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ORIGINAL ARTICLE

CLINICAL CYTOMETRY WILEY

Clinical application of flow cytometry in patients with unexplained cytopenia and suspected myelodysplastic syndrome: A report of the European LeukemiaNet International MDS-Flow Cytometry Working Group

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Abstract

This article discusses the rationale for inclusion of flow cytometry (FCM) in the diagnostic investigation and evaluation of cytopenias of uncertain origin and suspected myelodysplastic syndromes (MDS) by the European LeukemiaNet international MDS Flow Working Group (ELN *i*MDS Flow WG). The WHO 2016 classification recognizes that FCM contributes to the diagnosis of MDS and may be useful for prognostication, prediction, and evaluation of response to therapy and follow-up of MDS patients.

KEYWORDS
consensus, ELN, flow cytometry, myelodysplastic syndromes, standardization

1 | INTRODUCTION

The current gold standard for the diagnosis of myelodysplastic syndromes (MDS) is the detection of dysplastic features in the erythroid, myeloid, monocytic, and megakaryocytic cell lineages in the peripheral blood (PB) and bone marrow (BM) by cytomorphology in patients with one or more cytopenias, in the absence of other causes. Dysplasia is not specific for MDS, may be very mild and in the context of a normal karyotype or absence of other detectable genetic variants, the diagnosis of MDS is often challenging. Despite the extensive heterogeneity of cytogenetic and genomic variants associated with MDS (over 100 well described abnormalities) (Haferlach et al., 2014; Papaemmanuil et al., 2013; Schanz et al., 2011; Schanz et al., 2012) there is a commonality of abnormal phenotypic manifestations of cell proliferation, maturation and function which are detectable both by cytomorphology and immunophenotyping. Flow cytometry (FCM) can detect immunophenotypic aberrancies in the precursor populations and in the differentiation of erythroid, myeloid, and monocytic populations by subtle changes in antigen expression in the BM of patients with MDS (Loken et al., 2008; Loken & Wells, 2008; Van De Loosdrecht et al., 2009). Current key diagnostic criteria include the presence of dysplasia in >10% of cells within one or more cell lineages, the presence of >15% ring sideroblasts, the percentage of blasts in the PB and BM, the presence of cytogenetic abnormalities typical for MDS and presence of a mutation in the splicing factor SF3B1 in combination with >5% ring sideroblasts (Arber et al., 2016; Malcovati et al., 2020; Valent et al., 2007). Cytomorphological, histological and cytogenetic results are used to classify patients in different disease subtypes as described by the WHO-classification (Arber et al., 2016; Malcovati et al., 2013). The most recent WHO classification (2016) removed the terms 'refractory anemia' and 'refractory cytopenia', and replaced them by MDS, with 'unilineage' dysplasia or 'multilineage' dysplasia to provide a more accurate and comprehensive description (Arber et al., 2016).

Although the WHO criteria appear straightforward, the diagnosis of MDS can still be challenging, especially in lower-risk subtypes of MDS with no excess of blasts. Evaluation of dysplasia by cytomorphology is difficult and may be mistaken for and/or be associated with other non-neoplastic and malignant hematological disorders. Dyserythropoiesis and multilineage dysplasia may occur in non-neoplastic disorders, such as vitamin B12, folic acid, iron or copper deficiency, some heavy metal poisoning and HIV infection and can be drug induced or an in-vitro artifact from prolonged sample exposure to EDTA anticoagulant before slide preparation and staining. The WHO 2016 highlights the difficulties in assessing dyserythropoiesis and its lack of diagnostic specificity for myeloid neoplasms. When MDS is suspected, unilineage dyserythropoiesis must be assessed in the clinical context, with other associated features and awareness of other possible non-clonal diagnoses (Malcovati et al., 2013). In addition, it is well documented that inter-observer reproducibility of qualitative changes and

quantification of dysplasia is variable (Font et al., 2015). The WHO 2016 also states that if FCM is used to assess BM cells, the percentages of myeloid progenitor cells are informative but cannot replace a differential blast count on smears by routine cytomorphology. However, it accepts that abnormal immunophenotypes of $CD34^+$ cells may provide additional evidence of dysplasia. Aberrant (erythroid and myeloid) differentiation patterns may indicate dysplasia and aberrant findings in at least three tested features (not specified) and at least two cell compartments are highly associated with MDS or MDS/MPN. Finally, if FCM is not diagnostic in the absence of conclusive morphological and/or cytogenetic criteria, follow-up with repeated BM studies is recommended (Westers, Ireland, et al., 2012). We suggest that these observations and comments confirm the need for integration of FCM with routine cytomorphology results, which are complementary, although cytomorphology is still considered the current defined gold standard for the diagnosis of MDS.

1.1 | Idiopathic cytopenia, clonal cytopenia, and dysplasia of undetermined significance

The causes of cytopenias and dysplasia are not always revealed by standard diagnostic approaches. Patients with an unexplained cytopenia and without a clonal genetic marker are now categorized as idiopathic cytopenia of undetermined significance (ICUS) (Steensma, 2019; Valent et al., 2017). Patients with an unexplained cytopenia but with a clonal genetic abnormality are categorized as clonal cytopenia of undetermined significance (CCUS). Patients with apparent dysplasia by cytomorphological evaluation, but no cytopenia or other abnormalities in laboratory workup are termed idiopathic dysplasia of undetermined significance (IDUS). In patients with ICUS or CCUS, FCM may add phenotypic evidence of lineage dysplasia undetectable by cytomorphology. Although the exact role of FCM is not yet established, it is likely that FCM abnormalities may reveal a possible pre-MDS immunophenotype in these cases. In addition, the absence of FCM abnormalities in these cases may be instrumental in excluding MDS (Cremers et al., 2016; Kern et al., 2013).

2 | FLOW CYTOMETRY IN MYELODYSPLASTIC SYNDROMES

Improved diagnostic tools that add 'certainty of diagnosis' are needed to distinguish MDS from non-clonal causes of cytopenia and dysplasia. Furthermore, with the increasing availability of disease-modifying therapies, there is a growing need for standardized reassessment technologies that predict and monitor response to therapy. In the published European LeukemiaNet guidelines, FCM is added as a recommended tool for diagnostic purposes, if performed according to standard European LeukemiaNet guidelines (Della Porta et al., 2012; Porwit et al., 2014; Van De Loosdrecht et al., 2013; Van De Loosdrecht & Westers, 2013; Westers, Ireland, et al., 2012; Westers, van der Velden, et al., 2012). Using FCM, phenotypic and immunologic characteristics of hematopoietic cells can be evaluated. FCM evaluates the myeloid progenitor cells, B cell progenitors, maturing myeloid, monocytic, and erythroid cell subsets in BM aspirates. In 2009, the ELN *i*MDS Flow WG published its first guidelines of recommended methods for cell sampling, handling and processing (Westers, Ireland, et al., 2012). The same working group also described a minimum consensus panel of antibody combinations to study the myeloid, monocytic and erythroid cell subsets, which was followed by an update of recommendations in 2014 (Porwit et al., 2014). There is no single specific FCM marker for MDS, but the identification of multiple immunophenotypic abnormalities suggests the presence of an underlying clonal disorder. Knowledge of age-matched normal maturation patterns and expression levels of lineage identifying markers is therefore mandatory to enable evaluation of BM samples by FCM for possible MDS. Multiple MDS-FCM scoring systems have described the evaluation of different markers and different scoring strategies (Van De Loosdrecht & Westers, 2013; Westers, Ireland, et al., 2012). In this Special Issue of Cytometry Part B, Clinical Cytometry, the ELN *i*MDS Flow WG publishes updated recommendations for the analysis of patients with suspected MDS (Porwit et al., 2021; Van Der Velden et al., 2021). Pre-analytical, analytical and technical considerations are discussed including examples of antibody panels.

The most commonly used diagnostic strategy is a simple MDS-FCM scoring system (often referred to as the Ogata score) based on four parameters (Della Porta et al., 2012; Ogata et al., 2006; Porwit et al., 2021). Each abnormality scores 1 point and scores of ≥2 points are indicative of MDS. Most MDS-FCM scoring systems have been developed and validated in MDS patient cohorts, comparing groups of MDS patients with non-clonal cytopenias and normal controls (Cremers et al., 2016). Using this approach, FCM can identify different categories within separate WHO subcategories in MDS and may detect multilineage dysplasia in patients where standard cytomorphology has only observed unilineage dysplasia. Until recently, the evaluation of the erythroid cell lineage by FCM was challenging due to lack of validated markers. However, previous publications confirmed by the ELN *i*MDS Flow WG have demonstrated that CD45, CD36, CD71, CD105, CD117, and CD235a, can be used to study the erythroid lineage and derive an erythroid score (RED score) that evaluates specific erythroid FCM markers and hemoglobin levels (Mathis et al., 2013; Westers et al., 2017). An integrated FCM (*i*FS) comprising the Ogata score with additional defined dysplastic features of the immature, maturing granulocytic and monocytic compartments as well as erythroid cell analysis can now be derived (Cremers et al., 2017). In this issue of Cytometry Part B Clinical Cytometry, Oelschlaegel et al., show that the *i*FS is currently the best scoring system for MDS diagnosis with respect to diagnostic accuracy (Oelschlaegel et al., 2021). Analysis of megakaryocytes in FCM encounters technical challenges due to their size, scarcity and fragility and the megakaryocytic lineage is therefore best assessed by cytomorphology in combination with histology. In cases when cytomorphology did not meet the criteria for MDS but FCM features were consistent with MDS, approximately 50% of patients eventually evolved to overt MDS (Cremers et al., 2016; Kern et al., 2013; Kern et al., 2015). In contrast, only a small subset of patients with no

diagnostic results by FCM developed MDS (Kern et al., 2013). Finally, FCM has an important role in confirming the presence of neoplastic mast cells which may change the diagnosis, e.g., from MDS to systemic mastocytosis with an associated hematological neoplasm i.e., SM-MDS, or SM-CMML (Valent et al., 2019).

3 | FLOW CYTOMETRIC QUANTIFICATION AND IMMUNOPHENOTYPIC CHARACTERIZATION OF MYELOID PROGENITOR CELLS

FCM is an important complementary tool for both quantification and immunophenotypic characterization of myeloid progenitor cells and should be correlated with conventional cytomorphology. Various groups have shown numerical and immunophenotypic aberrancies of myeloid progenitor cells are helpful in differentiating myelodysplasia from non-neoplastic cytopenias with good correlation between FCM and cytomorphology for the total blast count and with respect to critical blast thresholds (Font et al., 2018; Kern et al., 2010; Sandes et al., 2013). FCM analysis of myeloid progenitor cells is an ideal tool for the diagnosis of MDS for several reasons: 1) myeloid progenitors are expected to be affected in almost all cases given the clonal stem cell nature of the disease; 2) both frequency and immunophenotypic abnormalities can be assessed by FCM; and 3) the myeloid progenitor cell immunophenotype is likely less affected by the coexistence of infection and/or inflammation than that of maturing cells and may therefore be more specific for the diagnosis of MDS (Behbehani et al., 2020; Kern et al., 2010). Aberrant patterns of the myeloid progenitor cell compartment include increased frequency of myeloid progenitor cells, decreased frequency of early B-cell progenitors, aberrant or asynchronous antigen expression of maturity markers and expression of lineage infidelity markers. Myeloid progenitor cell quantification is a critical diagnostic and prognostic marker in MDS, and FCM may become essential in situations where morphologic evaluation is difficult or unavailable. In routine clinical practice, combined cytomorphology and multiparameter FCM allow quick investigation to either rule out or to confirm a diagnosis of suspected MDS or acute myeloid leukemia (AML), particularly if smears are of poor quality and/or hemodiluted. Even when good quality BM smears are available, the reproducible identification and quantification of blast cells by cytomorphology can be a challenge. The FCM detection of immunophenotypic aberrancies in progenitor cells in cytopenic patients, with evidence of dysplasia but without blast increase, is a strong indicator of clonal disease, which may aid the selection of subsequent appropriate 'down-stream' diagnostic cytogenetic and molecular investigations for clinical decision-making.

4 | FLOW CYTOMETRY IN CMML

Flow cytometry studies have emerged as an essential diagnostic tool in patients with suspected classical chronic myelomonocytic leukemia (CMML), pre-CMML conditions and special CMML variants (Itzykson et al., 2018; Valent et al., 2019). Therefore, FCM of the PB is recommended in all cases with suspected CMML or a suspected pre-CMML condition. FCM studies are helpful to confirm monocyte and blast cell counts in these patients and to exclude AML. In addition, FCM is useful to confirm the presence of distinct monocyte populations. Monocytes are defined as $CD14^{+}$ cells in these analyses. Based on the expression of CD14 and CD16, monocytes are further divided into classical (cMo) ($CD14^{bright}/CD16^{-}$), intermediate (iMo) ($CD14^{bright}/CD16^{+}$) and non-classical (ncMo) monocytes ($CD14^{dim}/CD16^{+}$). Compared with healthy donors and patients with reactive monocytosis, the percentages of cMo in PB are higher and the percentage of ncMo is lower in patients with CMML (Selimoglu-Buet et al., 2015; Selimoglu-Buet et al., 2017; Vazquez et al., 2018). Using a cutoff value of >94% cMo monocytes by FCM CMML cases can be identified with a sensitivity of 92% and a specificity of 94%. In this Special Issue of Cytometry Part B, Clinical Cytometry, the ELN *i*MDS Flow WG shows the robustness of the monocyte assay with only limited variability of cMo percentages, validates the 94% cutoff value, confirms its high sensitivity and specificity and also confirms the possibility of its use in BM samples (Wagner-Ballon et al., 2021). Moreover, during successful therapy, the distribution of cMo, iMo, and ncMo reverts to a near normal or normal pattern. In cases with suspected CMML and concomitant infection and/or autoinflammation, the cMo fraction may be below 94% (Selimoglu-Buet et al., 2015; Selimoglu-Buet et al., 2017). A low percentage of $SLAN^{+}$ ncMo below 1.7% may add significantly to the accuracy of CMML diagnosis in these cases (Hofer et al., 2019; Tarfi et al., 2020).

5 | IMPLEMENTATION OF FLOW CYTOMETRY IN A DIAGNOSTIC ALGORITHM FOR PATIENTS WITH CYTOPENIA AND SUSPECTED MYELODYSPLASTIC SYNDROMES OR CMML

In the busy clinical and diagnostic laboratory setting, it is common for samples to be referred for 'cytopenia of unknown cause', which has a wide differential diagnosis. Common non-clonal causes of cytopenia such as hemolysis, iron or vitamin deficiencies and immune disorders must be excluded. This has usually been undertaken by the referring clinician, although the results may not all be known at the time of referral. The differential diagnosis of malignant conditions includes lymphoproliferative disorders, plasma cell neoplasia and acute myeloid and lymphoid leukemia as well as MDS in addition to congenital platelet disorders. It is therefore common to initiate a simultaneous array of investigations (see Figure 1). The starting point is usually cytomorphology and FCM. Combined with cytomorphology, FCM is an extremely useful screening tool in acute situations of unknown cytopenias, particularly if smears are of poor quality and lack specific cytomorphology, when MDS criteria are not met or in situations of inter-observer discrepancy between assessors. In addition to an MDS-directed flow panel, a FCM screening tube to detect immunophenotypically aberrant populations of blasts, mature B-, T- and NK-cells and plasma cells is recommended. Preliminary results

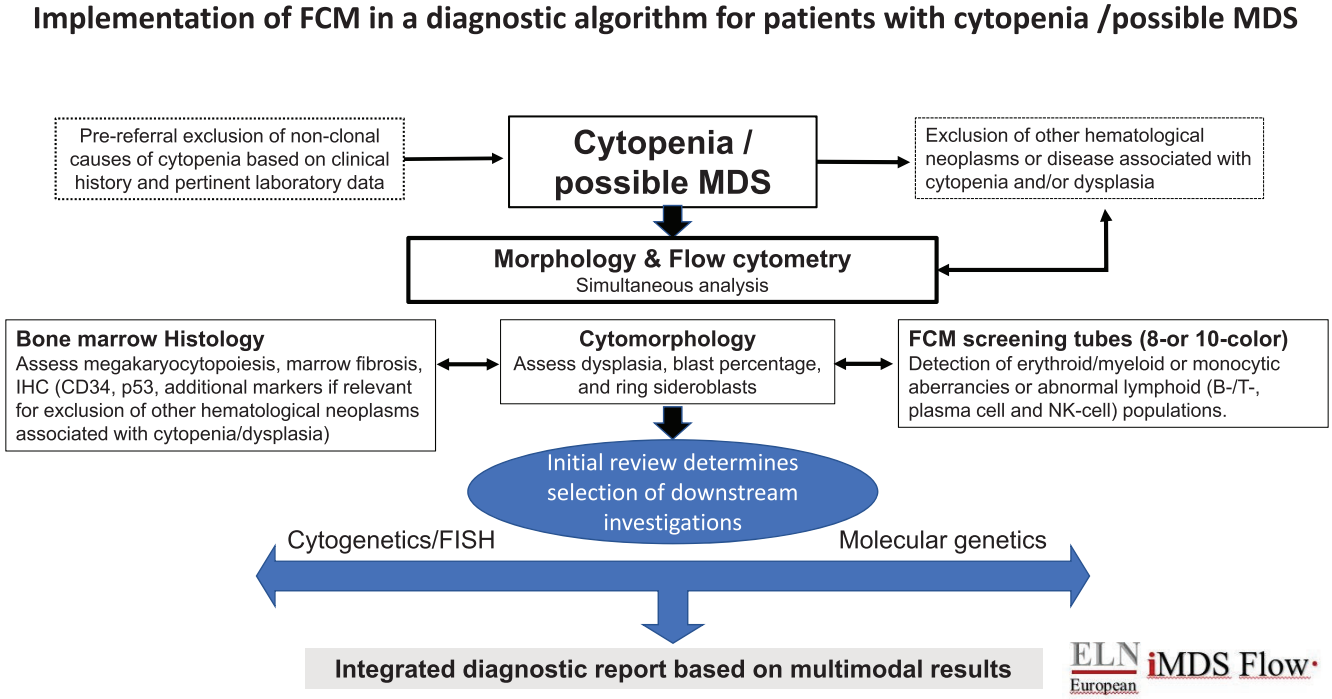


FIGURE 1 Diagnostic algorithm for patients with unexplained cytopenia and possible MDS by the ELN *i*MDS flow WG [Color figure can be viewed at wileyonlinelibrary.com]

from this approach then determine more appropriately directed 'downstream' cytogenetic, FISH or molecular variant investigations. Occasional patients have dual diagnoses of MDS and another clonal hematological malignancy. In other cases, MDS has emerged after treatment of a preceding hematological malignancy and is therapy related MDS with or without residuum or relapse of the original disease (Jonsdottir et al., 2021). Rarely, MDS may be associated with clonal or non-clonal proliferations of NK or T-NK cells. The latter are usually detected by excess large granular lymphocytes on cytomorphology and confirmed and quantified by an appropriate T-cell FCM panel (Bareau et al., 2010; Sauntharajah et al., 2001). Confirmation of this rare entity will direct a different clinical and therapeutic management plan. Depending on the cytomorphology and FCM results, other hematological malignancies should have been excluded. These results should form part of a final integrated report inclusive of histopathology, cytogenetics, and molecular analyses. Figure 1 shows the diagnostic algorithm for patients with unexplained cytopenia and possible MDS by the ELN *i*MDS Flow Working Group. The implementation of this algorithm in daily routine clinical and laboratory practice needs fine-tuning of logistics between laboratories.

6 | FLOW CYTOMETRIC IDENTIFICATION OF IMMUNE CELLS SUCH AS T- AND B-CELLS, MONOCYTES AND DENDRITIC CELLS IN MYELODYSPLASTIC SYNDROMES

Evidence for the role of immune dysregulation, including myeloid-derived inflammation and adaptive immune (lack) response, in the pathogenesis of MDS is increasingly demonstrated (Kordasti et al., 2007; Kotsianidis et al., 2009; Winter et al., 2020). Indeed, the number and functional status of $CD4^+$ and $CD8^+$ T-cells, NK cells, monocytes and dendritic cells are correlated with disease severity (Van Leeuwen-Kerkhoff et al., 2020; Van Leeuwen-Kerkhoff et al., 2021). In addition, an increased number of circulating myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) has been observed during disease progression (Kittang et al., 2016). Moreover, an independent prognostic value of Tregs (peripheral blood) and progenitor B cell (bone marrow) frequencies in patients with lower-risk MDS has been proposed (Kahn et al., 2015). The latter is already included as a single parameter in the Ogata score. Until now, there is no consensus about the analysis of immune anomalies in MDS. The role of (dysplastic) eosinophils and basophils is not yet defined although eosinophilia and/or basophilia may have prognostic implications. Multicenter studies, using agreed FCM panels, are required to better understand the predictive value of T and B cell subpopulations and design future robust diagnostic panels and an "MDS-related immune scoring system".

7 | FLOW CYTOMETRY IN MYELODYSPLASTIC SYNDROMES AND PROGNOSIS

It has been shown that granulocytic and monocytic dysplasia in MDS, identified as immunophenotypic abnormalities by FCM, correlates with the International Prognostic Scoring System (IPSS), the WHO-adjusted prognostic scoring system (WPSS), transfusion dependency

and time-to-progression to advanced MDS/AML as well as with outcome after hematopoietic stem cell transplantation (Alhan et al., 2016; Alhan, Westers, Cremers, et al., 2014; Alhan, Westers, van der Helm, et al., 2014; van de Loosdrecht et al., 2008). Immunophenotypic aberrancies on myeloid progenitors may also have an independent prognostic impact, even if the percentage of BM blasts is below 5%. The IPSS and IPSS-R represent the benchmark for clinical trials and treatment decision making in MDS (Della Porta et al., 2015; Greenberg et al., 2012; Van Spronsen et al., 2016). Currently, the next generation of prognostic scoring systems, i.e., the IPSS-molecular is currently under investigation by the IWG-PM group. It has already been shown that cytogenetic abnormalities typically associated with MDS, such as monosomy 7, del(5q) and complex cytogenetics are correlated with an increased FCM score, whereas chromosomal abnormalities such as trisomy 8, del(20q) and loss of Y, which may also occur in other hematological neoplasms more frequently display a lower FCM score (FCSS according to Wells) (Cutler et al., 2011). This confirms previous data reporting that among patients with lower risk MDS, FCM abnormalities were less prominent in cases of trisomy eight or del(20q). Moreover, the number of FCM aberrancies identified in MDS has been reported to be associated with overall survival (OS) (Alhan et al., 2016; Alhan, Westers, Cremers, et al., 2014; Alhan, Westers, van der Helm, et al., 2014). The FCSS extends the prognostic utility of FCM assessment especially in MDS-MLD. FCM has the advantage of being a rapid tool producing early results, which may further refine diagnostic, prognostic and monitoring models in parallel with the impact of molecular variant analysis as part of a complementary integrated diagnosis and prognostic strategy.

8 | FLOW CYTOMETRY FOR PREDICTING AND MONITORING TREATMENT RESPONSE AND DISEASE PROGRESSION IN MYELODYSPLASTIC SYNDROMES

Immunophenotypically aberrant myeloid progenitors are a key parameter in predicting the MDS response to growth factor treatment (Westers et al., 2010). Patients with low serum erythropoietin (EPO) levels and immunophenotypically normal myeloid progenitors have a high probability (94%) of responding to erythropoiesis stimulating agents (ESA). By contrast, patients with aberrant myeloid progenitors and/or high serum EPO levels have a poorer (11%) response. Similarly, an increase of CD117/c-kit-expressing erythroid precursors predicts ESA response and longer progression free survival (Raimbault et al., 2019). In addition, the degree of phosphorylation of ERK assessed by FCM correlates with response to erythroid stimulating agents and OS in low/int-1 risk MDS (Frisan et al., 2010). In SF3B1variant MDS, the single nucleotide variant is associated with distinct immunophenotypic features, and co-occurrence of both SF3B1 mutation and deletion of chromosome 5q affects the BM immunophenotype. These genotype-immunophenotype associations and immunophenotypic subtypes within SF3B1-MDS provide leads that may further refine therapeutic strategies for this particular MDS subgroup (Duetz, Van Gassen, et al., 2021). Disease monitoring by FCM may be important especially when other disease parameters such as hematological, molecular, and cytogenetic results are normal or uninformative. In MDS with a del(5q) abnormality, it has been shown that an aberrant immunophenotype reverses to normal upon treatment with lenalidomide (Oelschlaegel et al., 2015). Stable or increasing FCM aberrancies during treatment with hypomethylating agents may spare patients from expensive, long-term treatment with ineffective drugs which might otherwise benefit patient survival (Alhan, Westers, Cremers, et al., 2014; Alhan, Westers, van der Helm, et al., 2014; Subirá et al., 2021). Until now, no general recommendations have been made for the use of FCM in routine monitoring of treatment in patients with MDS except for MRD assessments in high-risk MDS according to AML strategies. In line with the latter, FCM may contribute to identify therapeutic targets (CD33, CD47, CD123, CD47, CLL-1, MDR-1, and PD-L1) on the surface of stem and progenitor cells in MDS.

9 | DATA ANALYSIS AND INTERPRETATION

In most clinical laboratories, FCM data are being analyzed manually, best known as "expert gating". While it is still the recommended and an acceptable approach, it has limitations, particularly the subjectivity of population gating strategies. Additionally, with modern flow cytometers, larger antibody panels can be used which makes manual gating very labor-intensive and increases the analytical complexity of the subsequent multidimensional data. There are several dimensionality reductions and clustering methods (i.e., SPADE, viSNE, and FlowSOM) that can be used for a more in-depth analysis of multidimensional FCM data and some of them are designed to simplify bioinformatics approaches for day-to-day use (Duetz et al., 2020; Lhermitte et al., 2018; Lhermitte et al., 2021; Opzoomer et al., 2021; Van Gassen et al., 2015; Vial et al., 2021). Artificial intelligence (AI) approaches are also being used to identify abnormal cell clusters (Duetz, Westers, et al., 2021). While most of these tools are not yet clinically accredited, the ELN *i*MDS Flow WG strongly encourages collaborative validation and subsequent implementation of these tools in clinical FCM laboratories. This strategy may also significantly reduce the costs of FCM by enabling a reduction of laboratory materials and time restraints on expert analysis (Duetz, Westers, et al., 2021).

10 | CONCLUSION

In summary, the working group has agreed that FCM is a rapid tool that complements and adds to routine diagnostic investigations in patients with undiagnosed cytopenias and suspected MDS and/or CMML. In this special issue of Cytometry Part B Clinical Cytometry, a series of case studies is presented to illustrate the role of FCM in the diagnostic workup of MDS (Westers et al., 2021). The value of FCM in assessing the prognosis of MDS beyond that of molecular variant

analysis, is not yet established but FCM may be useful in predicting and monitoring disease during treatment with standard therapeutic regimens such as erythropoiesis stimulating and hypomethylating agents. Repeated FCM assessments are strongly recommended not only in cases such as ICUS and IDUS, but also to monitor the natural course of the disease in patients with untreated low and intermediate-1 risk MDS. The same holds true for patients treated with currently available drugs, preferably within clinical trials as conducted by national and international collaborating groups.

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AUTHOR CONTRIBUTIONS

All persons listed as co-authors contributed to pre-conference and post-conference discussions and actively participated in the Standardization Conferences. All co-authors contributed equally by discussing criteria, standards and recommendations at one or all of the following Working Conferences: 14th/2nd virtual annual ELN MDS Flow Working Conference April 30, 2021; 13th/1st virtual meeting Nov 6, 2020; 12th ELN MDS Flow Working Conference Oct 31-Nov 2, 2019, Nijmegen, The Netherlands; 11th Annual meeting Nov 1–3, 2018, Munich, Germany; 10th Annual meeting, Nov 2–4, 2017, Lund, Sweden; 9th Annual meeting, Oct 27–29, 2016, Paris, France; 8th Annual meeting Oct 29–31, 2015, Athens, Greece. In addition, all persons listed as co-authors provided essential input by drafting parts of the manuscript and by approving the final version of the document.

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