In this issue: FLOW CYTOMETRY IN THE CLINICAL LABORATORY

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Flow cytometry in the diagnosis of myelodysplastic syndromes

Bettina Kárai, Eszter Szánthó, János Kappelmayer, Zsuzsa Hevessy
Department of Laboratory Medicine, University of Debrecen, Debrecen H-4032, Hungary

Corresponding Author:

Bettina Kárai
Department of Laboratory Medicine, University of Debrecen, Nagyerdei krt. 98,
Debrecen H-4032, Hungary
Tel: +36 30 386 2672
Fax: +36 52 417 631
e-mail: bettina.karai@gmail.com

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Abstract

Myelodysplastic syndromes are clonal hematopoietic stem cell disorders. Their exact etiology is unknown. Myelodysplastic syndromes cause progressive bone marrow failure resulting in pancytopenia and refractory, transfusion-dependent anemia. One can observe typical morphological alterations in the erythroid, myeloid and/or megakaryocytic cell lineage. Blast counts may also be increased. The pathologic cells are genetically unstable, and a myelodysplastic syndrome might transform into acute myeloid leukemia. The overall survival of these diseases range between few months to around ten years. Correct diagnosis and accurate prognostic classification is essential. In the past decades several scoring systems were established beginning with the French-American-British classification to the most recent Revised International Prognostic Scoring System. In all of these classifications bone marrow morphology is still the most important factor, though nowadays the genetic aberrations and flow cytometry findings are also included. The diagnosis and prognostic classification of myelodysplastic syndromes remain a great challenge for hematologists.

Introduction

Myelodysplastic syndromes (MDS) are clonal hematopoietic stem-cell disorders. The incidence of MDS is 3.4 per 100,000/per year in the United States, which increases with age. The median age at diagnosis is 76 years in the U.S. and 74 years in Europe. The incidence is slightly higher in men than in women [1, 2].

The exact etiology of MDS is unknown. MDS have two subtypes according to their etiology, a primary (de novo) and a secondary one. The development of the second type of MDS occurs more frequently after some environmental mutagenic event, such as the effect of toxic chemicals, e.g. benzene, or treatment of malignant tumor with radiation and/or chemotherapy. Several studies have examined the causes of MDS, which include environmental exposures, cytogenetic and epigenetic changes in stem cells and progenitors, altered bone marrow microenvironment, immune dysregulation, and abnormal cell cycle regulation and differentiation (Figure 1). Thus it has become commonly accepted that MDS is the result of a complex process [3].

Although MDS are a heterogeneous diseases group, there are some common characteristics to these pathological conditions. One of these is the progressive bone marrow failure, which manifests in peripheral cytopenia due to ineffective hematopoesis. In patient histories we often encounter anemia resistant to treatment (refractory anemia), while the bone marrow is hypercellular and erythroid hyperplasia can be detected. When examining these peripheral and bone marrow samples, typical morphological

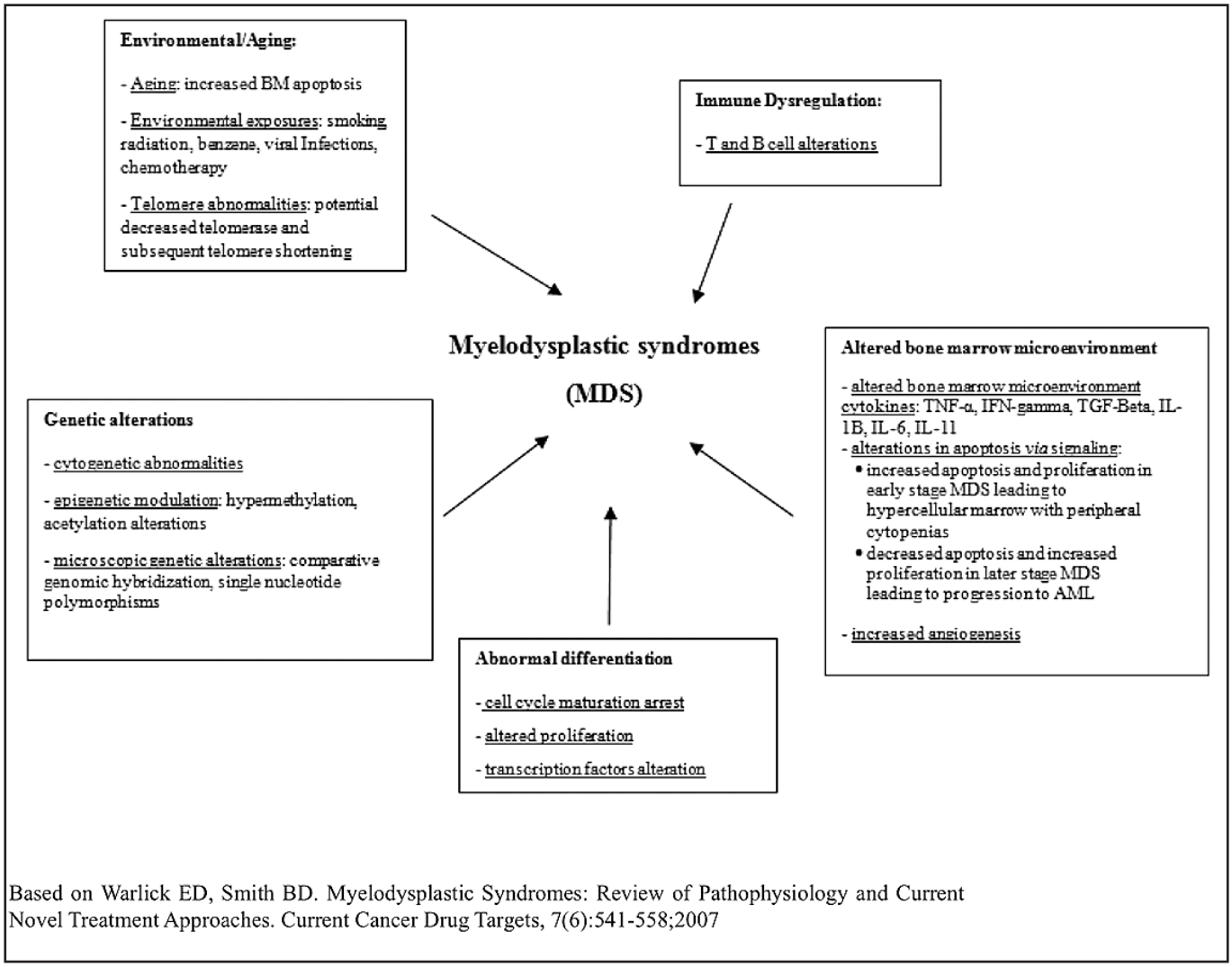

Figure 1
Theories of pathophysiology involved in MDS development.

alterations – dysplastic features – can be observed, which might affect the erythroid, myeloid and megakaryocytic cell lineages. In addition, blast counts might also be increased in severe cases. Another common characteristic is the genetic instability of the pathological cells, which results in an enhanced risk of MDS transforming into acute myeloid leukemia (AML). This transformation occurs in approximately 30 percent of the cases, and it is one of the most important causes of mortality of MDS. Further causes of mortality may include consequences of ineffective haematopiesis and the complications of cytopenia (e.g. infections, bleeding).

Overall survival time in MDS has a large interval from some months up to more than ten years, therefore correct diagnosis and accurate prognostic classification are essential for the
optimal treatment [4, 5].

CLASSIFICATION SYSTEM, PROGNOSTIC SCORING SYSTEM

In the past 30 years, several classification and prognostic scoring systems have been developed. The first widespread classification system was the French-American-British (FAB), which assigned patients to five categories: refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMML) [6]. This classification system is based on the histopathological examination of peripheral and bone marrow specimens (Table 1), where the percentage of sideroblasts and blasts are taken into consideration along with the morphologic features.

Table 1
Typical morphologic alteration in MDS

dyserythropoiesis	dysgranulopoiesis	dysmegakaryocytopoiesis
anisocytosis	nuclear/cytoplasmic asynchrony	large megakaryocytes with unsegmented nuclei
poikilocytosis	hypogranulation	micromegakaryocytes
macrocytosis	nuclear hyposegmentation pseudo Pelger- Huet cells	megakaryocytes with two or more small, unconnected nuclei
increased dacryocytes		giant hypogranular platelets
basophil stippling		
increased		
nucleated red blood cell		
nuclear fragmentation (karyiorrhexis)		
nuclear budding (bridging)		
ring sideroblasts		

The International Prognostic Scoring System (IPSS), published in 1997, was based on at least seven previous risk assessments, including the FAB classification as the most dominant source. In this study 816 primary MDS patients were examined in terms of survival and AML evolution, respectively. Patients who had previously received intensive chemotherapy and those with CMML (proliferative subtype) who had a higher white blood cell count (WBC) than 12000/µL were excluded from the analysis. All variables (blast percentage, peripheral cytopenia, cytogenetic abnormalities, age and gender) were weighed according to their statistical power. Finally three prognostic parameters – percentage of blasts, cytogenetic alteration, and the degree of peripheral cytopenia – were selected to develop a new prognostic scoring system that assigned patients into one of four risk groups: low, intermediate-1, intermediate-2, high (Table 2). There is significant difference between these groups in overall survival and in the probability of AML evolution. Patients older than 60 and assigned to the low and intermediate-1 groups exhibited significantly reduced overall survival [7].

Based on the results of IPSS the World Health Organization (WHO) made several changes to the FAB classification and introduced a new system. One of the major alterations concerned the criteria of AML. While the FAB classification established the diagnosis of AML when the blast percentage reached 30% in peripheral blood or bone marrow, the WHO reduced this threshold to 20%; furthermore, it established a new category within AML, namely, AML transformed from MDS. Consequently the former RAEB-T group is absent from the WHO classification. On the other hand, new groups were also created, such as MDS with isolated 5q deletion – MDS del(5q); refractory cytopenia with multiple cell lineage dysplasia (RCMD), and unclassified MDS – the RAEB group was also split on the basis of blast percentage (RAEB-1 and RAEB-2). The creation of the MDS del(5q) group is justified by the different therapy requirements, especially good prognosis and idiosyncratic clinical symptoms (anemia, normal or increased platelet count in the peripheral bloody, and increased count of hypolobulated megakaryocytes in the bone marrow) of these patients. According to the most recent (2008) WHO recommendations, the unclassified MDS group consists of patients with cytopenia and blast count under 1% in the peripheral blood and under 5% in the bone marrow, while upon analyzing the latter, no cell lineage can be declared dysplastic, yet characteristic cytogenetic alterations of MDS can be detected (Table 2). A cell lineage is dysplastic if clear dysplastic features are observed in at least 10% of its cells. Beyond these morphological criteria, factors causing secondary dysplasia must also be excluded (iron-, B12-, folic acid-, or copper-deficiency; infection (HIV), autoimmune disorders. [3,8,9,10,11].

The WHO Classification-Based Prognostic Scoring System (WPSS) was published in 2007, the advantage of which over IPSS is the exclusion of FAB RAEB-T- and CMML patients . These patients are currently classified in the AML and MDS/MPN (MPN: Myelo- Proliferative Neoplasm) category. Another advantage of WPSS is that it is a dynamic system that can be applied throughout the course of the illness. This is because while in the IPSS study patients were examined only at diagnosis, participants of the WPSS monitoring were repeatedly checked and re-classified if necessary. Furthermore, in addition to the WHO classification and the karyotype, the WPSS incorporated a new, independent prognostic factor that is transfusion dependency (Table 2) [12].

The above data demonstrate that morphology remains the basis for both diagnosis and prognostic classification but the current WHO recommendations (2008) and the WPSS also considers the cytogenetic and clinical features. Even if the quality of the sample is appropriate the examiners face a difficult task when looking for the minimum morphological criteria determined by the WHO and the International Working Group on Morphology of Myelodysplastic Syndrome (IWGM-MDS) (Figure 2) [13]. In

Table 2
Prognostic scoring systems in MDS

	IPSS		WPSS		R-IPSS	
prognostic variable	***BM blast(%)***	***point***	***WHO category***	***point***	***BM blast %***	***point***
	• <5	0	• RA, RARS, 5q-	0	• ≤2	0
	• 5-10	0,5	• RCMD, RCMD-RS	1	• >2-<5	1
	• 11-20	1,5	• RAEB-1	2	• 5-10	2
	• 21-30	2	• RAEB-2	3	• >10	3
	karyotype*	***point***	***karyotype****	***point***	***karyotype****	***point***
	• good	0	• good	0	• very good	0
	• intermediate	0,5	• intermediate	1	• good	1
	• poor	1	• poor	2	• intermediate	2
	cytopenias	***point***	***transfusion requirement***	***point***	• poor	3
	• 0/1	0	• no	0	• very poor	4
	• 2/3	0,5	• regular	1	***hemoglobin (g/dl)***	***point***
					• ≥10	0
					• 8-<10	1
					• <8	1,5
					platelets (G/L)	***point***
					• ≥100	0
					• 50--<100	0,5
					• <50	1
					ANC (G/L)	***point***
					• ≥0,8	0
					• <8	0,5
risk groups		***risk score***		***risk score***		***risk score***
	low	0	very low	0	very low	≤1,5
	intermediate-1	0,5-1	low	1	low	>1,5-3
	intermediate-2	1,5-2	intermediate	2	intermediate	>3-4,5
	high	≥2	high	3-4	high	>4,5-6
			very high	5-6	very high	>6
karyotype*					very good	• -Y alone • del(11q)
	good	• normal • -Y alone • del(5q) alone • del(20q)alone	good	• normal • -Y alone • del(5q) alone • del(20q) alone	good	• normal • del(5q) • del(20q) • del(12p) • double including del(5q)
	intermediate	• +8 • single miscellaneous • double abnormalities	intermediate	• +8 • single miscellaneous • double abnormalities	intermediate	• del(7q) • +8 • +19 • i(17q) • any other single/double independent clones
	poor	• ≥3 abnormalities • chrom. 7 anomalies	poor	• ≥3 abnormalities • chrom. 7 anomalies	poor	• -7 • inv(3)/t(3q)/del(3q) • double including-7/del(7q) • complex 3 abnormalities
					very poor	• complex >3 abnormalities

Based on
- *Greenberg P. et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 1997;89:2079-2088.*
- *Malcovati L. et al. Time-dependent prognostic scoring system for predicting survival and leukemic evolution in myelodysplastic syndromes. Journal of Clicinical Oncology 2007; 25:3503-3510.*
- *Greenberg PL et al. Revised international prognostic scoring system for myelodysplastic syndromes. Blood 2012; 120:2454-2465.*

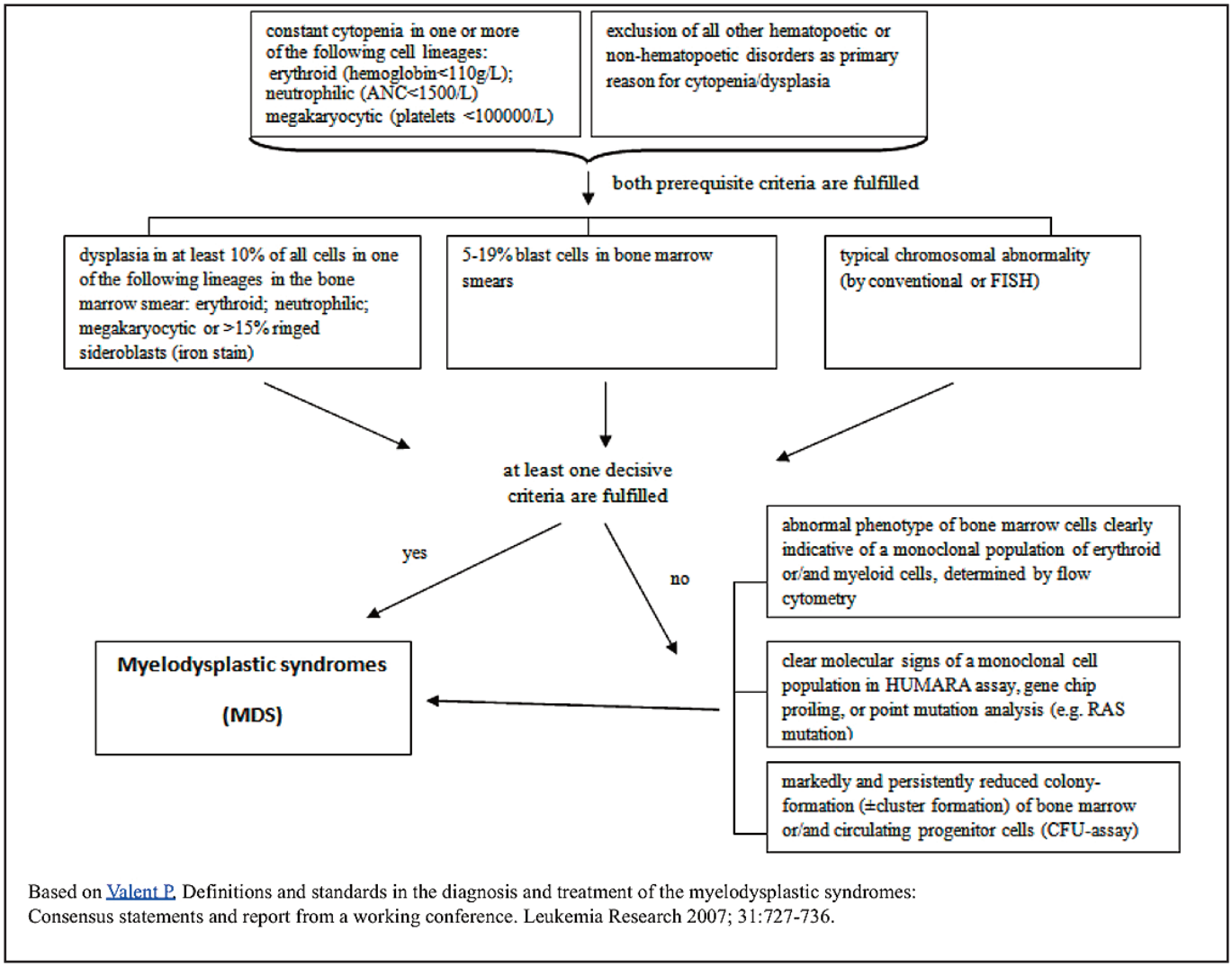

Figure 2
Minimal diagnostic criteria in MDS.

an attempt to provide an objective diagnosis and prognostic classification of MDS, in the last two decades several working groups have been trying to introduce new technologies and to establish a new system of criteria.

One of the most documented methods is the application of flow cytometry [14] (Figure 3). comparing the antigen-expression patterns of normal hematopoietic cells and of those taken from MDS patients reveals several characteristic distinctions on the blasts [15,16] as well as on cells of the myeloid [17,18], erythroid [19], and megakaryocytic lineage [20,21]. The most important are the followings: abnormal CD45 expression on the granulocytes and blast cells, decreased CD11b, HLA-DR, CD13, CD33, CD14 expression on the monocytes; attenuation or complete loss of CD11b, CD13, CD16, CD33 on the granulocytes; appearance of lymphoid markers (CD7, CD56) on granulocytes (Figure 4).

On this basis a flow-cytometric scoring system was created in 2003 (Flow Cytometric Scoring System, FCSS). The bone marrow patterns of 115 MDS and 104 other patients along with 25 healthy individuals were examined with three-color flow cytometric analysis. According to the pathological differences in the antigen-expression of the cells of the myeloid line, the intensity of the side-scatter, the myeloid-lymphoid ratio, and the blast percentage MDS patients were classified in three groups (mild, moderate, severe). Significant differences were found between the groups in terms of mean overall survival and relapse potential following allogeneic bone marrow transplantation (111 patients). Comparing the FCSS and IPSS results of MDS patients, the two systems showed good correlation, and the FCSS can offer extra information in the case of the IPSS intermedier-1 group, which facilitates prognostic stratification [17].

In the minimum diagnostic criteria system based on the agreements of the 2006 MDS conference, flow cytometry figures as a co-criterion. This way flow cytometry is indicated as a useful tool in cases where an unequivocal MDS diagnosis cannot be established on the basis of clinical data, morphology, and cytogenetics. Two such conditions are known today, namely, idiopathic cytopenia of undetermined significance (ICUS) and idiopathic dysplasia of uncertain significance (IDUS). In both

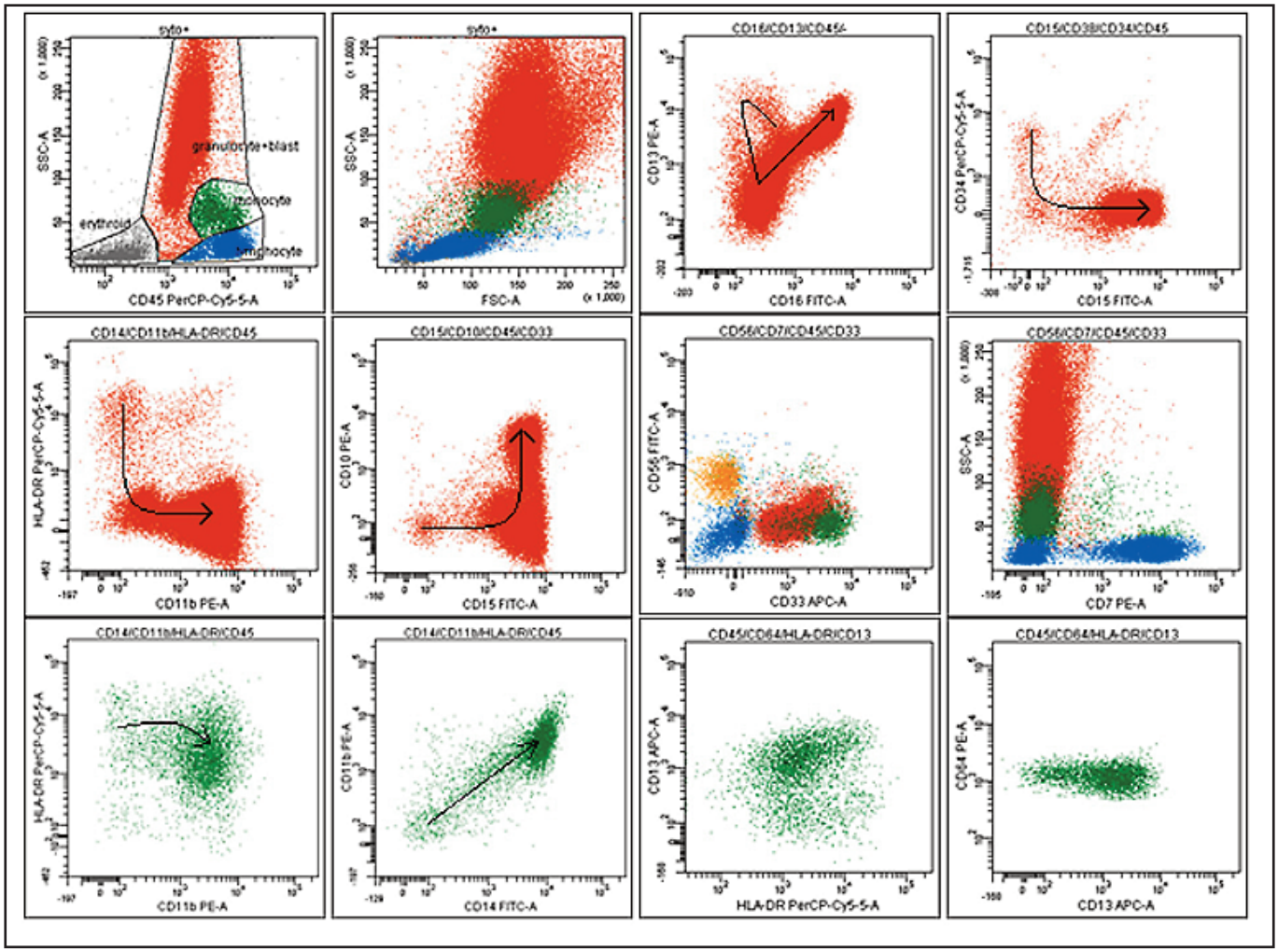

Figure 3
Normal granulocyte, monocyte maturation. The arrows indicate the maturation process.

cases, diseases causing chronic cytopenia and dysplasia can be ruled out, yet only some of the minimum criteria of MDS are met, therefore an MDS diagnosis cannot be established. In the case of ICUS, refractory cytopenia can be observed, accompanied by mildly or unmodified morphology and normal karyotype, while patients classified as having IDUS exhibit the reverse, that is, unequivocal dysplastic morphological features without the cytopenia necessary for diagnosis of MDS [13,22,23].

Summary

In summary, the diagnosis and prognostic classification of MDS seems to be the greatest challenge among all myeloid neoplasms. The uncertainty is sustained by several factors. On one hand, MDS is a rather heterogeneous group of diseases; on the other hand, the correct evaluation of morphology—which serves as the basis for diagnosis and prognosis—is a difficult task even for experienced examiners. Therefore in the past decades, to facilitate the more precise classification of patients with a number of objective studies, such as well-defined anamnestic data (e.g., number of transfusions), laboratory parameters (WBC, absolute neutrophil count, platelet count, lactate dehydrogenase value (LDH), ferritin, β2 microglobulin, etc.), cytogenetic, flow cytometric, and molecular genetic research were int he center of interest. The most recent prognostic scoring systems reflect these efforts. In case of the Revised International Prognostic Scoring System (R-IPSS), the prognostic power of several parameters (cytogenetic alterations, degree of cytopenia, LDH, ferritin, β2 microglobulin, myelofibrosis, age, sex, FAB, WHO classifications) was tested on numerous patients (IPSS n=816, R-IPSS n=7012). The analysis of such a large sample allowed the demonstration of the prognostic effect of less frequent cytogenetic alterations, thus, instead of the three cytogenetic groups of IPSS, here five groups facilitate the more precise anticipation of clinical outcome. Beside cytogenetics, the percentage of blasts, the hemoglobin concentration, platelet and absolute neutrophil count proved to be the most determining parameters. On the basis of these factors, patients are assigned to five risk groups, making the assessment of low-risk patients more precise [24,25] (Table 2).

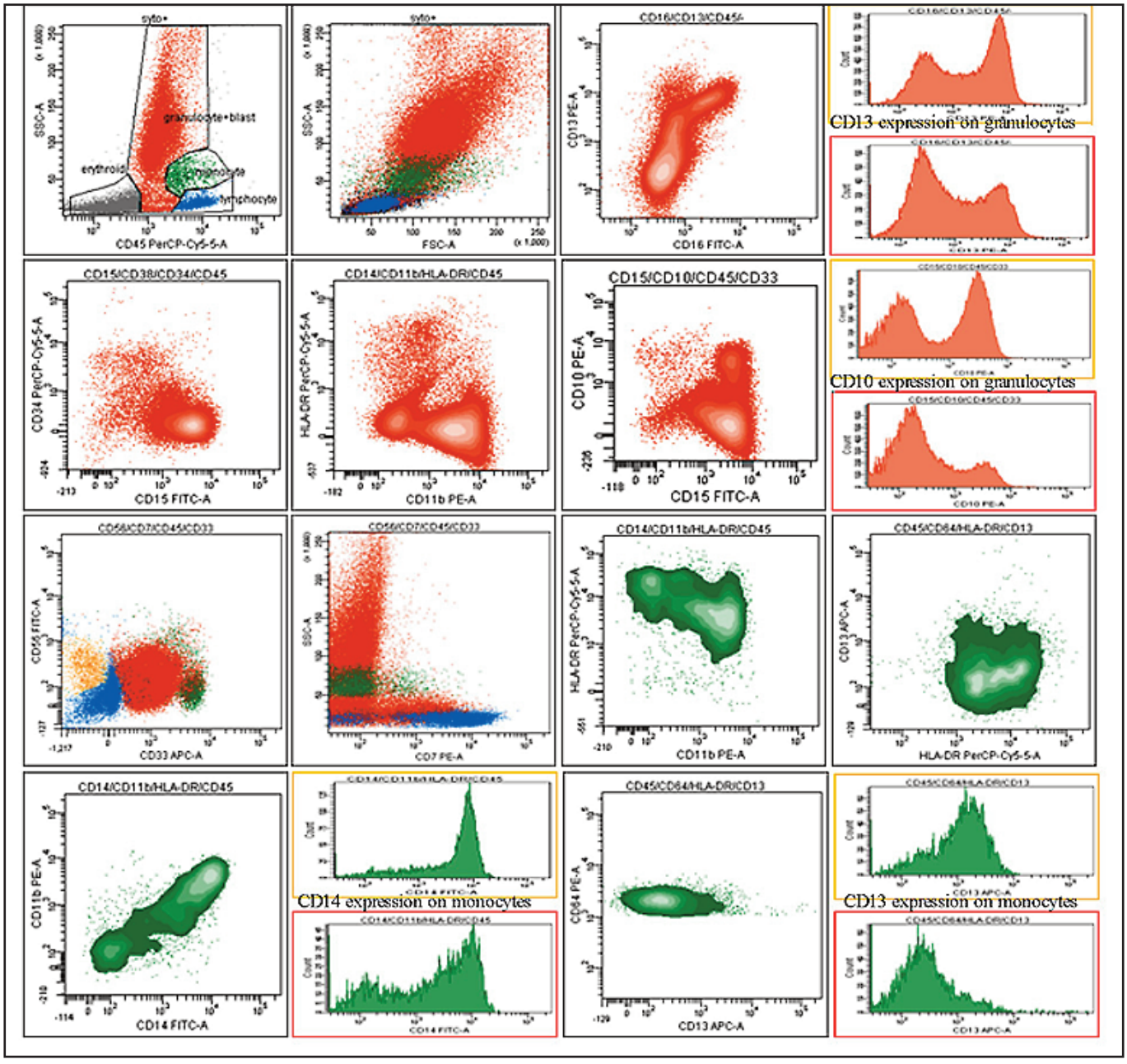

Figure 4
Characteristic distinctions of antigen-expression patterns in MDS. Histograms show one of the most important antigen expression on dysplastic (red frame) and normal (yellow frame) granulocytes or monocytes.

The most up-to-date flow-cytometric scoring scales – such as the one prepared by European LeukemiaNET – also aid in the diagnosis and prognostic classification of low-risk as well as ICUS and IDUS patients. In that study, 797 patient samples (417 low-risk MDS, 380 pathologic control samples) were analyzed by flow cytometry. According to the results, merely four cytometric parameters facilitate effectively the diagnosis of low-risk patients. These are the followings: the percentage of bone marrow blasts, the percentage of progenitor B cells within CD34 positive cells, the mean fluorescence intensity of CD45 expression in lymphocytes as compared to myeloblasts, and the granulocyte to lymphocyte side scatter ratio [18].
The results of these new studies contributed to the more objective and more precise diagnosis and clinical follow-up of MDS throughout a wider institutional spectrum.

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