SUPPLEMENTARY APPENDIX

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

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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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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^{TM} Kit (BD Biosciences, San Jose, CA, USA) and MPO-PE was added (clone 5B8) during the permeabilization phase. All antibodies, the BD FACS^{TM} 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.