BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-065850
Article Type:	Protocol
Date Submitted by the Author:	20-Jun-2022
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Keywords:	Leukaemia < HAEMATOLOGY, IMMUNOLOGY, STATISTICS & RESEARCH

	METHODS

Flow cytometry lyophilized-reagent tube for quantifying peripheral blood neutrophil myeloperoxidase expression in myelodysplastic syndromes (MPO-MDS-Develop). Protocol for a diagnostic accuracy study.

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Short title: Ruling out myelodysplastic syndromes

Word count: 3629

Abstract: 245

Tables: 1

Figures: 1

References: 42

ABSTRACT

Introduction — Suspicion of myelodysplastic syndromes (MDS) is the commonest reason for bone marrow aspirate in elderly patients. Peripheral blood neutrophil myeloperoxidase expression quantified by flow cytometric analysis might rule out MDS for up to 35% of patients referred for suspected disease, without requiring bone marrow aspiration. Yet laboratory-developed liquid antibody cocktails have practical limitations, because of lack of standardization and poor stability. This research project aims to estimate the level of agreement and comparative accuracy between a single-use flow cytometry tube of lyophilized reagents (BD Lyotube Stain 468) and its laboratory-developed liquid reagent counterpart in quantifying peripheral blood neutrophil myeloperoxidase expression, among adult patients referred for suspected MDS.

Methods and analysis — The MPO-MDS-Develop project is a cross-sectional diagnostic accuracy study of two index tests by comparison with a reference standard in consecutive unselected adult patients conducted at a single university hospital. Flow cytometry analysis of peripheral blood samples will be performed by independent operators who are blinded to the reference diagnosis, using either Lyotube Stain 468 or laboratory-developed liquid reagent cocktail. The reference diagnosis of MDS will be established by cytomorphological evaluation of bone marrow aspirate by two independent hematopathologists blinded to the index test results. Morphologic assessment will be complemented by bone marrow flow cytometric score, karyotype, and targeted next-generation sequencing panel of 43 genes, where relevant.

Ethics — An institutional review board (Comité de Protection des Personnes Sud Est III, Lyon, France) approved the protocol, prior to study initiation.

Trial registration — ClinicalTrials.gov Identifier: NCT04399018.

Keywords — Myelodysplastic syndromes; Flow cytometry; Diagnosis; Neutrophils; ROC curve.

ARTICLE SUMMARY

Strengths and limitations of this study

- Peripheral blood neutrophil myeloperoxidase expression quantified by flow cytometric analysis might rule out myelodysplastic syndromes in 29–35% of patients referred for suspected disease.
- This study will estimate the level of agreement and comparative accuracy between a single-use flow cytometry tube of lyophilized reagents (BD Lyotube Stain 468) and its laboratory-developed liquid reagent counterpart in quantifying peripheral blood neutrophil myeloperoxidase expression among unselected consecutive patients.
- The reference diagnosis of MDS will be established by cytomorphological evaluation of bone marrow aspirate by two independent hematopathologists and complemented by bone marrow flow cytometric score, karyotype, and targeted next-generation sequencing, where relevant.
- This study will be conducted at a single hospital laboratory and the findings may not apply to other settings.

INTRODUCTION

Background

Myelodysplastic syndromes (MDS) encompass a heterogeneous group of clonal bone marrow neoplasms, with a median age at diagnosis of 70 years (1). MDS are characterized by recurrent cytogenetic and molecular abnormalities, morphologic dysplasia for one or more hematopoietic cell lineage, and ineffective hematopoiesis (1, 2). Patients with MDS have poor prognosis, due to peripheral blood cytopenia-related complications and progression to acute myeloid leukemia (3, 4).

Cytomorphological evaluation of bone marrow is the reference standard for the diagnosis of MDS and may be complemented by information obtained from conventional cytogenetic, flow cytometry, and molecular profiling analysis (5). Hence suspicion of MDS is the commonest reason for bone marrow aspiration in older patients with persistent peripheral blood cytopenia of unclear etiology. However, many patients are exposed to unnecessary bone marrow aspiration-related discomfort and harms (6-8) because of the relatively low prevalence of disease among subjects who are referred for suspected MDS (9).

Peripheral blood neutrophil myeloperoxidase expression quantified by flow cytometric analysis has generated interest since it might rule out MDS in 29–35% of patients referred for suspected disease, without requiring invasive bone marrow aspiration (10). Three original studies totaling more than 200 patients support the accuracy of intra-individual robust coefficient of variation (RCV) of peripheral blood neutrophil myeloperoxidase expression in discriminating MDS from other cytopenia etiologies, with area under the receiver operating characteristics (ROC) curve point estimates ranging from 0.87 to 0.94 (11, 12). Yet this laboratory-developed test have practical limitations for routine application in busy clinical flow cytometry laboratories. Indeed, the preparation of liquid antibody cocktail within a laboratory is a time-consuming process (13), requires specific expertise (13), lacks standardization (14), is prone to errors (15), and faces decreased stability of tandem dyes (16, 17).

The use of premade stable standardized reagent panels may address the issues of reliability and efficiency inherent to laboratory-developed tests (16). Lyophilisation is a method which has been used to stabilize premixed multicolor reagent cocktails within flow cytometry tubes (Lyotube), for various routine clinical applications (16). Lyophilised reagent cocktails showed stability at room temperature for 12 to 18 months (16). They provide a simplified way of handling complex multicolor flow cytometry assays, with performance comparable to reference liquid cocktails (16). Indeed, the use of lyophilised reagent cocktails reduces batch-to-batch variations, prevents multiple sources of errors, and saves resources (13, 16). Additionally, standardization of reagent panels facilitates inter-laboratory comparisons and centralized interpretations of flow cytometric data (18).

In order to optimize the workflow for quantifying peripheral blood neutrophil myeloperoxidase expression in suspected MDS, BD Bioscience has manufactured a 5-color lyophilized cocktail, which consists of a single-use customized freeze dried cocktail within a standard 12 × 75 mm polystyrene flow cytometry tube (BD Lyotube Stain 468). It contains five reagents, including CD15-PerCP-Cy5.5 (clone HI98), CD11b-APC (clone D12), CD16-APC-H7 (clone 3G8), CD14-V450 (clone MΦP9), CD45-V500 (clone HI30). A difference with the original laboratory-developed test is that BD Lyotube Stain 468 does not include CD64 FITC antibody (clone 10.1) since this reagent does not contribute to the quantification of peripheral blood neutrophil myeloperoxidase expression (11, 12).

This protocol describes the rationale for the ongoing MPO-MDS-Develop study, explains how the index tests and the reference method are implemented, how data collection is conducted, and how the results will be analyzed and interpreted. This study enrolled participants from 27 July 2020 to 30 September 2021, with an anticipated study completion date of 31 December 2023.

Research hypothesis

The primary hypothesis guiding the project is that an approach based on a single-use flow cytometry tube of lyophilized reagents for quantifying peripheral blood neutrophil myeloperoxidase expression can accurately rule out MDS, with sensitivity and negative predictive value estimates approaching 100%. The secondary hypotheses are that quantification of peripheral blood neutrophil myeloperoxidase expression using a flow cytometry tube of lyophilized reagents yields 1) a high level of intra-laboratory reproducibility, 2) a substantial level of agreement with laboratory-developed liquid reagent cocktail, and 3) and comparable diagnostic accuracy in discriminating MDS to laboratory-developed liquid reagent cocktail.

Objectives

The broad aim of this study is to estimate the level of agreement and comparative accuracy between BD Lyotube Stain 468 and its laboratory-developed liquid reagent counterpart in quantifying peripheral blood neutrophil myeloperoxidase expression, among adult patients referred for suspected MDS. More specifically, the primary objective is to estimate the discriminative accuracy (i.e., area under the ROC curve) for the intra-individual RCV of peripheral blood neutrophil myeloperoxidase expression obtained with BD Lyotube Stain 468 and laboratory developed tests. The secondary objectives are 1) to assess intra- and inter-assay reproducibility, 2) to investigate unprocessed specimen stability at 24 h, 48 h, and 72h under two different storage conditions (i.e., room temperature and 4°C), respectively, and 3) to estimate the negative predictive value with a prespecified threshold of 30.0% for intra-individual RCV of peripheral blood neutrophil myeloperoxidase obtained with BD Lyotube Stain 468.

METHODS

Study design

The MPO-MDS-Develop project is a cross-sectional diagnostic accuracy study of two index tests by comparison with a reference standard in consecutive unselected adult patients referred for suspected disease (19). No specific intervention is assigned to participants. All diagnostic testing, procedures, and medication ordering are performed at the discretion of attending physicians. Compliance with current guidelines is advocated for the reference diagnostic work-up of patients with suspected MDS (1, 5).

Study sites

Although the study was planned to be conducted at three university-affiliated hospitals in France, only one site (Grenoble university hospital) has implemented the study protocol and is recruiting patients. The principal investigator at the two other study sites withdrew before the recruitment started, for personal convenience, logistical reasons, or resource shortage. The decision was made by mutual agreement to not conduct the project at these two study sites.

Patients

Eligibility. Eligible participants are unselected consecutive adults referred for suspected MDS. Suspicion of MDS relies on medical history and unexplained peripheral blood cytopenia. Peripheral blood cytopenia will be defined using standard laboratory values (20). To be eligible, patients will be required to meet all five inclusion criteria and none of the exclusion criteria. The inclusion criteria are:

- Age at enrollment ≥18 years
- Clinical suspicion of MDS
- Indication for bone marrow examination

- ≥1 peripheral blood cytopenia defined by hemoglobin concentration <12 g/dL for female and <13g/dL for male patients, platelet count <150 x10^9/L, or absolute neutrophil count <1.8 x10^9/L
- Inpatient or outpatient care

The exclusion criteria are:

- Refusal to participate
- History of or active documented MDS
- Enrollment in intensive or critical care unit
- Individuals protected by French regulation (incarcerated as prisoner, unability to understand research information because of language restriction, dementia, or altered mental status)
- Not affiliated with social security system
- Previous enrollment in the study.

Screening. All consecutive patients referred for suspicion of MDS will be prospectively screened for eligibility. Additionally, research staff will review inclusion and exclusion criteria, using computerized laboratory records on a daily basis.

Recruitment. Participants will be included in the study once all the screening activities have been conducted. A clinical research assistant is dedicated to assist investigators in recruiting patients and collecting baseline characteristics.

Index tests

Independent operators who are blinded to the reference diagnosis will perform flow cytometry analysis of peripheral blood samples, using either Lyotube Stain 468 or laboratory-developed liquid reagent cocktail.

Flow cytometer. The study uses a three-laser, eight-color BD FACSCanto-II™ flow cytometer (BD Biosciences, San José, CA, USA), which is maintained and quality controlled according to the

manufacturer instructions. The project complies with the Franceflow standard operating procedure in order to standardize instrument setup (21). Photomultiplier tubes are adjusted and checked daily using Rainbow Calibration Particles (BD Sphero ™ BD Biosciences). BD™ CompBeads (BD Biosciences) are used to establish the fluorescence compensation matrix.

Blood sample collection. Peripheral blood samples are collected in BD Vacutainer® 5 ml K2E (EDTA) anticoagulant plastic tubes (Ref 368861, BD Diagnostics, Le Pont de Claix Cedex, France), stored at ambient temperature, and processed on the same day of collection.

Lyotube Stain 468. Peripheral blood sample aliquot (50 µL) is stained with Lyotube Stain 468 for 15 min at room temperature in the dark, according to manufacturer's recommendation. The Lyotube Stain 468 contains five fluorochrome-conjugated dried reagents, including CD15-PerCP-Cy5.5 (clone HI98), CD11b-APC (clone D12), CD16-APC-H7 (clone 3G8), CD14-V450 (clone MΦP9), CD45-V500 (clone HI30).

Laboratory-developed test. In parallel, peripheral blood sample aliquot (50 µL) is incubated for 15 min at room temperature in the dark, with a panel of five fluorochrome-conjugated liquid reagents, including CD15-PerCP-Cy5.5 (clone HI98), CD11b-APC (clone D12), CD16-APC-H7 (clone 3G8), CD14-V450 (clone MΦP9), CD45-V500 (clone HI30).

Fixation and permeabilization. The fixation and permeabilization phases for lyophilized and liquid reagent experiments are performed using BD IntraSure™ Kit in three steps with incubation at room temperature in the dark, according to manufacturer's recommendation. Antibodies, Lyotube Stain 468, BD FACS™ Lysing Solution (BD Biosciences, San Jose, CA, USA), and BD IntraSure™ Kit are obtained from BD Biosciences (San Jose, CA, USA).

Data analysis. At least 10,000 neutrophils are acquired and analyzed using BD FACSDiva Software, as previously described (Figure 1). Myeloperoxidase expression in the peripheral blood neutrophil population within an individual subject is expressed as RCV (11). The intra-individual RCV is calculated as the robust standard deviation divided by the median fluorescence intensity. The

robust standard deviation is a function of the deviation of individual data points to the median of the study population (11). Intra-individual RCV is expressed as percentage and reflects the variability in myeloperoxidase expression in the peripheral blood neutrophil population within an individual subject (11).

Reproducibility assessment. Intra- and inter-assay precision and unprocessed specimen stability will be assessed according to current guidelines (22-24). Reproducibility will be quantified using coefficient of variation, computed as the standard deviation multiplied by 100 and divided by the mean. To assess intra-assay precision, blood samples will be obtained from five individuals (24). Each sample will be assayed in triplicate in a single analytical run by the same operator (22, 24). To assess inter-assay precision, a single blood sample will be assayed by five different operators using five independent analytical runs on the same day. To assess unprocessed specimen stability at room temperature and 4°C, blood samples obtained from 5 individuals will be assayed at baseline, 24 h, 48 h, and 72h (24).

Reference diagnosis

The reference diagnosis of MDS will be established according to current guidelines (5). Cellular morphology and percentage of excess blasts in bone marrow will be evaluated independently by two experienced hematopathologists who are blinded to the index test results. The criteria for MDS diagnosis are 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. Morphologic assessment can be complemented by bone marrow flow cytometric score (25), karyotype, and targeted next-generation sequencing panel of 43 genes, where relevant (1, 5). Consistent with the 2016 revision of the WHO classification (26), MDS subtype categorization is based on the numbers of dysplastic lineages, the percentages of blasts in bone marrow and peripheral blood, the percentages of ring

sideroblasts, and conventional karyotype analysis. Idiopathic cytopenia of uncertain significance (ICUS) is defined by unexplained mild cytopenia for 6 months of follow-up not fulfilling MDS criteria (27).

The criteria for chronic myelomonocytic leukemia (CMML) diagnosis are 1) the presence of persistent peripheral blood monocytosis $\geq 1 \times 10^9$/L and 2) monocytes accounting for more than 10% of the white blood cell differential count (28). Confirmed suspicions of MDS will be categorized, using the original (29) and revised (30) International Prognostic Scoring System (IPSS and IPSS-R), respectively.

Data collection, management, and confidentiality

Data are collected prospectively by the investigator or a designated representative, using a standardized case report form (CRF). Recorded data are listed in Table 1. Final data review will be performed, checking for validity, consistency, omission, or any apparent discrepancies prior to locking the database. Access to the study data will be restricted to clinical research assistants, investigators, and data managers.

Table 1. Recorded data.

Characteristics
Gender
Age at enrollment
History of hematological disease
Alternate diagnosis as likely as MDS
History of/ongoing or recent treatment with antimitotic chemotherapy, radiation therapy, immunosuppressive/biotherapy
History of occupational exposure
Complete blood count with reticulocyte count
Peripheral blood biochemical test results (C-reactive protein, creatinine, urea, liver profile [ASAT, ALAT, gamma glutamyl transferase, alkalin phosphatase, bilirubine], TSH, ferritin, haptoglobin, serum B12 vitamin, and serum folate)
Flow cytometry MO1 if CMML suspected
Date of peripheral blood sample
Storage conditions and date of index test processing
Robust coefficient of variation for neutrophil myeloperoxidase expression in peripheral blood, %
Cytomorphological evaluation of bone marrow aspirate
MDS / CMML status according to reference method
MDS subcategorization according to WHO classification
Bone marrow blasts (%)
Bone marrow megakaryocyte dysplasia (%)
Bone marrow erythroid dysplasia (%)
Bone marrow myeloid dysplasia (%)
Bone marrow ring sideroblasts (%)
Bone marrow karyotype
Fluorescence in-situ hybridization (FISH)
Flow cytometric score (Ogata) for low-grade MDS
International prognostic scoring system (IPSS)
Revised International Prognostic Scoring System (IPSS-R)
Next generation sequencing (NGS) panel of 43 genes

(Continued)

Table 1 (Continued).

Characteristics
Alternate diagnosis for unconfirmed cases of MDS
Repeated bone marrow aspirate findings for patients with ICUS or uninterpretable/inconclusive bone marrow aspirate at baseline

Abbreviations: ALAT = alanine transaminase; ASAT = aspartate aminotransferases; CMML = chronic myelomonocytic leukemia; ICUS = idiopathic cytopenia of undetermined significance; MDS = myelodysplastic syndrome; TSH = thyroid-stimulating hormone; WHO = World Health Organization

Outcomes

The primary outcome is the reference diagnosis of MDS or CMML established by bone marrow examination by two independent experienced hematopathologists blinded to the index test results. Disagreements will be solved by a third hematopathologist. Repeated bone marrow aspirate within 6 to 12 months of enrollment will be proposed to patients with ICUS (27) or inconclusive or uninterpretable bone marrow examination at baseline.

The secondary outcomes include intra-laboratory coefficient of variation for quantifying intra- and inter-assay precision; relative change from baseline, expressed in percentage, for assessing unprocessed specimen stability; and negative predictive value point estimates along with 95% confidence interval (CI) for intra-individual RCV of peripheral blood neutrophil myeloperoxidase expression. Although inter-laboratory coefficient of variation is a prespecified secondary outcome, it cannot be evaluated in this study which is conducted at a single site.

Sample size

Assuming an area under the ROC curve point estimate of 0.90, we estimated that an effective sample size of 82 participants with a 22% prevalence of MDS would provide a precision of ±0.10 (95% CI

ranging from 0.80 to 1.00). Anticipating a 20% rate of uninterpretable or inconclusive bone marrow aspirates, 21 additional patients will be recruited, leading to an overall sample size of 103 patients. The sample size was estimated with PASS version 15 (NCSS, LLC. Kaysville, Utah, USA).

Statistical analysis

A statistical analysis plan (SAP) will be developed prior to database lock and reviewed by the principal investigator and an independent statistician (31). Statistical analysis will be performed by a statistician in accordance with the SAP. Any post-hoc or unplanned analyses not specified in the SAP will be clearly identified as such in the final statistical report and manuscripts for publication.

The analytical sample will consist of all patients who have been included in the study. A *Standards for Reporting Diagnostic Accuracy* (STARD) statement style flow-chart will present graphically patient flow throughout the study (32). Descriptive summary statistics will be used for reporting continuous (arithmetic mean and standard deviation or median and 25^{th}-75^{th} percentiles) and categorical (numbers and percentages) variables.

Baseline patient characteristics and peripheral blood markers will be compared according to MDS status, using the χ^2 test, replaced by the Fisher exact test where appropriate, for categorical variables and the Student *t*-test or non-parametric Wilcoxon test for continuous variables. We will assess the independent associations of MDS status with intra-individual RCV for peripheral blood neutrophil myeloperoxidase expression, using multivariable logistic regression. Odds ratio estimates will be adjusted for imbalance in baseline patient characteristics. We will examined trends toward higher intra-individual RCV values across increasing IPSS and IPSS-R categories, using the nonparametric Wilcoxon-type test for trend (33).

We will assess comparative accuracy of intra-individual RCV obtained with Lyotube stain 468 and laboratory-developed tests in discriminating patients with confirmed versus unconfirmed MDS by the area under the ROC curve (34, 35). We will report sensitivity, specificity, positive

predictive value, negative predictive value, and likelihood ratio point estimates along with exact binomial (i.e., Clopper-Pearson) 95% CI for intra-individual RCV with a prespecified threshold of 30.0%. This threshold has been selected because an intra-individual RCV value for neutrophil myeloperoxidase expression lower than 30.0% accurately ruled out MDS, with both sensitivity and negative predictive value estimates of 100%, in previous studies (11, 12).

We will graphically appraise the agreement in continuous intra-individual RCV for Lyotube Stain 468 and laboratory-developed tests by examining a scatterplot of differences versus the means of the two variables with the limit of agreement superimposed (36). We will check for the absence of bias by performing regression analysis of the differences as a function of the means. Intra-class correlation coefficient will be used to quantify absolute agreement in continuous intra-individual RCV (37). We will also estimate Cohen's Kappa coefficient to quantify agreement in binary intra-individual RCV with a threshold of 30.0%.

For transparency purpose, the completeness of study data will be reported for baseline characteristics, index test, and reference standard. Missing values for baseline characteristics will be imputed using multiple imputations in multivariable analysis.

No formal interim analysis is planned in this study. Two-tailed *P*-values less than 0.05 will be considered statistically significant. All statistical analyses will be performed using Stata Special Edition version 16 or higher (Stata Corporation, College Station, TX, USA). Additional software may be used for the production of graphics and for statistical methodology not provided by this software package.

Patients and public involvement statement

A member of the Patient Representative Department at the Université Grenoble Alpes School of Medicine (38) reviewed the study protocol and will be involved in the interpretation and the

dissemination plan of the study results. Patients or their representatives will not be involved in the other aspects of the research project.

ETHICS AND DISSEMINATION

Research ethics approval

An institutional review board (Comité de Protection des Personnes CPP Sud Est III, Lyon, France) reviewed and approved the study protocol and the information form, prior to study initiation.

Protocol amendments

During the conduct of the study, protocol changes are not desirable and will not be made unless new information strongly suggests that such changes would strengthen the scientific validity of the findings. If substantive modifications are necessary that may impact on the study conduct or results, including changes of study objectives, eligibility criteria, data collection methods, variable definitions or significant administrative aspects, they will require a formal amendment to the protocol. Minor corrections or clarifications that have no effect on the way the study is to be conducted will be documented in a memorandum.

Protocol registration

The present protocol has been registered with the clinicaltrials.gov registry (NCT04399018, first posted on May 22, 2020). Recorded information will be updated on a regular basis.

Consent to participate

According to French regulations, the consent to participate is sought under a regime of "non-opposition" (opt-out): after appropriate written information is delivered, data are collected except in the case of opposition from the patient.

Dissemination policy

Efforts will be made to reduce the interval between data collection completion and the release of the primary study results. We expect that 6 months will be necessary to compile the primary study results before manuscript submission to an appropriate journal. All publications will comply with the STARD statement (32). A publication committee will review all manuscripts and abstracts for accuracy, quality, scientific priority, and style prior to submission (39). All investigators and subinvestigators that have actively participated in the study will be listed at the end of all manuscripts if this can be arranged with the publisher. Authors' names will be listed in order of contribution. Assistance for preparing and editing manuscripts (e.g., English language revision) provided by professional medical writers will be acknowledged. In accordance with French regulation, study participants will be provided with the overall study results on request to the principal investigator.

No later than 3 years after final acceptance of the primary study paper, a completely deidentified data set will be available for sharing purpose, on reasonable request to the principal investigator. Individual participant data that underlie the results reported in the published articles (i.e., main text, tables, figures, and appendices) will be supplied to researchers who submit a methodologically sound proposal.

DISCUSSION

This study will estimate accuracy attributes of intra-individual RCV using the BD Lyotube Stain 468 for the diagnosis of MDS. Previous studies have shown that laboratory-developed tests for quantifying intra-individual RCV of neutrophil myeloperoxidase expression in peripheral blood has sufficient sensitivity and negative predictive value to safely rule out MDS on their own (11, 12). This project will provide additional evidence on whether a single-use tube of lyophilized reagents is amenable to standardization in high-volume clinical flow cytometry laboratories, without

deteriorating diagnostic accuracy compared with its laboratory-developed liquid reagent counterpart (16). Ultimately, the BD Lyotube Stain 468 would have the potential to accelerate the diagnostic work-up for patients with suspected MDS and hasten their access to investigations for alternate diagnoses. Although a formal cost-effectiveness analysis is not in the scope of this project, we anticipate comparable unit costs for BD Lyotube Stain 468 (22 € per test) and liquid antibody cocktail (21 € per test) and the potential for volume-based cost saving with BD Lyotube Stain 468.

Our study has several strengths. First, adequate diagnostic reference of MDS will be used, with independent hematopathologists performing cytomorphological evaluation of bone marrow blinded to index test results. Second, the potential for spectrum bias will be minimized by enrolling unselected consecutive patients referred for suspected MDS and using broad inclusion criteria (40). Third, a prespecified threshold for intra-individual RCV (i.e., 30%) will be used to prevent optimistic diagnostic accuracy estimates (40).

The limitations of our study should be acknowledged. First, our study will be conducted at a single hospital laboratory and our findings may not apply to other settings. Although the original test based on liquid reagent cocktails showed satisfactory reproducibility estimates across operators, instrument setup procedures, and laboratories (11), inter-laboratory reproducibility for BD Lyotube stain 468 will deserve further investigation before promoting its use. Second, conventional cytogenetics and molecular profiling will not be available for all study participants since the reference diagnosis of MDS relies primarily on cytomorphological evaluation of bone marrow aspirate. Although gene sequencing is not required according to current guidelines (26), it may simplify the differential diagnosis of MDS (1, 41). Yet, many hospitals do not have extensive access to next generation sequencing analysis (41, 42). In the present study, indication of next generation sequencing analysis is restricted to challenging suspicions of MDS for which the detection of somatic mutations could help the diagnosis or prognosis assessment.

To conclude, the MPO-MDS-Develop study will provide evidence on diagnostic accuracy of intra-individual RCV using BD Lyotube stain 468 before implementing prospective management studies or randomized controlled trials designed to evaluate processes of care, short- and long-term patient outcomes, and resource utilization for ruling out MDS into routine practice.

ACKNOWLEDGMENTS:

The authors thank Drs. Claude Eric Bulabois, Clara Mariette, Martin Carré, Stéphane Courby, Anne Thiebaut-Bertrand, Rémy Gressin, Lysiane Molina for being involved in patient recruitment. The authors are indebted to Laure Dusset, Ghislaine Del-Vecchio, Richard Di Schiena, Claire Gasquez, Frédérique Martinez and Karine Nicolino for technical assistance.

AUTHOR CONTRIBUTIONS

Tatiana Raskovalova, Laura Scheffen, Marie-Christine Jacob, and José Labarère developed the protocol and drafted the initial manuscript.

Nicolas Gonnet provided technical and administrative support.

Simon Chevalier, Sylvie Tondeur, Bénédicte Bulabois, Mathieu Meunier, Gautier Szymanski, Frédéric Garban, Christine Lefebvre, Charlotte Planta, Chantal Dumestre-Perard, Raymond Merle, and Sophie Park contributed to critical revision of the manuscript.

José Labarère provided statistical expertise.

All authors critically reviewed the protocol and approved submission of the final manuscript.

FUNDING

This study is supported by Centre Hospitalier Universitaire Grenoble Alpes grant number 38RC19.425. The statistical analysis will be developed within the Grenoble Alpes Data Institute, which is supported by the French National Research Agency under the “Investissements d’avenir” program (ANR-15-IDEX-02). Becton Dickinson Biosciences provided flow cytometry tube of lyophilized reagents (BD Lyotube Stain 468) and antibodies free of charge.

COMPETING INTEREST STATEMENT:

None declared

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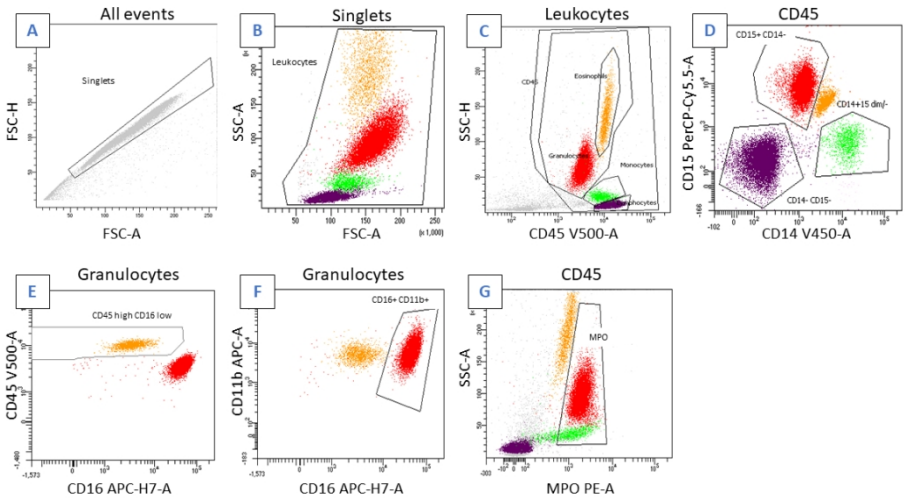
FIGURE CAPTION LIST

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



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338x190mm (96 x 96 DPI)

BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-065850.R1
Article Type:	Protocol
Date Submitted by the Author:	31-Aug-2022
Complete List of Authors:	Raskovalova, Tatiana; Grenoble Alpes University Hospital, Laboratoire d'immunologie; Université Grenoble Alpes, Institute for Advanced Biosciences (IAB), INSERM U1209, CNRS UMR 5309 Scheffen, Laura; Grenoble Alpes University Hospital, Laboratoire d'immunologie Jacob, Marie-Christine; Grenoble Alpes University Hospital, Laboratoire d'immunologie; Université Grenoble Alpes, Institute for Advanced Biosciences (IAB), INSERM U1209, CNRS UMR 5309 Chevalier, Simon; Grenoble Alpes University Hospital, Laboratoire d'Hématologie Biologique Tondeur, Sylvie; Grenoble Alpes University Hospital, Laboratoire d'Hématologie Biologique Bulabois, Bénédicte; Grenoble Alpes University Hospital, Laboratoire d'Hématologie Biologique Meunier, Mathieu; Grenoble Alpes University Hospital, Clinique Universitaire d'Hématologie; Université Grenoble Alpes, Institute for Advanced Biosciences (IAB), INSERM U1209, CNRS UMR 5309 Szymanski, Gautier; Grenoble Alpes University Hospital, Laboratoire d'Hématologie Biologique Lefebvre, Christine; Grenoble Alpes University Hospital, Laboratoire d'Hématologie Biologique Planta, Charlotte; Grenoble Alpes University Hospital, Laboratoire d'immunologie Dumestre-Perard, Chantal; Grenoble Alpes University Hospital, Laboratoire d'immunologie Gonnet, Nicolas; Grenoble Alpes University Hospital, CIC 1406, INSERM Garban, Frédéric; Grenoble Alpes University Hospital, Clinique Universitaire d'Hématologie; Université Grenoble Alpes, TIMC, UMR 5525, CNRS Merle, Raymond; Université Grenoble Alpes, Département Universitaire des Patients Grenoble Alpes Park, Sophie; Grenoble Alpes University Hospital, Clinique Universitaire d'Hématologie; Université Grenoble Alpes, Institute for Advanced Biosciences (IAB), INSERM U1209, CNRS UMR 5309 Labarère, José; Grenoble Alpes University Hospital, Clinical epidemiology unit; Université Grenoble Alpes, TIMC, UMR 5525, CNRS
Primary Subject	Haematology (incl blood transfusion)

Heading:	
Secondary Subject Heading:	Diagnostics, Geriatric medicine, Immunology (including allergy)
Keywords:	Leukaemia < HAEMATOLOGY, IMMUNOLOGY, STATISTICS & RESEARCH METHODS

Flow cytometry lyophilized-reagent tube for quantifying peripheral blood neutrophil myeloperoxidase expression in myelodysplastic syndromes (MPO-MDS-Develop): protocol for a diagnostic accuracy study

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Word count: 3994

Abstract: 274

Tables: 1

Figures: 1

References: 43

ABSTRACT

Introduction: Suspicion of myelodysplastic syndromes (MDS) is the most common reason for bone marrow aspirate in elderly patients. Peripheral blood neutrophil myeloperoxidase expression quantified by flow cytometric analysis might rule out MDS for up to 35% of patients referred for suspected disease, without requiring bone marrow aspiration. Yet laboratory-developed liquid antibody cocktails have practical limitations, because of lack of standardization and poor stability. This research project aims to estimate the level of agreement and comparative accuracy between a single-use flow cytometry tube of lyophilized reagents (BD Lyotube Stain 468) and its laboratory-developed liquid reagent counterpart in quantifying peripheral blood neutrophil myeloperoxidase expression, among adult patients referred for suspected MDS.

Methods and analysis: The MPO-MDS-Develop project is a cross-sectional diagnostic accuracy study of two index tests by comparison with a reference standard in consecutive unselected adult patients conducted at a single university hospital. Flow cytometry analysis of peripheral blood samples will be performed by independent operators blinded to the reference diagnosis, using either Lyotube Stain 468 or laboratory-developed liquid reagent cocktail. The reference diagnosis of MDS will be established by cytomorphological evaluation of bone marrow aspirate by two independent hematopathologists blinded to the index test results. Morphologic assessment will be complemented by bone marrow flow cytometric score, karyotype, and targeted next-generation sequencing panel of 43 genes, where relevant. The target sample size is 103 patients.

Ethics and dissemination: An institutional review board (Comité de Protection des Personnes Sud Est III, Lyon, France) approved the protocol prior to study initiation (reference number: 2020-028-B). Participants will be recruited using an opt-out approach. Efforts will be made to release the primary results within 6 months of study completion.

Study registration number: ClinicalTrials.gov, NCT04399018.

Keywords: Myelodysplastic syndromes; Flow cytometry; Diagnosis; Neutrophils; ROC curve.

ARTICLE SUMMARY

Strengths and limitations of this study

- Adequate diagnostic reference of myelodysplastic syndromes will be used, with independent hematopathologists performing cytomorphological evaluation of bone marrow blinded to index test results.
- The potential for spectrum bias will be minimized by enrolling unselected consecutive patients.
- A prespecified threshold will be used to prevent optimistic diagnostic accuracy estimates.
- Conventional cytogenetics and molecular profiling will not be available for all participants.
- This study will be conducted at a single hospital laboratory and the findings may not apply to other settings.

INTRODUCTION

Background

Myelodysplastic syndromes (MDS) encompass a heterogeneous group of clonal bone marrow neoplasms, with a median age at diagnosis of 70 years (1). MDS are characterized by recurrent cytogenetic and molecular abnormalities, morphologic dysplasia for one or more hematopoietic cell lineage, and ineffective haematopoiesis (1, 2). Patients with MDS have poor prognosis, due to peripheral blood cytopenia-related complications and progression to acute myeloid leukaemia (3, 4).

Cytomorphological evaluation of bone marrow is the reference standard for the diagnosis of MDS and may be complemented by information obtained from conventional cytogenetic, flow cytometry, and molecular profiling analysis (5). Hence suspicion of MDS is the most common reason for bone marrow aspiration in older patients with persistent peripheral blood cytopenia of unclear aetiology. However, many patients are exposed to unnecessary bone marrow aspiration-related discomfort and harm (6-8) because of the relatively low prevalence of disease among subjects who are referred for suspected MDS (9).

Peripheral blood neutrophil myeloperoxidase expression quantified by flow cytometric analysis has the potential to rule out MDS without requiring invasive bone marrow aspiration (10). Myeloperoxidase is an enzyme synthetized during myeloid differentiation and constitutes the major component of neutrophil azurophilic granules (11). Its cytoplasmic expression is associated with degranulation of mature granulocytes (12), a classical dysplastic feature of MDS (13). Using a retrospective case-control study design, we reported that the intra-individual robust coefficient of variation (RCV) for peripheral blood neutrophil myeloperoxidase expression discriminated MDS cases and healthy controls, with an area under the receiver operating characteristic (ROC) curve estimate of 0.94 (95% confidence interval [CI], 0.86–0.97) (14). In two prospective studies, intra-individual RCV values lower than 30% accurately rule out MDS, with 100% sensitivity and 100% negative predictive value estimates, suggesting that flow cytometric analysis of peripheral blood

neutrophil myeloperoxidase expression might obviate the need for bone marrow aspirate for 29–35% of patients referred for suspected MDS (14, 15). Yet this laboratory-developed test has practical limitations for routine application in busy clinical flow cytometry laboratories. Indeed, the preparation of liquid antibody cocktail within a laboratory is a time-consuming process (16), requires specific expertise (16), lacks standardization (17), is prone to errors (18), and faces decreased stability of tandem dyes (19, 20).

The use of premade stable standardized reagent panels may address the issues of reliability and efficiency inherent to laboratory-developed tests (19). Lyophilisation is a method which has been used to stabilize premixed multicolour reagent cocktails within flow cytometry tubes (Lyotube), for various routine clinical applications (19). Lyophilised reagent cocktails show stability at room temperature for 12 to 18 months (19). They provide a simplified way of handling complex multicolour flow cytometry assays, with performance comparable to reference liquid cocktails (19). Indeed, the use of lyophilised reagent cocktails reduces batch-to-batch variations, prevents multiple sources of errors, and saves resources (16, 19). Additionally, standardization of reagent panels facilitates inter-laboratory comparisons and centralized interpretations of flow cytometric data (21).

In order to optimize the workflow for quantifying peripheral blood neutrophil myeloperoxidase expression in suspected MDS, BD Bioscience has manufactured a 5-color lyophilized cocktail, which consists of a single-use customized freeze-dried cocktail within a standard 12 × 75 mm polystyrene flow cytometry tube (BD Lyotube Stain 468). It contains five reagents, including CD15-PerCP-Cy5.5 (clone HI98), CD11b-APC (clone D12), CD16-APC-H7 (clone 3G8), CD14-V450 (clone МΦР9), CD45-V500 (clone HI30).

This protocol describes the rationale for the ongoing MPO-MDS-Develop study, explains how the index tests and the reference method are implemented, how data collection is conducted, and how the results are analyzed and interpreted. This study enrolled participants from 27 July 2020 to 30 September 2021, with an anticipated study completion date of 31 December 2023.

Research hypothesis

The primary hypothesis guiding the project is that an approach based on a single-use flow cytometry tube of lyophilized reagents for quantifying peripheral blood neutrophil myeloperoxidase expression can accurately rule out MDS, with sensitivity and negative predictive value estimates approaching 100%. The secondary hypotheses are that quantification of peripheral blood neutrophil myeloperoxidase expression using a flow cytometry tube of lyophilized reagents yields 1) a high level of intra-laboratory reproducibility, 2) a substantial level of agreement with laboratory-developed liquid reagent cocktail, and 3) and comparable diagnostic accuracy in discriminating MDS to laboratory-developed liquid reagent cocktail.

Objectives

The broad aim of this study is to estimate the level of agreement and comparative accuracy between BD Lyotube Stain 468 and its laboratory-developed liquid reagent counterpart in quantifying peripheral blood neutrophil myeloperoxidase expression, among adult patients referred for suspected MDS. More specifically, the primary objective is to estimate the discriminative accuracy (i.e., area under the ROC curve) for the intra-individual RCV of peripheral blood neutrophil myeloperoxidase expression obtained with BD Lyotube Stain 468 and laboratory developed tests. The secondary objectives are 1) to assess intra- and inter-assay reproducibility, 2) to investigate unprocessed specimen stability at 24 h, 48 h, and 72h under two different storage conditions (i.e., room temperature and 4°C), respectively, and 3) to estimate the negative predictive value with a prespecified threshold of 30.0% for intra-individual RCV of peripheral blood neutrophil myeloperoxidase obtained with BD Lyotube Stain 468.

METHODS AND ANALYSIS

Study design

The MPO-MDS-Develop project is a cross-sectional diagnostic accuracy study of two index tests by comparison with a reference standard in consecutive unselected adult patients referred for suspected disease (22). No specific intervention is assigned to participants. All diagnostic testing, procedures, and medication ordering are performed at the discretion of attending physicians. Compliance with current guidelines is advocated for the reference diagnostic work-up of patients with suspected MDS (1, 5).

Study sites

Although the study was planned to be conducted at three university-affiliated hospitals in France, only one site (Grenoble university hospital) has implemented the study protocol and is recruiting patients. The principal investigator at the two other study sites withdrew before the recruitment started, for personal convenience, logistical reasons, or resource shortage. The decision was made by mutual agreement to not conduct the project at these two study sites.

Patients

Eligibility. Eligible participants are unselected consecutive adults referred for suspected MDS. Suspicion of MDS relies on medical history and unexplained peripheral blood cytopenia. Peripheral blood cytopenia will be defined according to current guidelines (23). To be eligible, patients will be required to meet all five inclusion criteria and none of the exclusion criteria. The inclusion criteria are:

- Age at enrolment ≥18 years
- Clinical suspicion of MDS
- Indication for bone marrow examination

- ≥1 peripheral blood cytopenia defined by haemoglobin concentration <12 g/dL for female and <13g/dL for male patients, platelet count <150 x10^9/L, or absolute neutrophil count <1.8 x10^9/L
- Inpatient or outpatient care

The exclusion criteria are:

- Refusal to participate
- History of or active documented MDS
- Enrolment in intensive or critical care unit
- Individuals protected by French regulation (incarcerated as prisoner, inability to understand research information because of language restriction, dementia, or altered mental status)
- Not affiliated with social security system
- Previous enrolment in the study.

Screening. All consecutive patients referred for suspicion of MDS will be prospectively screened for eligibility. Additionally, research staff will review inclusion and exclusion criteria, using computerized laboratory records on a daily basis.

Recruitment. Participants will be included in the study once all the screening activities have been conducted. A clinical research assistant is dedicated to assist investigators in recruiting patients and collecting baseline characteristics.

Index tests

Independent operators who are blinded to the reference diagnosis will perform flow cytometry analysis of peripheral blood samples, using either Lyotube Stain 468 or laboratory-developed liquid reagent cocktail.

Flow cytometer. The study uses a three-laser, eight-colour BD FACSCanto-II™ flow cytometer (BD Biosciences, San José, CA, USA), which is maintained and quality controlled

according to the manufacturer instructions. The project complies with the Franceflow standard operating procedure in order to standardize instrument setup (24). Photomultiplier tubes are adjusted and checked daily using Rainbow Calibration Particles (BD Sphero ™ BD Biosciences). BD™ CompBeads (BD Biosciences) are used to establish the fluorescence compensation matrix.

Blood sample collection. Peripheral blood samples are collected in BD Vacutainer® 5 ml K2E (EDTA) anticoagulant plastic tubes (Ref 368861, BD Diagnostics, Le Pont de Claix Cedex, France), stored at ambient temperature, and processed on the same day of collection.

Lyotube Stain 468. Peripheral blood sample aliquot (50 µL) is stained with Lyotube Stain 468 for 15 min at room temperature in the dark, according to manufacturer's recommendation. The Lyotube Stain 468 contains five fluorochrome-conjugated dried reagents, including CD15-PerCP-Cy5.5 (clone HI98), CD11b-APC (clone D12), CD16-APC-H7 (clone 3G8), CD14-V450 (clone MΦP9), CD45-V500 (clone HI30).

Laboratory-developed test. In parallel, peripheral blood sample aliquot (50 µL) is incubated for 15 min at room temperature in the dark, with a panel of five fluorochrome-conjugated liquid reagents, including CD15-PerCP-Cy5.5 (clone HI98), CD11b-APC (clone D12), CD16-APC-H7 (clone 3G8), CD14-V450 (clone MΦP9), CD45-V500 (clone HI30). A difference with the original test is that BD Lyotube Stain 468 and the liquid laboratory-developed test used for the present study do not include CD64 FITC antibody (clone 10.1) since this reagent does not contribute to the individualization of neutrophils (14, 15).

Fixation and permeabilization. The fixation and permeabilization phases for lyophilized and liquid reagent experiments are performed using BD IntraSure™ Kit in three steps with incubation at room temperature in the dark, according to manufacturer's recommendation. 10 µL anti-myeloperoxidase antibody (MPO-PE, clone 5B8) is added during the permeabilization phase. Antibodies, Lyotube Stain 468, BD FACS™ Lysing Solution (BD Biosciences, San Jose, CA, USA), and BD IntraSure™ Kit are obtained from BD Biosciences (San Jose, CA, USA).

Data analysis. At least 10,000 neutrophils are acquired and analysed using BD FACSDiva Software, as previously described (Figure 1). Myeloperoxidase expression in the peripheral blood neutrophil population within an individual subject is expressed as RCV (14). The intra-individual RCV is calculated as the robust standard deviation divided by the median fluorescence intensity. The robust standard deviation is a function of the deviation of individual data points to the median of the study population (14). Intra-individual RCV is expressed as percentage and reflects the variability in myeloperoxidase expression in the peripheral blood neutrophil population within an individual subject (14).

Reproducibility assessment. Intra- and inter-assay precision and unprocessed specimen stability will be assessed according to current guidelines (25-27). Reproducibility will be quantified using coefficient of variation, computed as the standard deviation multiplied by 100 and divided by the mean. To assess intra-assay precision, blood samples will be obtained from five individuals (27). Each sample will be assayed in triplicate in a single analytical run by the same operator (25, 27). To assess inter-assay precision, a single blood sample will be assayed by five different operators using five independent analytical runs on the same day. To assess unprocessed specimen stability at room temperature and 4°C, blood samples obtained from 5 individuals will be assayed at baseline, 24 h, 48 h, and 72h (27).

Reference diagnosis

The reference diagnosis of MDS will be established according to the 5th edition of the WHO Classification of Haematolymphoid Tumours (23). Cellular morphology and percentage of excess blasts in bone marrow will be evaluated independently by two experienced hematopathologists who are blinded to the index test results. The criteria for MDS diagnosis are 1) the presence of ≥10% dysplastic cells in any hematopoietic lineage, 2) the exclusion of acute myeloid leukaemia (defined by the presence of ≥20% peripheral blood or bone marrow blasts), and 3) the exclusion of reactive

aetiologies of cytopenia and dysplasia. Morphologic assessment can be complemented by bone marrow flow cytometric score (28), karyotype, and targeted next-generation sequencing panel of 43 genes, where relevant (1, 5). Consistent with (23), MDS subtype categorization includes those with defining genetic abnormalities and those morphologically defined. Confirmed suspicions of MDS will be categorized, using the original (29) and revised (30) International Prognostic Scoring System (IPSS and IPSS-R), respectively. Idiopathic cytopenia of uncertain significance (ICUS) is defined by unexplained cytopenia for 6 months of follow-up not fulfilling MDS criteria and with no MDS-related mutations (31). Clonal cytopenia of undetermined significance (CCUS) is defined by unexplained persistent cytopenia not fulfilling MDS criteria but with MDS-related mutations.

The prerequisite criteria for chronic myelomonocytic leukaemia (CMML) diagnosis are 1) the presence of persistent peripheral blood monocytosis $\geq 0.5 \times 10^9$/L and 2) monocytes accounting for more than 10% of the white blood cell differential count (23). Depending on peripheral blood monocytosis, one or more supporting criteria are required among 1) dysplasia involving on or more myeloid lineages, 2) acquired clonal cytogenetic or molecular abnormality, and 3) detection of increased peripheral blood classical monocytes (flow cytometry MO1 >94%) (23).

Data collection, management, and confidentiality

Data are collected prospectively by the investigator or a designated representative, using a standardized case report form (CRF). Recorded data are listed in Table 1. Final data review will be performed, checking for validity, consistency, omission, or any apparent discrepancies prior to locking the database. Access to the study data will be restricted to clinical research assistants, investigators, and data managers.

Table 1. Recorded data

Characteristics
Gender
Age at enrolment
History of haematological disease
Alternate diagnosis as likely as MDS
History of/ongoing or recent treatment with antimitotic chemotherapy, radiation therapy, immunosuppressive/biotherapy
History of occupational exposure
Complete blood count with reticulocyte count
Peripheral blood biochemical test results (C-reactive protein, creatinine, urea, liver profile [ASAT, ALAT, gamma glutamyl transferase, alkaline phosphatase, bilirubin], TSH, ferritin, haptoglobin, serum B12 vitamin, and serum folate)
Flow cytometry MO1 if CMML suspected
Date of peripheral blood sample
Storage conditions and date of index test processing
Robust coefficient of variation for neutrophil myeloperoxidase expression in peripheral blood, %
Cytomorphological evaluation of bone marrow aspirate
MDS / CMML status according to reference method
MDS subcategorization according to WHO classification
Bone marrow blasts (%)
Bone marrow megakaryocyte dysplasia (%)
Bone marrow erythroid dysplasia (%)
Bone marrow myeloid dysplasia (%)
Bone marrow ring sideroblasts (%)
Bone marrow karyotype
Fluorescence in-situ hybridization (FISH)
Flow cytometric score (Ogata) for MDS
International prognostic scoring system (IPSS)
Revised International Prognostic Scoring System (IPSS-R)
Next generation sequencing (NGS) panel of 43 genes

(continued)

Table 1 (continued)

Characteristics
Alternate diagnosis for unconfirmed cases of MDS
Repeated bone marrow aspirate findings for patients with ICUS or uninterpretable/inconclusive bone marrow aspirate at baseline

Abbreviations: ALAT = alanine transaminase; ASAT = aspartate aminotransferases; CMML = chronic myelomonocytic leukaemia; ICUS = idiopathic cytopenia of undetermined significance; MDS = myelodysplastic syndrome; TSH = thyroid-stimulating hormone; WHO = World Health Organization

Outcomes

The primary outcome is the reference diagnosis of MDS or CMML established by bone marrow examination by two independent experienced hematopathologists blinded to the index test results. Disagreements will be solved by a third hematopathologist. Repeated bone marrow aspirate within 6 to 12 months of enrolment will be proposed to patients with ICUS (31) or inconclusive or uninterpretable bone marrow examination at baseline.

The secondary outcomes include intra-laboratory coefficient of variation for quantifying intra- and inter-assay precision; relative change from baseline, expressed in percentage, for assessing unprocessed specimen stability; and negative predictive value point estimates along with 95% CI for intra-individual RCV of peripheral blood neutrophil myeloperoxidase expression. Although inter-laboratory coefficient of variation is a prespecified secondary outcome, it cannot be evaluated in this study which is conducted at a single site.

Sample size

Assuming an area under the ROC curve point estimate of 0.90, we estimated that an effective sample size of 82 participants with a 22% prevalence of MDS would provide a precision of ±0.10 (95% CI

ranging from 0.80 to 1.00). Anticipating a 20% rate of uninterpretable or inconclusive bone marrow aspirates, 21 additional patients will be recruited, leading to an overall sample size of 103 patients. The sample size was estimated with PASS version 15 (NCSS, LLC. Kaysville, Utah, USA).

Statistical analysis

A statistical analysis plan (SAP) will be developed prior to database lock and reviewed by the principal investigator and an independent statistician (32). Statistical analysis will be performed by a statistician in accordance with the SAP.

The analytical sample will consist of all patients who have been included in the study. A *Standards for Reporting Diagnostic Accuracy* (STARD) statement style flow-chart will present graphically patient flow throughout the study (33). Descriptive summary statistics will be used for reporting continuous (arithmetic mean and standard deviation or median and 25^{th}-75^{th} percentiles) and categorical (numbers and percentages) variables.

Baseline patient characteristics and peripheral blood markers will be compared according to MDS status, using the χ^2 test, replaced by the Fisher exact test where appropriate, for categorical variables and the Student *t*-test or non-parametric Wilcoxon test for continuous variables. We will assess the independent associations of MDS status with intra-individual RCV for peripheral blood neutrophil myeloperoxidase expression, using multivariable logistic regression. Odds ratio estimates will be adjusted for imbalance in baseline patient characteristics. We will examined trends toward higher intra-individual RCV values across increasing IPSS and IPSS-R categories, using the nonparametric Wilcoxon-type test for trend (34).

We will assess comparative accuracy of intra-individual RCV obtained with Lyotube stain 468 and laboratory-developed tests in discriminating patients with confirmed versus unconfirmed MDS by the area under the ROC curve (35, 36). We will report sensitivity, specificity, positive predictive value, negative predictive value, and likelihood ratio point estimates along with exact

binomial (i.e., Clopper-Pearson) 95% CI for intra-individual RCV with a prespecified threshold of 30.0%. This threshold is derived from two previous studies showing that intra-individual RCV values for neutrophil myeloperoxidase expression lower than 30.0% accurately ruled out MDS, with both sensitivity and negative predictive value estimates of 100% (14, 15).

We will graphically appraise the agreement in continuous intra-individual RCV for Lyotube Stain 468 and laboratory-developed tests by examining a scatterplot of differences versus the means of the two variables with the limit of agreement superimposed (37). We will check for the absence of bias by performing regression analysis of the differences as a function of the means. Intra-class correlation coefficient will be used to quantify absolute agreement in continuous intra-individual RCV (38). We will also estimate Cohen's Kappa coefficient to quantify agreement in binary intra-individual RCV with a threshold of 30.0%.

For transparency purpose, the completeness of study data will be reported for baseline characteristics, index test, and reference standard. Missing values for baseline characteristics will be imputed using multiple imputations in multivariable analysis.

No formal interim analysis is planned in this study. Two-tailed *P*-values less than 0.05 will be considered statistically significant. All statistical analyses will be performed using Stata Special Edition version 16 or higher (Stata Corporation, College Station, TX, USA). Additional software may be used for the production of graphics and for statistical methodology not provided by this software package.

Patients and public involvement

A member of the Patient Representative Department at the Université Grenoble Alpes School of Medicine (39) reviewed the study protocol and will be involved in the interpretation and the dissemination plan of the study results. Patients or their representatives will not be involved in the other aspects of the research project.

ETHICS AND DISSEMINATION

Research ethics approval

An institutional review board (Comité de Protection des Personnes CPP Sud Est III, Lyon, France) reviewed and approved the study protocol and the information form, prior to study initiation (reference number for ethics approval: 2020-028-B).

Protocol amendments

During the conduct of the study, protocol changes are not desirable and will not be made unless new information strongly suggests that such changes would strengthen the scientific validity of the findings. If substantive modifications are necessary that may impact on the study conduct or results, including changes of study objectives, eligibility criteria, data collection methods, variable definitions or significant administrative aspects, they will require a formal amendment to the protocol. Minor corrections or clarifications that have no effect on the way the study is to be conducted will be documented in a memorandum.

Protocol registration

The present protocol has been registered with the clinicaltrials.gov registry (NCT04399018, first posted on May 22, 2020). Recorded information will be updated on a regular basis.

Consent to participate

According to French regulations, the consent to participate is sought under a regime of "non-opposition" (opt-out): after appropriate written information is delivered, data are collected except in the case of opposition from the patient.

Dissemination policy

Efforts will be made to reduce the interval between data collection completion and the release of the primary study results. We expect that 6 months will be necessary to compile the primary study results before manuscript submission to an appropriate journal. All publications will comply with the STARD statement (33). A publication committee will review all manuscripts and abstracts for accuracy, quality, scientific priority, and style prior to submission (40). All investigators and sub-investigators that have actively participated in the study will be listed at the end of all manuscripts if this can be arranged with the publisher. Authors' names will be listed in order of contribution. Assistance for preparing and editing manuscripts (e.g., English language revision) provided by professional medical writers will be acknowledged. In accordance with French regulation, study participants will be provided with the overall study results on request to the principal investigator.

No later than 3 years after final acceptance of the primary study paper, a completely deidentified data set will be available for sharing purpose, on reasonable request to the principal investigator. Individual participant data that underlie the results reported in the published articles (i.e., main text, tables, figures, and appendices) will be supplied to researchers who submit a methodologically sound proposal.

DISCUSSION

This study will estimate accuracy attributes of intra-individual RCV using the BD Lyotube Stain 468 for the diagnosis of MDS. Previous studies have shown that laboratory-developed tests for quantifying intra-individual RCV of neutrophil myeloperoxidase expression in peripheral blood has sufficient sensitivity and negative predictive value to safely rule out MDS on their own (14, 15). This project will provide additional evidence on whether a single-use tube of lyophilized reagents is amenable to standardization in high-volume clinical flow cytometry laboratories, without deteriorating diagnostic accuracy compared with its laboratory-developed liquid reagent counterpart

(19). Ultimately, the BD Lyotube Stain 468 would have the potential to accelerate the diagnostic work-up for patients with suspected MDS and hasten their access to investigations for alternate diagnoses. Although a formal cost-effectiveness analysis is not in the scope of this project, we anticipate comparable unit costs for BD Lyotube Stain 468 (22 € per test) and liquid antibody cocktail (21 € per test) and the potential for volume-based cost saving with BD Lyotube Stain 468.

Our study has several strengths. First, adequate diagnostic reference of MDS will be used, with independent hematopathologists performing cytomorphological evaluation of bone marrow blinded to index test results. Second, the potential for spectrum bias will be minimized by enrolling unselected consecutive patients referred for suspected MDS and using broad inclusion criteria (41). Third, a prespecified threshold for intra-individual RCV (i.e., 30%) will be used to prevent optimistic diagnostic accuracy estimates (41).

The limitations of our study should be acknowledged. First, our study will be conducted at a single hospital laboratory and our findings may not apply to other settings. Although the original test based on liquid reagent cocktails showed satisfactory reproducibility estimates across operators, instrument setup procedures, and laboratories (14), inter-laboratory reproducibility for BD Lyotube stain 468 will deserve further investigation before promoting its use. Second, conventional cytogenetics and molecular profiling will not be available for all study participants since the reference diagnosis of MDS relies primarily on cytomorphological evaluation of bone marrow aspirate. Although gene sequencing is not required according to current guidelines, it may simplify the differential diagnosis of MDS (1, 42). Yet, many hospitals do not have extensive access to next generation sequencing analysis (42, 43). In the present study, indication of next generation sequencing analysis is restricted to challenging suspicions of MDS for which the detection of somatic mutations could help the diagnosis or prognosis assessment.

To conclude, the MPO-MDS-Develop study will provide evidence on diagnostic accuracy of intra-individual RCV using BD Lyotube stain 468 before implementing prospective management

studies or randomized controlled trials designed to evaluate processes of care, short- and long-term patient outcomes, and resource utilization for ruling out MDS into routine practice.

Acknowledgments

The authors thank Drs. Claude Eric Bulabois, Clara Mariette, Martin Carré, Stéphane Courby, Anne Thiebaut-Bertrand, Rémy Gressin, Lysiane Molina for being involved in patient recruitment. The authors are indebted to Laure Dusset, Ghislaine Del-Vecchio, Richard Di Schiena, Claire Gasquez, Frédérique Martinez and Karine Nicolino for technical assistance.

Contributors

Tatiana Raskovalova, Laura Scheffen, Marie-Christine Jacob, and José Labarère developed the protocol and drafted the initial manuscript. Nicolas Gonnet provided technical and administrative support. Simon Chevalier, Sylvie Tondeur, Bénédicte Bulabois, Mathieu Meunier, Gautier Szymanski, Frédéric Garban, Christine Lefebvre, Charlotte Planta, Chantal Dumestre-Perard, Raymond Merle, and Sophie Park contributed to critical revision of the manuscript. José Labarère provided statistical expertise. All authors critically reviewed the protocol and approved submission of the final manuscript.

Funding

This study is supported by Centre Hospitalier Universitaire Grenoble Alpes grant number 38RC19.425. The statistical analysis will be developed within the Grenoble Alpes Data Institute, which is supported by the French National Research Agency under the "Investissements d'avenir" program (ANR-15-IDEX-02). Becton Dickinson Biosciences provided flow cytometry tube of lyophilized reagents (BD Lyotube Stain 468) and antibodies free of charge.

Competing interests

None declared.

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FIGURE TITLES

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

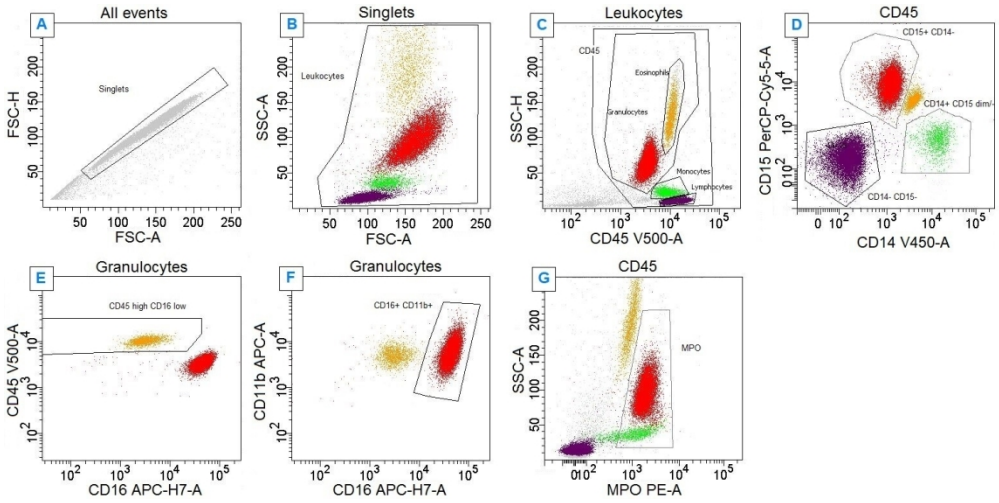

Figure 1. Gating strategy for quantifying peripheral blood neutrophil myeloperoxidase expression.

131x64mm (300 x 300 DPI)