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SPECIAL REPORT

Standardization of flow cytometry in myelodysplastic syndromes: a report from an international consortium and the European LeukemiaNet Working Group

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Flow cytometry (FC) is increasingly recognized as an important tool in the diagnosis and prognosis of myelodysplastic syndromes (MDS). However, validation of current assays and agreement upon the techniques are prerequisites for its widespread acceptance and application in clinical practice. Therefore, a working group was initiated (Amsterdam, 2008) to discuss and propose standards for FC in MDS. In 2009 and 2010, representatives from 23, mainly European, institutes participated in the second and third European LeukemiaNet (ELN) MDS workshops. In the present report, minimal requirements to analyze dysplasia are refined. The proposed core markers should enable a categorization of FC results in cytopenic patients as 'normal', 'suggestive of', or 'diagnostic of' MDS. An FC report should include a description of validated FC abnormalities such as aberrant marker expression on myeloid progenitors and, furthermore, dysgranulopoiesis and/or dysmonocytopoiesis, if at least two abnormalities are evidenced. The working group is dedicated to initiate further studies to establish robust diagnostic and prognostic FC panels in MDS. An ultimate goal is to refine and improve diagnosis and prognostic scoring systems. Finally, the working group stresses that FC should be part of an integrated diagnosis rather than a separate technique.

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INTRODUCTION

Morphology is not always clear-cut in the diagnosis of myelodysplastic syndromes (MDS).[1] In the clinical context, cytogenetics is currently considered as the most important parameter, because the karyotype has diagnostic, prognostic and therapeutic implications. However, especially in cytopenic patients with normal or inconclusive cytogenetics, there is a need for additional diagnostic markers. Analysis by flow cytometry (FC) of bone marrow cells has been introduced as an important co-criterion in the diagnosis of MDS.[2] FC can identify specific aberrations on both immature and maturing compartments among different hematopoietic lineages. The information obtained by FC analysis is moreover clearly complementary to cytomorphology. For instance, abnormal myeloid progenitor cells can be detected in the absence of morphological evidence of increased myeloblasts.[3-5] The first international workshop from the European LeukemiaNet (ELN; WP8 and WP10) on Standardization of FC in MDS (Amsterdam, The Netherlands, 2008) reported the first steps in defining minimal criteria for the diagnosis and prognostic evaluation of MDS.[6] The current World Health Organization (WHO) 2008 recommendations recognize multiple FC aberrancies (>3) in maturation patterns as indicative of MDS.[7] The significance of increased percentages of progenitor cells and expression of CD34 and/or CD117 on these cells is also acknowledged. The task of our working party is to provide laboratories with guidelines to define the precise role of FC in the diagnosis and prognosis of MDS. The major goals of the second and third working conferences were as follows: (a) define the minimal requirements to assess bone marrow dysplasia by FC in immature progenitor cells and in the maturing myelomonocytic lineage in known or suspected MDS; (b) define how these data are

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to be captured, that is, how to focus on the population of interest and how they should be interpreted objectively; (c) consider the specificity of FC analysis in MDS related to a series of other (non) clonal hematological diseases; (d) define the role of FC in the diagnosis and prognosis of MDS related to the currently validated French-American-British, WHO, International Prognostic Scoring System and the WHO-based Prognostic Scoring System, including relevant prognostic cytogenetic and molecular markers.[1]

MINIMAL REQUIREMENTS TO ASSESS DYSPLASIA BY FC

MDS comprises a heterogeneous group of myeloid neoplasms; therefore, no single specific marker can indicate MDS. The presence of multiple aberrancies has been shown to be of higher predictive value for MDS than single aberrancies.[4,8–10] Thus, multiparametric assessment of an accumulation of anomalies might strongly support the diagnosis of MDS by FC. Table 1 summarizes the group's current agreement on the minimal FC parameters that are considered most relevant for the diagnosis of MDS. Analysis of these parameters is thought to be achievable in any center, using, as a minimal requirement, four-color FC. The FC analyses should at least focus on the immature myeloid progenitor cells. Among the parameters to measure are their percentage, evaluation of their plasticity ($CD34^+CD45^+$) profile in combination with forward and sideward light scatter (FSC and SSC, respectively), expression of CD117, and expression of maturation and lineage infidelity markers. As such, FC allows to differentiate normal and abnormal progenitor cells. All participants agreed on analyzing maturing neutrophils for SSC and the patterns of CD13/CD11b/CD16 expression. Analysis of monocytes was also considered valuable. The way in which this can be accomplished with respect to the subpopulations of interest is discussed below. Next to that, enumeration of progenitor B cells and analysis of erythroid differentiation might add valuable information, particularly within low-grade MDS.

Little applications have been described for the FC analysis of the megakaryocytic lineage. Standard morphology and immunohistochemistry are therefore still recommended for the analysis of dysplasia in this cell lineage.[11]

IMPLEMENTATION OF MULTIPARAMETER FC ANALYSIS IN MDS

FC can be applied for the diagnosis, prediction of prognosis and monitoring of response towards disease-modifying drugs in MDS.[2] This approach requires consensus regarding sample processing, antibody combinations and data analysis. The optimal methods for processing and handling samples for FC in MDS were previously published by the ELN Working Group in 2009[6] and are summarized in Table 2. Table 3 presents the core markers that should enable to evidence the abnormal expression of specified antigens and the relation between antigens of relevance in specific cell populations. CD45 acts as a backbone marker in all combinations discussed. There are no restrictions regarding fluorochrome conjugates and antibody clones. However, caution

Table 1. Recommended minimal requirements to assess dysplasia by flow cytometry

Bone marrow subset	*Recommended analyses*	*Aberrancy*
Immature myeloid and monocytic progenitors	Percentage of cells in nucleated cell fraction[a]	Increased percentage
	Expression of CD45	Lack of/decreased/increased
	Expression of CD34	Lack of/decreased/increased
	Expression of CD117	Homogenous under/overexpression
	Expression of HLA-DR	Lack of/increased expression
	Expression of CD13 and CD33	Lack of/decreased/increased
	Asynchronous expression of CD11b, CD15	Presence of mature markers
	Expression of CD5, CD7, CD19, CD56[b]	Presence of lineage infidelity markers
Maturing neutrophils	Percentage of cells as ratio to lymphocytes	Decreased
	SSC as ratio vs SSC of lymphocytes	Decreased
	Relationship of CD13 and CD11b	Altered pattern[c]
	Relationship of CD13 and CD16	Altered pattern[c]
	Relationship of CD15 and CD10	Altered pattern[c]; for example, lack of CD10 on mature neutrophils
Monocytes	Percentage of cells	Decreased/increased
	Distribution of maturation stages	Shift towards immature
	Relationship of HLA-DR and CD11b	Altered pattern[c]
	Relationship of CD36 and CD14	Altered pattern[c]
	Expression of CD13 and CD33	(Homogenous) under/overexpression
	Expression of CD56[b]	Presence of lineage infidelity marker
Progenitor B cells	Enumeration as fraction of total CD34+ based on CD45/CD34/SSC in combination with CD10 or CD19	Decreased or absent
Erythroid compartment[d]	Percentage of nucleated erythroid cells	Increased
	Relationship CD71 and CD235a	Altered pattern[c]
	Expression of CD71	Decreased
	Expression of CD36	Decreased
	Percentage of CD117-positive precursors	Increased

[a]Discrepancies in counts between several definitions indicate aberrancies. [b]To be used with caution, as CD56 can be upregulated upon activation, be aware of normal cut-off values (also in stressed marrow). [c]Altered patterns can include altered distribution of maturation stages and/or altered expression levels of indicated antigens. [d]Under evaluation. Examples of several flow cytometric aberrancies in myelodysplastic syndrome can be found on the European LeukemiaNet website: www.leukemia-net.org.

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Table 2. Processing of bone marrow samples for flow cytometric analysis of dysplasia

	Recommendations[a]
Anticoagulant in bone marrow sample	Preferably heparin (alternative: EDTA)
Storage temperature	Room temperature
Processing time	<24 h
Pre-treatment before staining (optional[b])	Bulk-lysis of erythrocytes by ammoniumchloride (without a fixative)
Washing buffer (optional)	Phosphate-buffered saline with 0.5% bovine or human serum albumin
Staining	Minimal four-color flow cytometry Minimum of 500 000 cells incubated per antibody combination
Preservation of stable antigen expression after staining	Paraformaldehyde fixation (0.5%)
Gating for immature myeloid cells	Called progenitors rather than blasts
Doublets	To be excluded by a FSC height/FSC area graph
Degranulated neutrophils	Assessed by comparing their SSC (in linear or logarithmic scale) to that of lymphocytes; of note, these cells will overlap with progenitors and/or monocytes and must be identified by appropriate markers
Glycosyl-phospatidyl-inositol-anchored antigens (i.e., CD14, CD16, CD24)	Expression will be altered if PNH is present, but this does not impair MDS diagnosis

Abbreviations: ELN, European LeukemiaNet; MDS, myelodysplastic syndrome; PNH, paroxysmal nocturnal hemoglobinuria. [a]Summary of ELN recommendations as published in 2009.[6] [b]A stain/lysis no wash procedure can also be used, though care must be taken when interpreting expression of markers that are present on mature erythrocytes, such as glycophorin A.

Table 3. Proposed core markers in the analysis of dysplasia by flow cytometry

General core markers	*Erythroid*	*Progenitors*	*Maturing neutrophils*	*Monocytes*
CD45	CD45	CD45	CD45	CD45
–	CD71	–	–	–
–	CD235a	–	–	–
CD34	–	CD34	CD34	CD34
CD117	CD117	CD117	CD117	CD117
HLA-DR	–	HLA-DR	HLA-DR	HLA-DR
CD11b	–	CD11b	CD11b	CD11b
CD13	–	CD13	CD13	CD13
CD16	–	–	CD16	CD16
CD33	–	–	CD33	CD33
CD14	–	–	CD14	CD14
–	CD36	–	–	CD36
–	–	–	CD64	CD64
CD7	–	CD7	–	–
CD56	–	CD56	CD56	CD56
CD19	–	CD19	–	–
–	–	CD5	–	–
–	–	–	–	CD2
–	–	CD15	CD15	–
–	–	–	CD10	–

Abbreviation: MDS, myelodysplastic syndrome. CD2 and CD25 can be added to analyze aberrant mast cells. In about 1–3% of all patients with MDS, a co-existing systemic mastocytosis (SM) is found.[61] The diagnosis then changes to SM-MDS as per the WHO criteria. Aberrant mast cells in SM are CD117^{bright} and aberrantly express CD2 and/or CD25.

is needed when using antibodies of IgM isotype, which may cause cell aggregates.

Analysis of the immature myeloid progenitor compartment

Definition and enumeration of myeloid progenitor cells. The term 'progenitor cells' was agreed upon by the working group to distinguish FC results from the morphological assessment of blasts. Hemodilution can result in differences between progenitor cell counts as assessed by morphology and FC. Moreover, there are usually fewer spicules in the bone marrow aspirates processed for FC than in the fraction used to prepare smears. Therefore, the amount of progenitor cells assessed by FC has to be regarded as an independent marker.

The quantification of progenitor cells by FC requires a definition of both reagents and gating procedures. The combination of CD45 and SSC provides a means to identify progenitor cells (CD45^{dim} and SSC$^{low/int}$; Figures 1a and b).[12] However, the immature cell compartment is very heterogeneous. Antibody combinations, such as CD45/CD34/CD117/HLA-DR and CD45/CD34/CD123/HLA-DR (Figure 1), are recommended to distinguish myeloid progenitor cells from other populations, such as B-cell precursors, monoblasts, basophils, erythroblasts and plasmacytoid dendritic cell precursors, which might show overlapping CD45 and SSC features.[6] Thus, multiple strategies must be applied to identify and enumerate the myeloid progenitor cells present in MDS: (a) CD45^{dim}SSC$^{low/int}$; (b) CD45^{dim}SSC$^{low/int}$CD34^{+} (negative for lymphoid markers such as CD19); (c) CD45^{dim}SSC$^{low/int}$HLA-DR^{+}CD11b^{-} (B-cell precursors excluded by SSC^{low} and/or CD19 expression); and (d) CD45^{dim}SSC$^{low/int}$HLA-DR^{+}CD117^{+}.[13] The percentages of myeloid progenitor cells obtained with these definitions should correlate, unless the aberrant myeloid progenitor cells lack a particular antigen (e.g., loss of HLA-DR, CD34, or occasionally CD45) or aberrantly gained expression of, for example, CD19. This stresses the importance of redundant combinations. Of note, CD117 is lost early in the monocytic lineage, and monocytic precursors might therefore be underestimated with this strategy. Furthermore, granular progenitors or degranulated neutrophils may overlap and should be defined accordingly. The use of combinations including antibodies defining mature neutrophils (CD15, CD24, CD10) can be useful, especially to appreciate the presence of degranulated neutrophils by backgating on the CD45/SSC scattergram as shown in Figures 1c and d.

Identification of the denominator of choice necessitates a comparative pilot study to evaluate 'per non-erythroid' versus 'per all-nucleated'. At present, the consensus is to use the total number of nucleated cells (i.e., erythroid precursors, progenitor cells, neutrophils, lymphocytes and monocytes) as the denominator for myeloid progenitor enumeration by FC. This requires elimination of residual mature non-nucleated erythroid cells and large platelets from the analysis; otherwise, results will be falsely low. The use of a nuclear dye (such as Draq5 or Draq7) could be helpful in this respect.[14,15] For the morphological assessment of bone marrow smears of acute myeloid leukemia, it is common to

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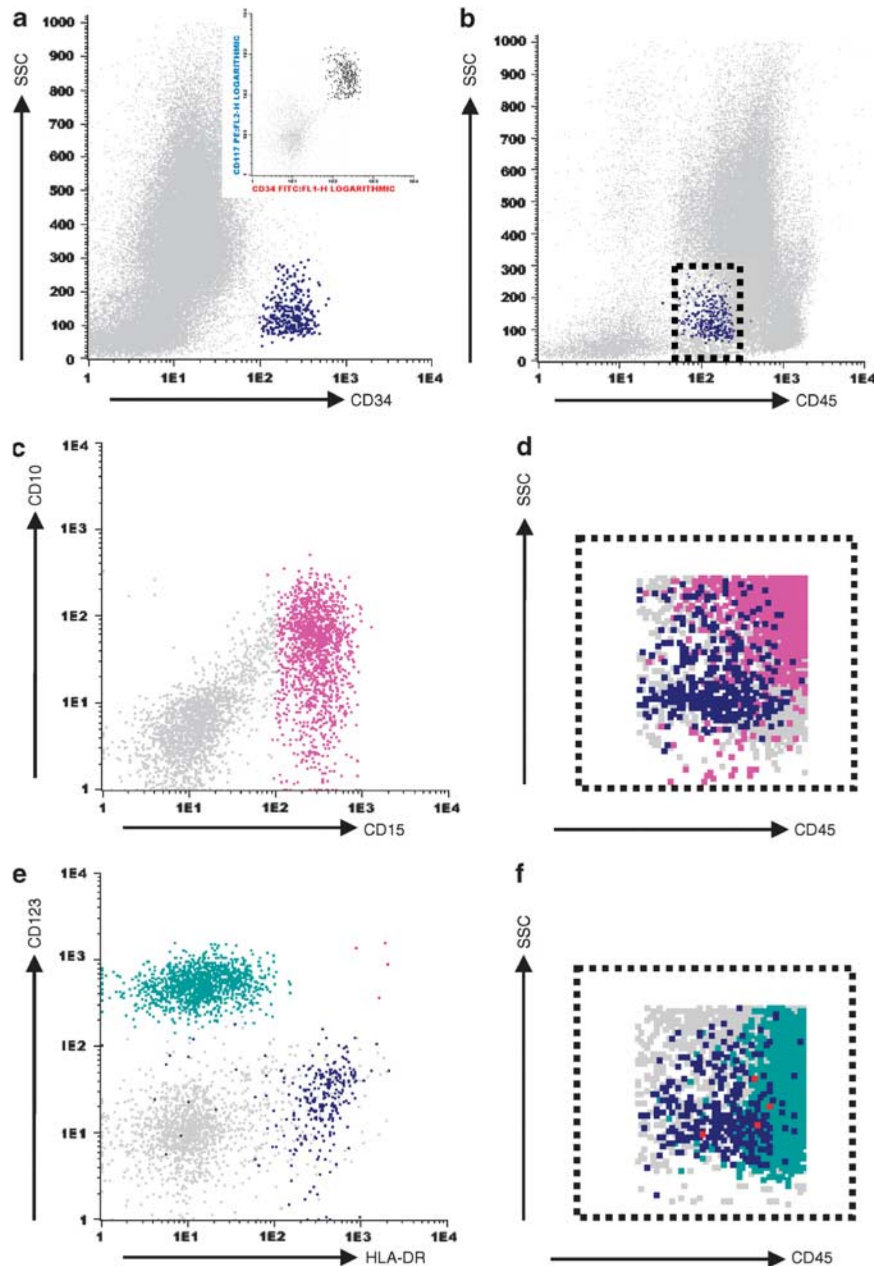

Figure 1. Heterogeneity of the CD45^{dim}SSC$^{low\text{-}int}$ population in a MDS bone marrow sample as assessed by FC. In panel (**a**), progenitor cells are selected based on CD34$^+$SSC$^{low\text{-}int}$ (highlighted in blue), in a CD34 (*x* axis) versus SSC (*y* axis) plot. The insert in panel (**a**) shows CD34 (*x* axis) versus CD117 (*y* axis) staining within the CD45^{dim} section; double-positive cells in this plot are CD34$^+$CD117$^+$ myeloid progenitors (in blue). In panel (**b**), these CD34$^+$ progenitors are back-gated in a CD45 (*x* axis) versus SSC (*y* axis) plot. In this panel, a gate is placed around the CD45^{dim}SSC$^{low\text{-}int}$ population. In panels (**c**–**f**), solely this CD45^{dim}SSC$^{low\text{-}int}$ section is depicted. Contamination of (hypogranular) neutrophils can be observed in a CD15 (*x* axis) versus CD10 (*y* axis) staining (**c**); neutrophils express CD15 from the promyelocyte stage and CD10 from the band stage; these cells are highlighted in pink, CD34$^+$ cells are displayed in blue. In panel (**d**), the CD45^{dim}SSC$^{low\text{-}int}$ section as defined in panel (**b**) is magnified to show the backgating of these neutrophils and CD34$^+$ progenitors (in pink and blue, respectively). Contamination of basophils and plasmacytoid dendritic cells can be observed in a HLA-DR (*x* axis) versus CD123 (*y* axis) staining (**e**). Basophils are HLA-DR$^-$CD123$^+$ (in green); the plasmacytoid dendritic cells express both HLA-DR and CD123 (only few present in this sample, depicted in red); CD34$^+$ are again highlighted in blue. In panel (**f**), the magnified CD45^{dim}SSC$^{low\text{-}int}$ section demonstrates the backgating of the cells as defined in panel (**e**): basophils, plasmacytoid dendritic cells and CD34$^+$ progenitors in green, red and blue, respectively. Note that aberrant loss or gain of defining markers complicates gating, and hence, analysis of myeloid progenitor cells (section 'Definition and enumeration of myeloid progenitor cells').

enumerate the blasts per non-erythroid as a denominator if the percentage of erythroid progenitors exceeds 50% (especially in the French-American-British M6 subtype). By applying this denominator in FC (thus selecting for CD45 positivity), care must be taken in case of the presence of CD45-negative progenitors.

Other caveats in the FC enumeration of progenitor cells in bone marrow aspirates are the degree of peripheral blood contamination and processing of the sample for FC, which requires the lysis of erythrocytes or density separation of the nucleated cells. The latter is associated with a variable and significantly impaired

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cell recovery from specimen to specimen. Centrifugation steps are also typically associated with cell loss. Of note, several methods can be applied to evaluate the degree of hemodilution (e.g., the proportion of mature, CD16^{+} neutrophils).[16,17]

Within the current WHO proposal,[7] the presence of circulating blasts is included in the classification. It is associated with a worse clinical outcome, that is, increased risk of leukemic transformation and post-transplant relapse.[10,18,19] Therefore, the detection and enumeration of myeloid progenitor cells in the peripheral blood is also of increasing interest. In primary myelofibrosis, circulating myeloid progenitor cells are often present because of extramedullary hematopoiesis. The effect of secondary fibrosis on the presence of circulating myeloid progenitor cells is not yet clear. It has not been fully investigated whether or not these circulating progenitor cells display specific aberrant FC patterns, although karyotypic aberrancies can be detected by fluorescence *in situ* hybridization.[19-21] FC on peripheral blood alone is not yet recommended in MDS, but it should be a part of separate clinical research studies.

Identification of flow cytometric aberrancies in myeloid progenitor cells. In addition to quantitative aberrancies, dysplastic immature myeloid progenitors in MDS may have an aberrant immunophenotype that distinguishes them from normal progenitors.[6] In low-risk MDS in particular, percentages of progenitor cells are low. Hence, an appropriate amount of immature progenitor cells should be acquired (i.e., at least 250) to ensure reliable assumptions regarding an aberrant profile by FC. The most widely recognized aberrancies in the immature myeloid compartment in MDS are an abnormal intensity or lack of expression of CD45, CD34, CD117, HLA-DR, CD13, CD33, asynchronous presence of CD11b or CD15, and/or the expression of lineage infidelity markers, such as CD5, CD7, CD19 or CD56 (Table 1).[3-6,9,22-25] The latter markers should not only be analyzed on CD34^{+} progenitor cells, but also among CD117^{+}/CD34^{-} precursors, because some precursor cells lack CD34 expression.

Aberrancies per marker or marker combination are indicated in Table 1, and some examples are depicted in Figure 2. Several markers, such as CD7, can be expressed on a small subset of early, normal myeloid immature progenitor cells, especially in recovering hematopoiesis.[4,6,26] This necessitates knowledge of expression levels in appropriate bone marrow controls.

Examples of other potentially valuable markers that warrant further investigation are TdT and CD38. A few TdT^{+} progenitor cells lacking expression of other B-cell-associated markers, such as CD19 and/or CyCD79a, have been reported, but it is not clear yet

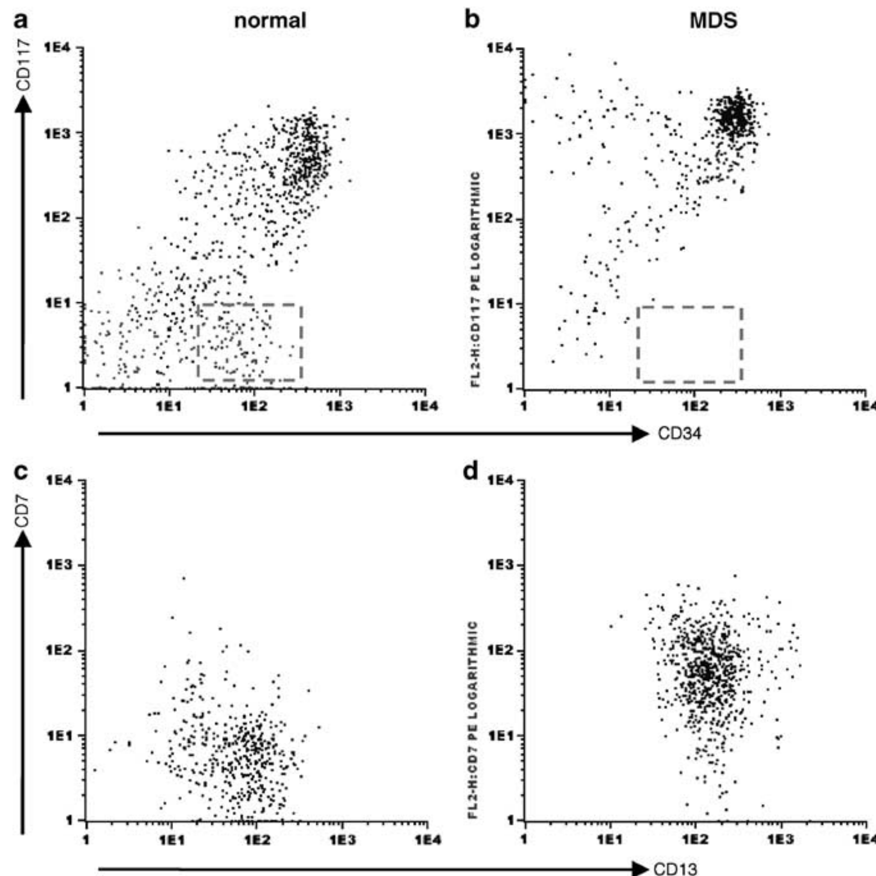

Figure 2. Immunophenotypic patterns in the progenitor population of an MDS bone marrow sample as compared with a normal control. In panels (**a**) and (**b**), progenitor cells (selected based on CD45^{dim}SSC$^{low-int}$) are displayed. In a normal bone marrow sample, the CD34 (*x* axis) versus CD117 (*y* axis) staining results in a heterogeneous pattern (**a**); in the example of an MDS case, the CD34^{+}CD117^{+} cells represent an aberrantly homogeneous population with aberrant overexpression of CD117. The marked regions in the plots (dashed square) indicate CD34^{+}CD117^{-} cells that may represent lymphoid progenitors. In contrast to the normal sample, no progenitor B cells are present in the MDS bone marrow sample displayed (**b**); this observation should be verified by an appropriate staining using CD34 in combination with CD19 and/or CD10. In panels (**c**) and (**d**), CD34^{+} myeloid progenitor cells were selected based on their CD45, SSC and CD34 properties. In this subpopulation, CD7 expression (*y* axis) on CD13-positive (*x* axis) myeloid progenitors was analyzed. Normally, only a very small fraction of myeloid progenitor cells express CD7 (**c**). In the MDS case displayed, CD7 is aberrantly expressed on the myeloid progenitor population (**d**).

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whether or not TdT expression in myeloid progenitor cells should be regarded as a marker for MDS.[27,28] A decrease in CD38 expression on $CD34^{+}$ myeloid progenitor cells has also been reported as a rather typical aberrancy in MDS.[29,30]

Analysis of the maturing myeloid and monocytic compartment

Definition of neutrophils. The combination of CD45 and SSC is regularly applied to identify maturing neutrophils by FC ($CD45^{int}SSC^{int\text{-}bright}$).[12] CD33 and CD64 are useful in distinguishing monocytes and hypogranular neutrophils.[31] Otherwise, a computer-assisted multidimensional, instead of simply multiparameter, analysis might overcome problems encountered when subpopulations overlap.

Identification of aberrancies in the maturing myeloid compartment. The amount of maturing neutrophils reflect, in part, the ability of the progenitor compartment to proliferate and differentiate, thereby supporting successful hematopoiesis. Conversely, a decreased amount of neutrophils might indicate disturbed granulopoiesis. An equal or decreased percentage of maturing neutrophils as compared with lymphocytes is a component of one of the MDS–FC scoring systems, although its clinical relevance is currently unknown.[4] It is also observed in aplastic anemia (data not shown).[32]

One of the most frequently reported FC aberrancies in the maturing neutrophil compartment is an abnormally decreased SSC reflecting hypogranularity, a well-known phenomenon in MDS.[13,22] The SSC of maturing neutrophils is recommended to be expressed as a ratio relative to that obtained for lymphocytes as an internal reference. The peak channel value for determination of the SSC ratio was reported to have less inter-operator variability,[33] although this parameter is not available in all analysis software.

The group discussed whether data should be acquired using a logarithmic or a linear display of the SSC parameter. In linear SSC display, eosinophils and other highly granular cells can be off scale. SSC in log scale might provide a better distinction between maturing neutrophils, monocytes and progenitor cells. It should be mentioned that linear versus log amplification during data acquisition is different from linear versus log display in analysis. Nevertheless, the cut-off for neutrophils SSC to be called aberrant is probably more important than the choice for linear or log scale.

In normal bone marrow, SSC increases from the most immature to mature neutrophils.[34] Consequently, the SSC may be affected by hemodilution. To avoid a pitfall in such cases, SSC evaluation of specific maturation-associated compartments, such as CD10-negative (immature) neutrophils, might be helpful.[33] Of note, mature neutrophils in MDS can aberrantly lack CD10.[22,35,36] Moreover, differences in SSC may be observed for different sample preparation procedures (i.e., distinct lysing solutions and fixatives). Despite the fact that fixation procedures, as recommended by our working group,[6] can stabilize the expression of vulnerable antigens, experience with paraformaldehyde has demonstrated an increment of neutrophil SSC. An even higher increase was observed after an extended period between staining and data acquisition (data not shown). Therefore, processing of samples needs to be exactly the same in patients and controls.

Dysplastic neutrophils can be recognized by increased or decreased expression of antigens or an aberrant relationship among two or more antigens. Most frequently reported are the aberrant relationships between CD13 and CD11b, and/or CD13 and CD16 (Table 1, and some examples in Figure 3).[6] Additionally, aberrant relationships between CD15 and CD10 are observed; for instance, mature neutrophils expressing CD15, but lacking or expressing only low levels of CD10 (Table 1). Besides, variations in the expression patterns of CD10 and CD15 on myeloid progenitors have been reported to be associated with prognosis.[3]

Because of genetic polymorphisms, the expression of some antigens, such as CD16 or CD33, may be non-aberrantly low. Furthermore, decreased expression of CD16 as well as CD11b has been reported to coincide with apoptosis. Assessment of the CD11b versus CD16 pattern next to that of CD16 versus CD13 can therefore be helpful.[13,24,37–41] Of note, loss of glycosyl-phospatidyl-inositol-associated cell surface proteins, such as CD16, is observed in paroxysmal nocturnal hemoglobinuria (PNH). PNH is a complementary indicator for aplastic anemia or bone marrow failure in cytopenic patients. Small (as low as 10^{-5}) to large PNH clones can also be found in MDS and do not help in distinguishing MDS from aplastic anemia.[42–45] The current opinion of the group is that the presence of a small PNH clone does not affect MDS bone marrow analysis. Noteworthy, the preferred material to analyze PNH is peripheral blood.[46]

Other possible dysplastic features in the neutrophil subpopulation as assessed by FC are altered expression of CD45 or CD33, asynchronous expression of CD34 and expression of lineage infidelity markers such as CD56.[4,5,9,22–24,36] All of these markers are currently not part of the recommendations (Table 1), but under investigation within the ELN.

Definition of monocytes. Morphological assessment of dysmonopoiesis in MDS is difficult unless there is marked monocytosis. In FC, monocytes can be defined on the basis of their CD45 expression (intermediate bright[12]), SSC (intermediate) and useful additional markers, such as CD14, CD64, CD36 and CD33. A combination of markers makes it easier to quantify this subpopulation. The use of CD14 alone may underestimate the percentage of monocytic cells, particularly when immature forms are present. As noted above, maturing neutrophils with an abnormally low SSC can interfere in the analysis of the monocytic population, depending on the gating strategies. CD33 or the combination of CD64 and CD24 might be useful to separate these two subpopulations (examples in Westers *et al.*[31]). Of note, doublets of cells from both populations might also hamper analysis and should be excluded in an FSC-height versus FSC-area dot plot. The position of monocytes as compared with lymphocytes with respect to CD45 and SSC may also be of importance, although this can also change upon cell activation.

Identification of aberrancies in the monocytic compartment. Recommended points of interest regarding aberrancies in the monocytic lineage are the proportion of monocytes (abnormally decreased or increased related to non-erythroid cells), an abnormal distribution of maturation stages, abnormal relationships of HLA-DR versus CD11b, and/or CD36 versus CD14, abnormal intensity of CD13 or CD33, and overexpression of CD56 (Table 1). Some examples are depicted in Figure 3.[4,5,9,22–24,36]

One caveat is that CD14 recognizes a glycosyl-phospatidyl-inositol-anchored protein that can be absent due to co-existence of PNH. In addition, CD56 expression is frequently seen upon hematopoiesis regeneration, activation and inflammatory responses, and therefore, the clinical context is of utmost importance.[4,26,47,48] CD56 upregulation due to activation often coincides with an increased expression of HLA-DR and CD64. An increase in the expression of either of these markers should therefore be interpreted in the context of that of other markers. In some cases, severe monocytopenia hampers solid information on dyspoiesis in monocytes.

Other aberrancies that might be of interest are an abnormal intensity of CD64 expression, asynchronous presence of CD34 or expression of lineage infidelity markers.[4,5,9,22–24,36] CD56 and CD2 may even contribute to discriminate chronic myelomonocytic leukemia from MDS and myeloproliferative neoplasms.[48–50] Notably, the diagnosis of chronic myelomonocytic leukemia is made on bone marrow examination, whereas absolute monocytosis is established in peripheral blood.

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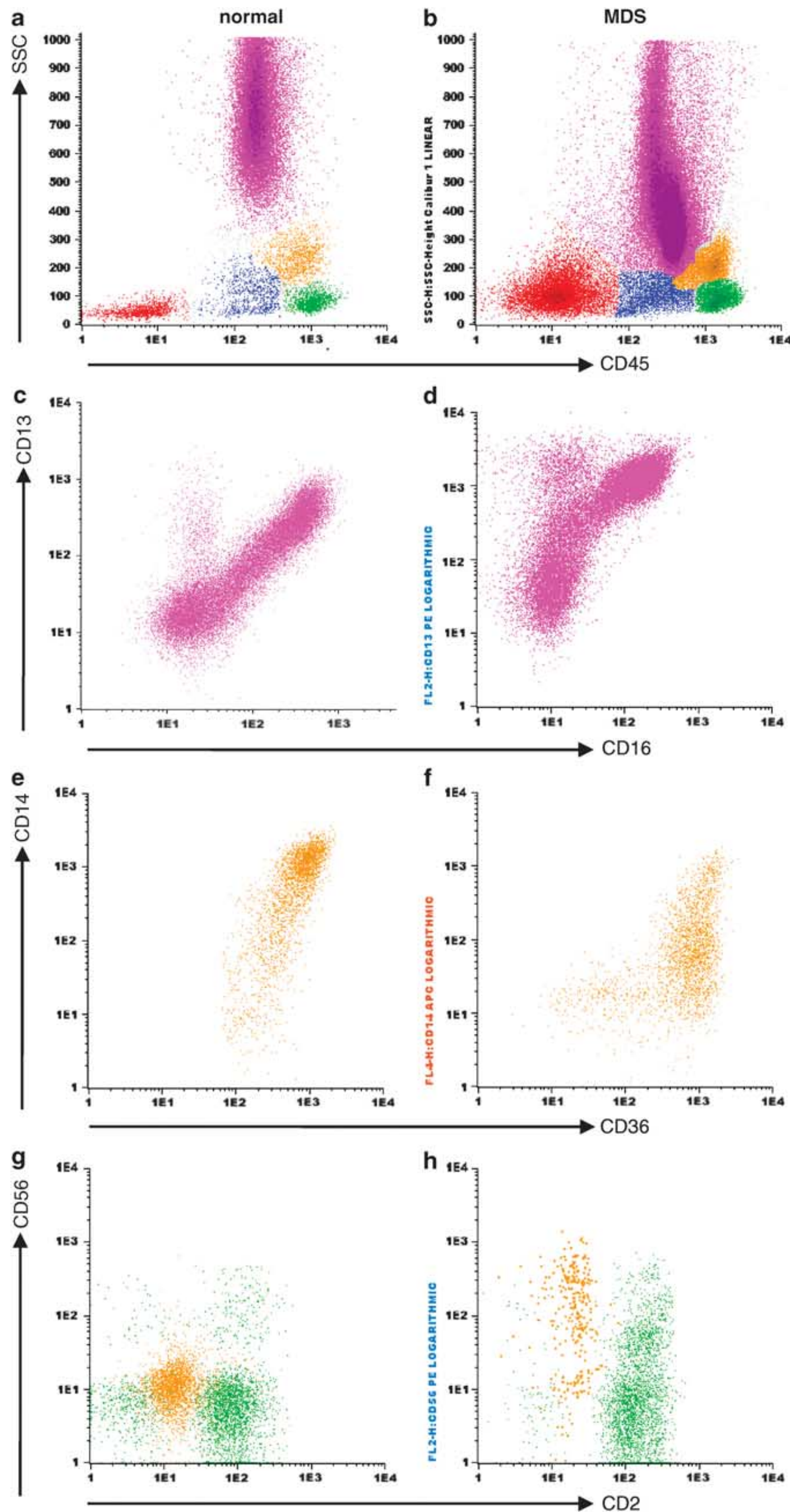
Definition and enumeration of lymphoid progenitor cells
A decrease in B-cell progenitor cells is frequently observed in MDS.[5,51-53] These cells can be identified in the $CD45^{dim/low}SSC^{low}$ region and by their $CD34^+CD19^+$ or $CD34^+CD10^+$ immunophenotype. All three quantification methods will work well in experienced laboratories. The



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$CD45^{low}/SSC^{low}$ quantification method demonstrates less inter-operator variability; however, most participants prefer specific markers to enumerate progenitor B cells. The $CD34^+CD19^+$ and/or $CD10^+$ population should be backgated in a CD45 versus SSC plot to confirm scatter characteristics. To circumvent problems regarding hemodilution in the enumeration of progenitor B cells, it is recommended to express these cells as fraction of all $CD34^+$ blast cells.[33,54]

The relevance of a decreased percentage of B-cell progenitors is not yet known, as it is also seen in the elderly population without MDS. Interestingly, a decreased relative amount of progenitor B cells (<5% of $CD34^+$ cells) was recently introduced as one of the cardinal parameters in a model to distinguish low-risk MDS from non-clonal cytopenias.[33] It should be noted that a relative decrease in B-cell progenitors can result from an increase in myeloid progenitor cells, especially in high-risk MDS.

Analysis of the erythroid compartment

Definition of the erythroid compartment. The erythroid population can be defined by its very low to lack of CD45 expression, and low FSC and SSC properties.[55]

Identification of aberrancies in the erythroid compartment. Participants of the working party stressed that erythroid analysis is useful according to their own experience; however, there is not enough published information on the erythroid lineage yet. A proposal regarding the aberrancies to be analyzed in the erythroid compartment will therefore be put together after exploring data from several groups in a future meeting.

FC patterns of normal erythroid development were originally described in 1987.[55] Commonly applied markers are CD45, CD117, CD71, CD235a and CD36. The endoglin marker CD105 is also used, but data on this particular marker are scarce.[9,22,28,36,56] Some of the aberrancies that are currently considered relevant (Table 1), and their accompanying pitfalls, are discussed here.

One of the most frequently observed aberrancies is an increased number of erythroid progenitors associated with a larger proportion of immature erythroid cells ($CD117^+$). This is observed in approximately 80% of low-risk MDS, although it is probably not specific for this disease. By contrast, a decrease in erythroid progenitors, due to apoptosis or erythropoietin deficiency, can also be observed.[9,22,28,36,56] Moreover, quantification of the amount of erythroid progenitors may be affected by red-cell lysing procedures, which may potentially lyse some of the nucleated red blood cells. It might therefore be worthwhile to analyze the erythroid/myeloid ratio before and after lysis. Nucleated erythroid cells can be separated from non-nucleated ones by including a DNA dye as discussed above (section 'Analysis of the immature myeloid progenitor compartment'), although this is not routinely applied thus far.

Furthermore, asynchronous expression of CD71 versus CD235a and a decreased reactivity for CD36 have been reported as signs of erythroid dysplasia in MDS bone marrow samples, both in approximately 70% of the patients.[9,22,28,36,56] An example is displayed in Figure 4. It should be kept in mind that the transferrin receptor (CD71) has a role in iron uptake and can also be present on non-erythroid immature cells, as it is required for their metabolism. Interestingly,

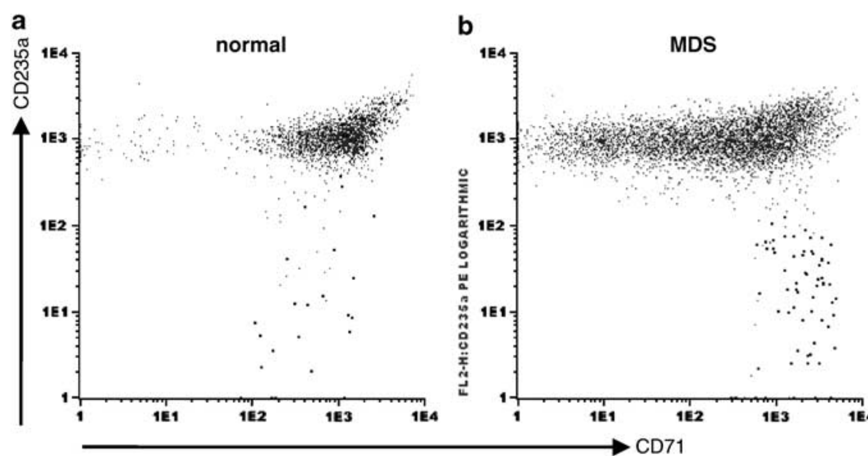

Figure 4. Immunophenotypic patterns in the erythroid subpopulation of an MDS bone marrow sample as compared with a normal control. In panel (**a**) and (**b**), maturation patterns of erythroid cells are shown in CD71 (*x* axis) versus CD235a (*y* axis) plots. In panel (**a**), the normal maturation from $CD71^+CD235a^+$ immature cells towards $CD71^-CD235a^+$ mature erythrocytes is displayed. More early precursors ($CD117^+$) can be found in the $CD71^+CD235a^-$ and $CD71^+CD235a^+$ region. In panel (**b**), an example of aberrantly decreased CD71 expression or sustained CD71 expression upon maturation of erythroid cells in MDS is depicted.

Figure 3. Immunophenotypic patterns in the maturing myeloid-monocytic population of an MDS bone marrow sample as compared with a normal control. Multi-color density plots of a normal bone marrow sample and a case of MDS are illustrated in panels (**a**) and (**b**), respectively, (CD45 (*x* axis) vs SSC (*y* axis)). The cell populations displayed are erythroid cells ($CD45^-SSC^{low}$ indicated in red), progenitor cells ($CD45^{dim}SSC^{low\text{-}int}$ blue), lymphocytes ($CD45^{bright}SSC^{low}$ green), monocytes ($CD45^{int\text{-}bright}SSC^{int}$ orange) and neutrophils ($CD45^{dim}SSC^{int\text{-}high}$ purple). The granularity (presented as SSC) of MDS neutrophils in panel (**b**) is aberrantly decreased as compared with that of the neutrophils in a normal control (**a**), indicating hypogranularity. In panels (**c**) and (**d**), maturation patterns of the selected neutrophil subpopulations are shown in CD16 (*x* axis) versus CD13 (*y* axis) plots. In panel (**c**), the normal maturation from $CD13^+CD16^-$ immature neutrophils, via a $CD13^{dim}$ interphase towards $CD13^+CD16^+$ mature neutrophils is displayed; panel (**d**) demonstrates an example of an aberrant maturation profile of MDS neutrophils. In panels (**e**) and (**f**), maturation patterns of monocytes are shown in a CD36 (*x* axis) versus CD14 (*y* axis) plot. Panel (**e**) presents the normal maturation from $CD36^+CD14^-$ immature monocytes towards $CD36^+CD14^+$ mature monocytes. In the example of an MDS case shown in panel (**f**), either aberrantly increased numbers of immature monocytes are present or CD14 expression is aberrantly low or lost on mature monocytes. In panels (**g**) and (**h**), expression patterns of CD56 on monocytes (orange) are shown in a CD2 (*x* axis) versus CD56 (*y* axis) plot. As an internal reference, $CD2^+$ lymphocytes are shown in green. In a normal bone marrow, no to weak expression of CD56 is observed (**g**); in the displayed example of an MDS case, monocytes aberrantly express CD56 (>1 log, (**h**)).

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one study reported an association between high levels of CD71 expression on neutrophils and the refractory anemia with ring sideroblasts subcategory of MDS.[57] Of note, platelets express CD36 and can interfere in the analysis of the erythroid lineage.

Another well-known feature of erythroid dysplasia is the presence of ring sideroblasts. These cells are definitely of diagnostic relevance; however, the antibody that can identify these cells via detection of mitochondrial ferritin is not commercially available.[39] Currently, there is no additional value of FC for the quantification of ring sideroblasts.

FUTURE DIRECTIONS FOR FC IN MDS

Validation of the specificity of FC aberrancies in MDS

The incidence of MDS is highest in the elderly. Yet, age-related changes, such as shortening of telomeres, might affect FC patterns.[58] Current knowledge on what is normal in the elderly, especially in the myeloid series is insufficient. Only those aberrancies or combination of aberrancies that exclusively occur in MDS will be conclusive for the diagnosis of MDS. A high degree of confidence is needed to convince clinicians that it might be worthwhile to start treating the patient. Therefore, validation of analyzed parameters is of utmost importance. Thus far, all reports on FC in MDS show a fair spread in the FC scores of normal versus MDS. To provide dependable data in the specificity and sensitivity of the observations in MDS with respect to controls, all aberrancies mentioned in Table 1 and discussed in the section 'Identification of aberrancies in the monocytic compartment' should ideally be analyzed versus age-matched normal controls, which is not easy to achieve. Comparisons should also be made versus non-clonal cytopenias, stressed marrows (e.g., regeneration after chemotherapy) and other malignant hematological diseases.[6] Some issues regarding the specificity of certain aberrancies for MDS, such as CD56 over-expression also seen upon activation, have been mentioned in section 'Identification of aberrancies in the monocytic compartment', but further validation in large multicenter cohorts is necessary. Using this approach, a better understanding will be gained in dealing with disease-specific FC aberrancies.

Flow cytometric scoring systems in the diagnosis of MDS

In many circumstances, it remains a struggle to dissect MDS from other conditions with cytopenia and normal karyotype. Numerous FC markers are currently being explored by several centers, and multicenter analyses of retrospective data sets should elucidate

Table 4. Summary of scoring models for flow cytometric evaluation of dysplasia

Year	*Reference*	*Diagnosis/ prognosis*	*Cohort MDS/ path. control/ normal*	*Subpopulations analyzed*	*Lineages analyzed*	*Parameters*	*Specificity*	*Sensitivity*	*Concordance with ELN recommendations*
2001	Stetler-Stevenson *et al.*[22]	D	45/25/4	ImmatMy/ matMy/Mo/ ery/MK	3	>15	100	88	+++
2005	Kussick *et al.*[62]	D	69/46/0	ImmatMy/ matMy/Mo	1	>15	88	89	+++
2005	Malcovati *et al.*[9]	D	103/27/19	ImmatMy/ matMy/Mo/ery	2	7	100	87	++
2005	Cherian *et al.*[35]	D	26/20/16	MatMy (blood)	1	>15	90	73	++
2006	Della Porta *et al.*[56]	D	104/69/19	Ery	1	6	98.5	>95	+
2006	Ogata *et al.*[52]	D	27/76/14	ImmatMy/B	1+1	13	100	41	++
2008	Stachurski *et al.*[24]	D	180/37/0	ImmatMy/ matMy/Mo	1	>15	97	84	+++
2008	Satoh *et al.*[63]	D	27/90/0	ImmatMy/B	1+1	3	83	78	+
2008	Matarraz *et al.*[25]	D	50/29	ImmatMy/ matMy/B	1+1	>15	100	100	+++
2009	Goardon *et al.*[30]	D	100/70/5	ImmatMy	1	1	92	95	–
2009	Ogata *et al.*[33]	D	134/106/0 multicenter	ImmatMy/ matMy/B	1+1	4	92–98	44–71	++
2009	Truong *et al.*[64]	D	12/61/0	ImmatMy/ matMy/Mo	1	>15	94	75	+++
2011	Della Porta *et al.* (model according to reference 33)	D	416/380/0 multicenter	ImmatMy/ matMy/B	1+1	4	93	72	+
2003	Wells *et al.*[4]	D/P	115/104/25	ImmatMy/ matMy/Mo	1	>15	93	70	+++
2007	Lorand-Metze *et al.*[36]	D/P	31/11/11	ImmatMy/ matMy/Mo/ery	2	5	~87	NE	++
2010	Matarraz *et al.*[28]	D/P	56/20/20	ImmatMy/ matMy/Mo/ ery/B	2+1	>15	100	100	+++
2010	Kern *et al.*[65]	D/P	459/266/11	ImmatMy/ matMy/Mo/ery	2	>15	95	70	+++
2011	Chu *et al.*[59] (model according to reference 4)	D/P	56/27/0	ImmatMy/ matMy/Mo	1	>15	100	75	+++
1987	Clark *et al.*[60]	P	33/4/16	MatMy/Mo (blood)	1	3	NE	NE	+
2004	Arroyo[8]	P	77/0/0	ImmatMy/ matMy/Mo	1	7	NE	NE	++
2011	Falco *et al.*[66]	P	424/0/0	ImmatMy/ matMy/Mo/ery	2	4	NE	NE	+

Abbreviations: D, diagnosis; ELN, European LeukemiaNet; P, prognosis; path. control, pathologic control; immatMy, immature myeloid progenitor cells; matMy, maturing neutrophils; MDS, myelodysplastic syndrome; Mo, monocytes; Ery, erythroid cells; MK, megakaryocytes; NE, not evaluable. All analyses are performed in bone marrow samples unless indictaed otherwise. Analysis of B-cell progenitors is indicated as (+1); concordance of analyzed markers with current ELN recommendations is expressed as: (–) no, (+) 1–5, (++) 5–10 and (+++) >10 markers.

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the most important markers and their combinations in multiparameter analyses.

To design a broadly applicable scoring system for FC evaluation of dysplasia, validated aberrancies should be ranked and weighed for their clinical relevance, and prospective data are needed for validation. Different groups have designed several scoring systems for the diagnosis and prediction of prognosis of MDS (summarized in Table 4). These models differ with respect to the origin of the sample (blood or bone marrow), processing and handling of the samples, number of cell lineages analyzed, subpopulations evaluated and amount of parameters included in the model. Nevertheless, most proposed models show a high concordance with the current ELN recommendations (Tables 1 and 4). On average, the sensitivity of the different proposed models is around 75% with a specificity of nearly 90% (Table 4). Inclusion of only those FC parameters that are highly reproducible and have an acceptable diagnostic power is warranted to increase the specificity and sensitivity of FC in the diagnosis of MDS. Specific markers or combinations of markers with better validation might lead to an upgrade of FC parameters to decisive, so-called B criteria in current guidelines.[2] The ELN Working Group is an ideal platform to collect data, and after analyses, to design, evaluate and implement a diagnostic FC scoring model. To illustrate this, multicenter validation (on behalf of the ELN) recently confirmed the ability to distinguish low-risk MDS without other specific markers (i.e., cytogenetics or ring sideroblasts) from non-clonal cytopenias by application of only four parameters.[33] These parameters are an increased percentage of $CD34^{+}$ progenitor cells in a bone marrow sample, a decreased number of progenitor B cells within the $CD34^{+}$ compartment, a decreased or increased CD45 expression on myeloid progenitor cells and a decreased SSC of neutrophils. In this study, additional useful markers were CD10, CD15, CD11b and CD56. The presence of two or more of these specific aberrancies could identify 70% of low-risk MDS cases with a specificity of 94%.[33] In line with this, the myeloid lineage-associated model by Wells *et al.*,[4] (originally designed to study prognosis) also showed consistent results with respect to specificity and sensitivity upon application in other centers.[5,59]

Flow cytometric scoring systems in the prognosis of MDS

A prognostic FC score for MDS patients was developed as early as 1987.[60] Variability in the currently reported prognostic models is as large as in the models proposed for FC diagnosis of MDS (Table 4). Thus far, the only validated prognostic flow score is the FC scoring system designed by Wells *et al*.[4,5,10,59] However, large multicenter validation studies are not available yet, which indicates a future role for the ELN Working Group.

CONCLUDING REMARKS

In MDS, FC is regarded as a new forthcoming standard. In the present report, minimal requirements to analyze dysplasia are refined. This core data set should enable to categorize bone marrow FC as normal or possibly consistent with MDS. It should however be stressed that FC in MDS can only be used as a part of an integrated diagnosis. Repeated FC assessments are highly recommended, not only in inconclusive cases, but also to monitor the course of the disease in untreated, mainly low-risk MDS patients, and during treatment with current available drugs. A report from the FC analysis should include descriptions of validated FC abnormalities and lineage-infidelity marker expression. Finally, coincidence of MDS with other disorders should be indicated, such as clonal lymphoproliferative disease, mastocytosis, autoimmune diseases and PNH-associated glycosyl-phospatidyl-inositol-deficient cells.

The ELN–MDS Working Group is convinced that consensus on antibody combinations and agreement on the interpretation of antigen expression patterns will contribute to solid information on the diagnosis, prognosis and treatment monitoring in MDS. The working group is dedicated to initiate further studies, including data collection and analyses, to establish commonly accepted standards in MDS. The ultimate goal will be to refine and improve diagnosis and prognostic scoring systems.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

All persons listed as co-authors contributed to pre-conference and post-conference discussions (June 2009 until November 2010), and actively participated in the Standardization Conferences (Munich, Germany, 30–31 October 2009; London, UK, 5–6 November 2010). All co-authors contributed equally by discussing criteria, standards, algorithms and recommendations at the Working Conference. 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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