


BMJ Open Flow cytometry lyophilised-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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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 standardisation and poor stability. This research project aims to estimate the level of agreement and comparative accuracy between a single-use flow cytometry tube of lyophilised 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 haematopathologists 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

STRENGTHS AND LIMITATIONS OF THIS STUDY

- ⇒ Adequate diagnostic reference of myelodysplastic syndromes will be used, with independent haematopathologists performing cytomorphological evaluation of bone marrow blinded to index test results.
- ⇒ The potential for spectrum bias will be minimised 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.

to release the primary results within 6 months of study completion.

Trial registration number NCT04399018.

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 characterised by recurrent cytogenetic and molecular abnormalities, morphologic dysplasia for one or more haematopoietic 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 synthesised 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 intraindividual 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% CI, 0.86 to 0.97).¹⁴ In two prospective studies, intraindividual 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 standardisation,¹⁷ is prone to errors¹⁸ and faces decreased stability of tandem dyes.^{19 20}

The use of premade stable standardised 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 stabilise premixed multicolour reagent cocktails within flow cytometry tubes (Lyotube), for various routine clinical applications.¹⁹ Lyophilised reagent cocktails show stability at room temperature for 12–18 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, standardisation of reagent panels facilitates interlaboratory comparisons and centralised interpretations of flow cytometric data.²¹

In order to optimise the workflow for quantifying peripheral blood neutrophil myeloperoxidase expression in suspected MDS, BD Bioscience has manufactured a 5-colour lyophilised cocktail, which consists of

a single-use customised 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) and 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 analysed 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 lyophilised 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 lyophilised reagents yields (1) a high level of intralaboratory reproducibility, (2) a substantial level of agreement with laboratory-developed liquid reagent cocktail and (3) 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 (ie, area under the ROC curve) for the intraindividual 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-assay and interassay reproducibility, (2) to investigate unprocessed specimen stability at 24, 48 and 72 hours under two different storage conditions (ie, room temperature and 4°C), respectively and (3) to estimate the negative predictive value with a prespecified threshold of 30.0% for intraindividual 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.¹⁵

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 as follows:

- ▶ Age at enrolment ≥ 18 years.
- ▶ Clinical suspicion of MDS.
- ▶ Indication for bone marrow examination.
- ▶ ≥ 1 peripheral blood cytopenia defined by haemoglobin concentration < 120 g/L for female and < 130 g/L for male patients, platelet count $< 150 \times 10^9$ /L or absolute neutrophil count $< 1.8 \times 10^9$ /L.
- ▶ Inpatient or outpatient care.

The exclusion criteria are as follows:

- ▶ 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 computerised 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 France-flow standard operating procedure in order to standardise 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 5mL 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 μ L) 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 M Φ P9) CD45-V500 (clone HI30).

Laboratory-developed test

In parallel, peripheral blood sample aliquot (50 μ L) 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 M Φ P9) and 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 individualisation of neutrophils.^{14 15}

Fixation and permeabilisation

The fixation and permeabilisation phases for lyophilised 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 μ L antimyeloperoxidase antibody (MPO-PE, clone 5B8) is added during the permeabilisation 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 intraindividual RCV is calculated as the robust SD divided by the median fluorescence

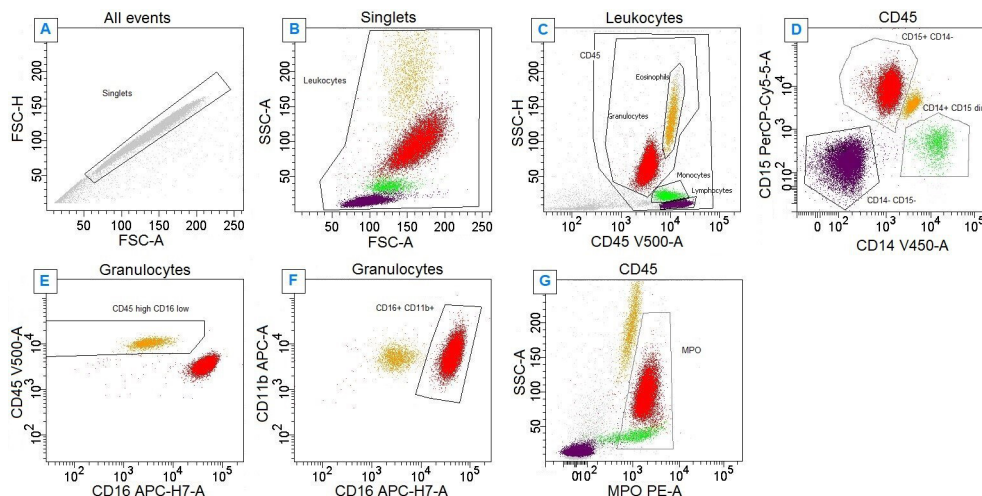


Figure 1 Gating strategy for quantifying peripheral blood neutrophil myeloperoxidase expression. Panel A: Singlet gate. Panel B: FSC–SSC leukocytes. Panel C: CD45 positive gate. Panel D: Population of granulocytes (CD15+ CD14-), monocytes (CD14+CD15dim/-), and lymphocytes (CD14-CD15-). Panel E: Population of eosinophils (CD45high+ CD16low). Panel F: Population of mature neutrophils (CD16+CD11b+). Panel G: MPO expression of mature neutrophils. Mature neutrophils were individualized by Boolean intersections as follows: [CD15+ CD14-] (D) AND NOT [CD45high CD16 low] (E) AND NOT [CD14+ CD15low/-] (D) AND NOT [CD15- CD14-] (D) AND [CD16+ CD11b+] (F). CD, cluster of differentiation; FSC-H, forward scatter height; FSC-A, forward scatter area; MPO, myeloperoxidase; SSC-A, side scatter area; SSC-H, side scatter height.

intensity. The robust SD is a function of the deviation of individual data points to the median of the study population.¹⁴ Intraindividual 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-assay and interassay 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 SD 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 five individuals will be assayed at baseline, 24 hours, 48 hours and 72 hours.²⁷

Reference diagnosis

The reference diagnosis of MDS will be established according to the fifth 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 haematopathologists who are blinded to the index test results. The criteria for MDS diagnosis are (1) the presence of $\geq 10\%$ dysplastic cells in any haematopoietic lineage, (2) the exclusion of acute myeloid leukaemia (defined by the presence of $\geq 20\%$ 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} MDS subtype categorisation includes those with defining genetic abnormalities and those morphologically defined.²³ Confirmed suspicions of MDS will be categorised, using the original²⁹ and revised³⁰ 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 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 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 standardised case report form. Recorded data are listed in [box 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

Box 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 antimetabolic 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 subcategorisation 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 hybridisation.
 Flow cytometric score (Ogata) for MDS.
 International Prognostic Scoring System.
 Revised International Prognostic Scoring System.
 Next-generation sequencing panel of 43 genes.
 Alternate diagnosis for unconfirmed cases of MDS.
 Repeated bone marrow aspirate findings for patients with ICUS or uninterpretable/inconclusive bone marrow aspirate at baseline.
 ALAT, alanine transaminase; ASAT, aspartate aminotransferases; CMML, chronic myelomonocytic leukaemia; ICUS, idiopathic cytopenia of undetermined significance; MDS, myelodysplastic syndrome; TSH, thyroid-stimulating hormone.

restricted to clinical research assistants, investigators and data managers.

Outcomes

The primary outcome is the reference diagnosis of MDS or CMML established by bone marrow examination by two independent experienced haematopathologists blinded to the index test results. Disagreements will be solved by a third haematopathologist. Repeated bone marrow aspirate within 6–12 months of enrolment will be proposed to patients with ICUS³¹ or inconclusive or uninterpretable bone marrow examination at baseline.

The secondary outcomes include intralaboratory coefficient of variation for quantifying intra-assay 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 intraindividual RCV of peripheral

blood neutrophil myeloperoxidase expression. Although interlaboratory 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 V.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 SD or median and 25th–75th 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 intraindividual RCV for peripheral blood neutrophil myeloperoxidase expression, using multivariable logistic regression. OR estimates will be adjusted for imbalance in baseline patient characteristics. We will examine trends towards higher intraindividual RCV values across increasing IPSS and IPSS-R categories, using the non-parametric Wilcoxon-type test for trend.³⁴

We will assess comparative accuracy of intraindividual 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 (ie, Clopper-Pearson) 95% CI for intraindividual RCV with a prespecified threshold of 30.0%. This threshold is derived from two previous studies showing that intraindividual 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 intraindividual 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. Intraclass correlation coefficient will be used to quantify absolute agreement in continuous intraindividual RCV.³⁸ We will also estimate Cohen's Kappa coefficient to quantify agreement in binary intraindividual 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 V.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 Medicine³⁹ 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

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 subinvestigators who 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 (eg, 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 (ie, 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 intraindividual RCV using the BD Lyotube Stain 468 for the diagnosis of MDS. Previous studies have shown that laboratory-developed tests for quantifying intraindividual 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 lyophilised reagents is amenable to standardisation 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 haematopathologists performing cytomorphological

evaluation of bone marrow blinded to index test results. Second, the potential for spectrum bias will be minimised by enrolling unselected consecutive patients referred for suspected MDS and using broad inclusion criteria.⁴¹ Third, a prespecified threshold for intraindividual RCV (ie, 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 interlaboratory 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.¹⁴² 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 intraindividual RCV using BD Lyotube stain 468 before implementing prospective management studies or randomised controlled trials designed to evaluate processes of care, short-term and long-term patient outcomes, and resource utilisation for ruling out MDS into routine practice.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting or dissemination plans of this research. Refer to the Methods section for further details.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

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REFERENCES

- Cazzola M. Myelodysplastic syndromes. *N Engl J Med* 2020;383:1358–74.
- Gondek LP, DeZern AE. Assessing clonal haematopoiesis: clinical burdens and benefits of diagnosing myelodysplastic syndrome precursor states. *Lancet Haematol* 2020;7:e73–81.
- Nachtkamp K, Stark R, Strupp C, et al. Causes of death in 2877 patients with myelodysplastic syndromes. *Ann Hematol* 2016;95:937–44.
- Zeidan AM, Shallis RM, Wang R, et al. Epidemiology of myelodysplastic syndromes: why characterizing the beast is a prerequisite to taming it. *Blood Rev* 2019;34:1–15.
- Fenaux P, Haase D, Santini V, et al. Myelodysplastic syndromes: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up[†]. *Ann Oncol* 2021;32:142–56.
- Bain BJ. Morbidity associated with bone marrow aspiration and trephine biopsy - a review of UK data for 2004. *Haematologica* 2006;91:1293–4.
- Gendron N, Zia Chahabi S, Poenou G, et al. Pain assessment and factors influencing pain during bone marrow aspiration: a prospective study. *PLoS One* 2019;14:e0221534.
- Vanhelleputte P, Nijs K, Delforge M, et al. Pain during bone marrow aspiration: prevalence and prevention. *J Pain Symptom Manage* 2003;26:860–6.
- Buckstein R, Jang K, Friedlich J, et al. Estimating the prevalence of myelodysplastic syndromes in patients with unexplained cytopenias: a retrospective study of 322 bone marrows. *Leuk Res* 2009;33:1313–8.
- Raskovalova T, Jacob M-C, Park S. Myelodysplastic syndromes. *N Engl J Med* 2020;383:2590.
- McKenna E, Mhaonaigh AU, Wubben R, et al. Neutrophils: need for standardized nomenclature. *Front Immunol* 2021;12:602963.
- Bedouhène S, Dang PM-C, Hurtado-Nedelec M, et al. Neutrophil degranulation of azurophil and specific granules. *Methods Mol Biol* 2020;2087:215–22.
- Germsing U, Strupp C, Giagounidis A, et al. Evaluation of dysplasia through detailed cytomorphology in 3156 patients from the Düsseldorf registry on myelodysplastic syndromes. *Leuk Res* 2012;36:727–34.
- Raskovalova T, Berger MG, Jacob M-C, et al. Flow cytometric analysis of neutrophil myeloperoxidase expression in peripheral blood for ruling out myelodysplastic syndromes: a diagnostic accuracy study. *Haematologica* 2019;104:2382–90.
- Raskovalova T, Jacob M-C, Bulaboïs C-E, et al. Flow cytometric analysis of peripheral blood neutrophil myeloperoxidase expression for ruling out myelodysplastic syndromes: a prospective validation study. *Ann Hematol* 2021;100:1149–58.
- Hedley BD, Cheng G, Luider J, et al. Initial flow cytometric evaluation of the Clearlab lymphoid screen. *Cytometry B Clin Cytom* 2018;94:863–9.
- Bouriche L, Bernot D, Nivaggioni V, et al. Detection of minimal residual disease in B cell acute lymphoblastic leukemia using an eight-color tube with dried antibody reagents. *Cytometry B Clin Cytom* 2019;96:158–63.



- 18 van der Velden VHJ, Flores-Montero J, Perez-Andres M, *et al.* Optimization and testing of dried antibody tube: the EuroFlow LST and PIDOT tubes as examples. *J Immunol Methods* 2019;475:112287.
- 19 Chan RC-F, Kotner JS, Chuang CM-H, *et al.* Stabilization of pre-optimized multicolor antibody cocktails for flow cytometry applications. *Cytometry B Clin Cytom* 2017;92:508–24.
- 20 Johansson U, Macey M. Tandem dyes: stability in cocktails and compensation considerations. *Cytometry B Clin Cytom* 2014;86:n/a–74.
- 21 Hedley BD, Cheng G, Keeney M, *et al.* A multicenter study evaluation of the ClearLLab 10C panels. *Cytometry B Clin Cytom* 2021;100:225–34.
- 22 Sackett DL, Haynes RB. The architecture of diagnostic research. *BMJ* 2002;324:539–41.
- 23 Khoury JD, Solary E, Abla O, *et al.* The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. *Leukemia* 2022;36:1703–19.
- 24 Solly F, Angelot-Delettre F, Ticchioni M, *et al.* Standardization of flow cytometric immunophenotyping for hematological malignancies: the FranceFlow group experience. *Cytometry A* 2019;95:1008–18.
- 25 Davis BH, McLaren CE, Carcio AJ, *et al.* Determination of optimal replicate number for validation of imprecision using fluorescence cell-based assays: proposed practical method. *Cytometry B Clin Cytom* 2013;84:329–37.
- 26 Ticchioni M, Brouzes C, Durrieu F, *et al.* Acceptable "real-life" variability for lymphocyte counts by flow cytometry. *Cytometry B Clin Cytom* 2019;96:379–388.
- 27 Wood B, Jevremovic D, Béné MC, *et al.* Validation of cell-based fluorescence assays: practice guidelines from the ICSH and ICCS - part V - assay performance criteria. *Cytometry B Clin Cytom* 2013;84:315–23.
- 28 Della Porta MG, Picone C, Pascutto C, *et al.* Multicenter validation of a reproducible flow cytometric score for the diagnosis of low-grade myelodysplastic syndromes: results of a European LeukemiaNET study. *Haematologica* 2012;97:1209–17.
- 29 Greenberg P, Cox C, LeBeau MM, *et al.* International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood* 1997;89:2079–88.
- 30 Greenberg PL, Tuechler H, Schanz J, *et al.* Revised international prognostic scoring system for myelodysplastic syndromes. *Blood* 2012;120:2454–65.
- 31 Valent P. ICUS, IDUS, CHIP and CCUS: diagnostic criteria, separation from MDS and clinical implications. *Pathobiology* 2019;86:30–8.
- 32 Gamble C, Krishan A, Stocken D, *et al.* Guidelines for the content of statistical analysis plans in clinical trials. *JAMA* 2017;318:2337–43.
- 33 Bossuyt PM, Reitsma JB, Bruns DE, *et al.* STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *BMJ* 2015;351:h5527.
- 34 Cuzick J. A Wilcoxon-type test for trend. *Stat Med* 1985;4:87–90.
- 35 Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* 1982;143:29–36.
- 36 Hanley JA, McNeil BJ. A method of comparing the areas under receiver operating characteristic curves derived from the same cases. *Radiology* 1983;148:839–43.
- 37 Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307–10.
- 38 Kottner J, Audigé L, Brorson S, *et al.* Guidelines for reporting reliability and agreement studies (GRRAS) were proposed. *J Clin Epidemiol* 2011;64:96–106.
- 39 Merle R, Pépin J-L, Palombi O, *et al.* Successful training of patients to intervene in health education and clinical research at Grenoble patient school. *J Patient Exp* 2022;9:237437352110698.
- 40 Chan A-W, Tetzlaff JM, Gøtzsche PC, *et al.* SPIRIT 2013 explanation and elaboration: guidance for protocols of clinical trials. *BMJ* 2013;346:e7586.
- 41 Whiting PF, Rutjes AWS, Westwood ME, *et al.* QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. *Ann Intern Med* 2011;155:529–36.
- 42 Estey E, Hasserrjian RP, Döhner H. Distinguishing AML from MDS: a fixed blast percentage may no longer be optimal. *Blood* 2022;139:323–32.
- 43 Vantuyghem S, Peterlin P, Thépot S, *et al.* Diagnosis and prognosis are supported by integrated assessment of next-generation sequencing in chronic myeloid malignancies. A real-life study. *Haematologica* 2021;106:701–7.