setup procedures (i.e., manufacturer recommendations [Cytometer setup and tracking research beads], FranceFlow and EuroFlow instrument setups) at two laboratories, 24 h after collection. Values are robust coefficients of variation for neutrophil myeloperoxidase expression in peripheral blood and coefficients of variation across instrument setup procedures within each laboratory.

† Values are inter-laboratory coefficients of variation for each instrument setup procedure.

Supplemental Table 10. Robust coefficient of variation for neutrophil myeloperoxidase expression measured by flow cytometric analysis in peripheral blood for patients with evidence of cytopenia according to WHO definition.

	MDS			Controls / unconfirmed MDS					
Study sample	*N*	MPO RCV, median (IQR), %		*N*	MPO RCV, median (IQR), %		*P*	AUC (95% CI)	
Retrospective case–control study*	33	41.1	(37.9–47.2)	44	30.9	(29.7–31.9)	<.001	0.94	(0.85–0.98)
Prospective validation study	11	36.4	(32.3–40.5)	42	30.8	(28.7–32.2)	<.001	0.85	(0.72–0.93)

Abbreviations: AUC, area under the receiver operating characteristic curve; CI, confidence interval; IQR, interquartile range (25–75th percentiles); MDS, myelodysplastic syndrome; MPO, myeloperoxidase; RCV, robust coefficient of variation.

* The analytical sample for the retrospective case–control study was restricted to 33 myelodysplastic syndrome cases with evidence of cytopenia according to WHO definition and 44 controls.

† The analytical sample for the prospective validation study was restricted to 53 consecutive patients with evidence of cytopenia according to WHO definition, including 11 confirmed and 42 unconfirmed suspected cases of myelodysplastic syndrome, respectively.