Leukemia (2015) **29,** 66–75

www.nature.com/leu

ORIGINAL ARTICLE

Minimal morphological criteria for defining bone marrow dysplasia: a basis for clinical implementation of WHO classification of myelodysplastic syndromes

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The World Health Organization classification of myelodysplastic syndromes (MDS) is based on morphological evaluation of marrow dysplasia. We performed a systematic review of cytological and histological data from 1150 patients with peripheral blood cytopenia. We analyzed the frequency and discriminant power of single morphological abnormalities. A score to define minimal morphological criteria associated to the presence of marrow dysplasia was developed. This score showed high sensitivity/specificity (>90%), acceptable reproducibility and was independently validated. The severity of granulocytic and megakaryocytic dysplasia significantly affected survival. A close association was found between ring sideroblasts and SF3B1 mutations, and between severe granulocytic dysplasia and mutation of *ASXL1*, *RUNX1*, *TP53* and *SRSF2* genes. In myeloid neoplasms with fibrosis, multilineage dysplasia, hypolobulated/multinucleated megakaryocytes and increased CD34+ progenitors in the absence of *JAK2*, *MPL* and *CALR* gene mutations were significantly associated with a myelodysplastic phenotype. In myeloid disorders with marrow hypoplasia, granulocytic and/or megakaryocytic dysplasia, increased CD34+ progenitors and chromosomal abnormalities are consistent with a diagnosis of MDS. The proposed morphological score may be useful to evaluate the presence of dysplasia in cases without a clearly objective myelodysplastic phenotype. The integration of cytological and histological parameters improves the identification of MDS cases among myeloid disorders with fibrosis and hypocellularity.

Leukemia (2015) **29,** 66–75; doi:10.1038/leu.2014.161

INTRODUCTION

The pathological hallmark of myelodysplastic syndromes (MDS) is marrow dysplasia, which represents the basis of the World Health Organization (WHO) classification of these disorders.[1,2] This classification provides clinicians with a useful tool for defining the different subtypes of MDS and determining individual prognosis, and is able to guide clinical decision-making regarding therapeutic choices.[3,4]

The current diagnostic approach to MDS includes cytology to evaluate morphological abnormalities of hematopoietic cells, bone marrow biopsy to assess cellularity, fibrosis and topography, and cytogenetics to identify non-random chromosomal abnormalities.[1,5] The combination of overt marrow dysplasia (that is, the presence of dysplastic changes in at least 10% of cells of the lineage under consideration) and clonal cytogenetic abnormality allows a conclusive diagnosis of MDS, but this is found in only a portion of patients. In many instances, cytogenetics is not informative and the diagnosis of MDS is entirely based on morphological criteria.[6]

The WHO proposal raised some concern regarding minimal criteria to define marrow dysplasia, as morphological abnormalities are also present in non-clonal cytopenias and in healthy subjects.[7–9] Although in clinical trials a centralized morphological review of the diagnosis is usually performed, in routine practice the inter-observer agreement in recognition of dysplasia is still unsatisfactory.[10,11] Diagnosis of MDS may be particularly difficult in patients with early-stage disease, especially those who do not have robust morphological markers, such as ring sideroblasts.[12,13] Moreover, the diagnostic process may be challenging in the one-fifth of MDS patients with hypoplastic or fibrotic bone marrow partially overlapping the disease phenotype of aplastic anemia and primary myelofibrosis, respectively.[14,15]

In the present study we performed a systematic review of cytological and histological data from patients with peripheral blood cytopenia, who underwent a marrow examination for a clinical suspect of MDS, with the aim to identify minimal reproducible criteria to define marrow dysplasia.

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Received 19 March 2014; revised 19 April 2014; accepted 7 May 2014; accepted article preview online 20 May 2014; advance online publication, 17 June 2014

SUBJECTS AND METHODS

Patients

Procedures were in accordance with the ethical standards of the Institutional Committee on Human Experimentation, IRCCS Policlinico San Matteo, Pavia, Italy, and with the Helsinki Declaration of 1975, as revised in 2000.

Cytomorphologic and histologic findings were reviewed in different patient populations (Figure 1). Diagnostic procedures were performed according to the recommendations of the European LeukemiaNet.[5]

A first patient cohort included subjects with peripheral blood cytopenia, who underwent bone marrow examination at the Department of Hematology Oncology, IRCCS Policlinico San Matteo, Pavia, from 2001 to 2012. Patients with absolute monocytosis ($>1\times10^9$/l), prominent myeloproliferative features (that is, platelet count $\geqslant450\times10^9$/l and/or white blood cell count $\geqslant13\times10^9$/l), marrow blast percentage $\geqslant$20%, marrow hypocellularity (that is, $<$30% in individuals younger than 60 years and $<$20% in those over 60 years of age) and/or grade 2–3 marrow fibrosis according to European consensus criteria[16] were excluded. This patient population included a 'learning cohort' whose examination was aimed at defining minimal morphological criteria associated with the presence of marrow dysplasia and a 'validation cohort' in which the diagnostic value and reproducibility of the proposed criteria was to be confirmed. Seventy-four subjects with normal whole blood count, including healthy donors as well as patients who underwent marrow examination for lymphoma staging without evidence of disease marrow involvement were also included as non-pathological controls.

We then analysed two additional patient populations including cytopenic patients seen at the Department of Hematology Oncology, Pavia, in the same time period with grade 2–3 fibrosis or hypocellular marrow, respectively. The aim of these additional analyses was to define criteria for the differential diagnosis of myeloid neoplasms with marrow fibrosis and of hypocellular myeloid disorders.

Cytomorphological review

Marrow smears were morphologically reviewed using a May–Grunwald–Giemsa and iron staining by two panels of expert cytologists (panel 1 included ET, RM and RI, while panel 2 included GMR and AC) blinded to clinical data. Evaluation of bone marrow dysplasia was performed by applying 2008 WHO criteria.[1] At least 200 cells in peripheral blood smears and 500 cells in bone marrow smears, including at least 100 erythroblasts, 100 granulocytic cells and 30 megakaryocytes, were evaluated. In each case, the frequency of all morphological abnormalities observed in erythroid, granulocytic and megakaryocytic lineage was recorded.

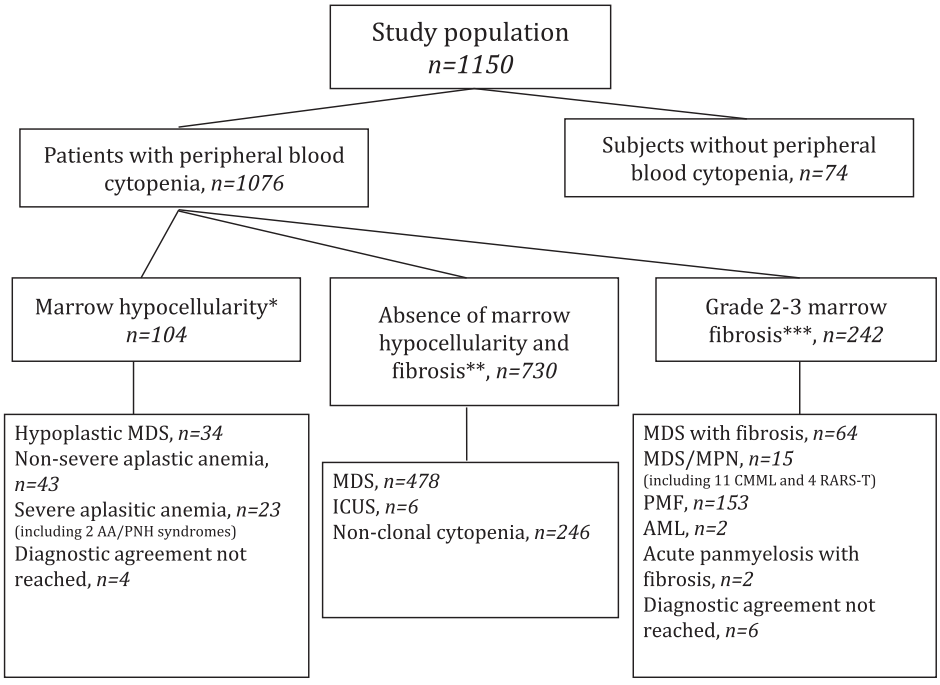
Histological evaluation

Biopsies were studied in a blinded manner by an independent pathologist panel (EB, MP, UG and GC).[1] Cellularity was evaluated in relation to age following the European consensus guidelines.[16] CD34 immunostaining was performed as previously described.[14] The tendency of CD34+ cells to form aggregates was also considered, with a cluster being defined as a group of $\geqslant$3 positive cells.[14] For the assessment of marrow fibrosis, paraffin sections were stained with Gomori's silver impregnation technique, and fibrosis was assessed semiquantitatively following the European consensus guidelines.[16]

Molecular analyses

Targeted gene sequencing of 111 genes implicated in the pathogenesis of myeloid malignancies was performed as previously described.[17] Briefly, genomic DNA samples underwent whole-genome amplification. Sequencing libraries were generated robotically in a 96-well format, each carrying a unique DNA barcode. Pools of 16 libraries were made and hybridized to RNA baits (custom Agilent SureSelect system) for all coding exons of target genes. Pools of 96 cases were sequenced on two lanes of an IlluminaHiSeq machine using the 75-bp paired-end protocol. Base substitutions and small insertions or deletions were identified using established algorithms.[18]

In addition, JAK2, MPL and CALR mutation analysis was carried out in patients affected with myeloid neoplasms with marrow fibrosis.[19]



* i.e. <30% in individuals younger than 60 years and <20% in those over 60 years of age)

** Patients with absolute monocytosis (>1x10^9/L), prominent myeloproliferative features (i.e. platelet count ≥ 450x10^9/L and/or white blood cell [WBC] count ≥ 13x10^9/L), marrow blast percentage ≥ 20% were excluded from the analysis

*** grade 2-3 marrow fibrosis according to EUMNET criteria (Haematologica. 2005 Aug;90(8):1128-32)

Abbreviations: MDS myelodysplastic syndrome; AA, aplastic anemia; PNH, paroxysmal nocturnal hemoglobinuria; ICUS, idiopathic cytopenia of undetermined significance; MPN, myeloproliferative neoplasms; PMF, primary myelofibrosis; AML, acute myeloid leukemia

Figure 1. Study population.

Statistical analysis

To define minimal criteria associated to the presence of marrow dysplasia, for each morphological parameter an optimal cutoff value to discriminate between MDS and controls was identified by adopting the receiver-operator characteristic curve method. As a second step, the weight of each parameter in the recognition of dysplasia was tested by a multivariable general logistic regression model. A score to define minimal morphological criteria for marrow dysplasia was developed based on regression coefficients from that model. Inter-operator reproducibility of morphological and histological analyses was assessed by Cohen's *K* coefficient. Acceptable reproducibility was defined as *K*-test >0.80.

Univariable and multivariable survival analyses were performed using Cox proportional hazards regression. In order to compare different multivariable models, we used the Akaike information criterion. Among a set of candidate models, a lower Akaike information criterion value indicates a better trade-off between fit and complexity. A difference of three or more in criterion values sustains a substantial difference in favor of the model with the lowest Akaike information criterion value.[20]

Detailed statistical methodology is reported in Supplementary File 1.

RESULTS

Review of MDS diagnosis and classification according to WHO 2008 criteria

Among 527 patients of the learning cohort, 324 subjects received a conclusive diagnosis of MDS at the Department of Hematology Oncology, Pavia, while 203 patients were affected with non-clonal cytopenia.

Bone marrow aspirates were reviewed by morphologist panel 1 by applying 2008 WHO criteria. The presence of morphological dysplasia was confirmed in 314 cases diagnosed with MDS, while 4 patients were diagnosed with MDS-unclassified and 6 patients were reclassified as idiopathic cytopenia of undetermined significance (Table 1A). Cytogenetics analysis was successful in 289 MDS patients, 119 of whom (41%) presented clonal chromosomal abnormalities. Oncogenic mutations were identified in 238 subjects. Most frequently mutated genes were *SF3B1* (26.5%), *TET2* (19.3%), *SFRS2* (13.9%), *ASXL1* (13.9%), *DNMT3A* (9.7%), *EZH2* (7.6%) and *RUNX1* (6.3%) (Supplementary Figure 1) Overall, oncogenic mutations and/or cytogenetic lesions sustained the presence of clonal hematopoiesis in 258 cases (82%).

Two hundred and three subjects with non-clonal cytopenia were diagnosed with iatrogenic cytopenia (including chemotherapy-induced cytopenia, $n=27$), anemia associated to chronic disease ($n=33$), anemia associated to iron and/or B12/folate deficiency ($n=53$), anemia associated to renal failure ($n=6$), hemolytic anemia ($n=15$), cytopenia associated with marrow infiltration ($n=5$), cytopenia in transplant recipients ($n=5$), infective cytopenia ($n=16$) and immune cytopenia (including idiopathic thrombocytopenic purpura, $n=43$).

Cytomorphological evaluation of erythroid dysplasia

Erythroid dysplasia according to the WHO 2008 criteria was detected in 312 MDS subjects (99%), and in 98% of cases was associated with the presence of anemia ($P<0.001$). Median number of dysplastic erythroid cells was 53% (range 10–94%). Multiple morphological abnormalities were detected in 94% of MDS patients.

As a first step we analysed the frequency of single morphological erythroid abnormalities in both MDS patients and controls (Supplementary Table 1A). Patients with non-clonal cytopenia showed morphological abnormalities in erythroid lineage in 191 cases (94%). In 131 cases (69%) the number of abnormal cells was $\geqslant$10%. Multiple cytological abnormalities were detected in 76 patients (40%). Morphological features associated with specific clinical conditions were reported in Supplementary Table 1B.

Non-cytopenic controls showed morphological abnormalities in erythroid lineage in four cases (6%), including megaloblastoid changes, cytoplasmic bridges and incomplete hemoglobinization. In one case the number of abnormal cells was $>10\%$.

Table 1A. Clinical characteristics of patients with a definitive diagnosis of MDS in the learning and validation cohorts

Clinical variable	*Learning cohort*	*Validation cohort*	P-*value*
Number of patients	318	160	
Median age (years)	70 (37–81)	69 (43–92)	NS
Sex (male/female)	183 (58%)/135 (42%)	96 (60%)/64 (40%)	NS
WHO classification			
RCUD	46 (15%)	19 (12%)	NS
Refractory anemia	44	18	
Refractory neutropenia	1	1	
Refractory thrombocytopenia	1	—	
RARS	35 (11%)	31 (19%)	
MDSdel5q	17 (5%)	6 (4%)	
RCMD	124 (39%)	47 (30%)	
RAEB-1	39 (12%)	23 (14%)	
RAEB-2	53 (17%)	32 (20%)	
MDS unclassified	4 (1%)	2 (1%)	
Absolute neutrophil count ($\times 10^9$/l)	1.46 (0.01–10.2)	1.31 (0.1–8.4)	NS
Hemoglobin (g/dl)	9.1 (5.9–12.1)	8.7 (6.2–11.8)	NS
Platelet count ($\times 10^9$/l)	92 (2–391)	85 (3–339)	NS
Transfusion-dependency	199/318 (63%)	108/160 (68%)	NS
IPSS risk	286/318 (90%)	149/160 (93%)	
Low	99 (35%)	54 (36%)	NS
Intermediate-1	122 (43%)	58 (39%)	
Intermediate-2	48 (17%)	27 (18%)	
High	17 (5%)	10 (7%)	

Abbreviations: IPSS, International Prognostic Scoring System; MDS myelodysplastic syndrome; NS, not significant; RAEB, refractory anemia with excess blasts; RARS, refractory anemia with ring sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RCUD, refractory cytopenia with unilineage dysplasia.

Megaloblastoid changes, multinuclearity, nuclear lobulation/irregular nuclear contours, pyknosis, cytoplasmic granules/inclusions, basophilic stippling, cytoplasmic vacuolization, cytoplasmic fraying, incomplete hemoglobinization and sideroblasts were more frequently reported in MDS patients than in controls (*P* from 0.007 to <0.001, Supplementary Table 2A).

A score to define minimal morphological criteria for erythroid dysplasia was developed based on multivariable general logistic regression model (Table 2 and Supplementary Figure 2). Inter-observer reproducibility in recognition of cytological parameters included in the score was independently evaluated by the two morphologist panels on bone marrow smears from 203 patients (*K*-test ranging from 0.81 to 0.95).

We tested the diagnostic value of the morphological score for recognition of erythroid dysplasia in the learning cohort. Erythroid dysplasia was correctly detected in 285 cases (sensitivity 91%). In patients stratified according to the WHO criteria, sensitivity ranged from 85% in subjects with refractory cytopenia with unilineage dysplasia to 100% in subjects with refractory anemia with ring sideroblasts. Twenty-five false-positive cases were noticed among 277 controls mainly including megaloblastic anemias ($n=8$, specificity 91%). None of the non-cytopenic controls was incorrectly classified. The positive and negative predictive value of the erythroid score were 92% and 90%, respectively.

There was a significant positive correlation between the score value and the number of dysplastic erythroblasts ($r=0.73$, $P<0.001$). No significant correlation was noticed between the erythroid score value and cytogenetic risk stratified according to MDS cytogenetic scoring system.[21] No correlation was found between the erythroid score value and the number of somatic mutations as detected by next-generation sequencing analysis. A significant association was found between the presence of ring sideroblasts and SF3B1 mutations ($P<0.001$).

Table 1B. Patients with peripheral blood cytopenia and grade 2–3 marrow fibrosis

	MDS-F	*PMF*	P-*value*
Number of patients	64	153	
Median age (years)	69 (27–78)	51 (31–87)	<0.001
Sex (male/female)	39 (61%)/25 (49%)	87 (57%)/66 (43%)	NS
Clinicobiological features			
Absolute neutrophil count ($\times 10^9$/l)	1.1 (0.01–3.2)	5.96 (1.1–10.7)	<0.001
Hemoglobin (g/dl)	8.1 (5.9–9.2)	10.3 (7.1–13.9)	<0.001
Platelet count ($\times 10^9$/l)	64 (6–198)	271 (65–919)	<0.001
Leukoerythroblastic blood smear			
Patients studied	61	150	
Positive patients	21 (35%)	137 (91%)	<0.001
Splenomegaly			
Patients studied	59	151	
Positive patients	14 (24%)	134 (89%)	<0.001
Circulating CD34+ cell count $\times 10^6$/l			
Patients studied	36	121	
Median (range)	1.5 (0–123)	26 (1–4320)	<0.001
JAK2 or MPL mutations			
Patients studied	51	145	
Positive patients	3 (6%)	98 (67%)	<0.001
CALR mutations			
JAK2/MPL-negative patients studied	12	26	
Positive patients	1	20 (77%)	<0.001
Cytological parameters			
Erythroid dysplasia			
Evaluable patients	48	47	
Positive patients	43 (90%)	16 (34%)	<0.001
Granulocytic dysplasia			
Evaluable patients	26	31	
Positive patients	20 (77%)	2 (6%)	<0.001
Megakaryocytc dysplasia			
Evaluable patients	8	13	
Positive patients	4	1	NS
Histological parameters			
Cellularity with respect to age			
Reduced/normal/increased	4 (7%)/18 (28%)/42 (65%)	38 (25%)/34 (22%)/81 (53%)	<0.001
Leukoerythroblastic ratio			
<1:1/normal/>2:1	39 (61%)/17 (26%)/8 (13%)	29 (19%)/12 (8%)/112 (73%)	<0.001
CD34 + progenitor cells (%)	5% (2–19)	<1% (0–12)	<0.001
CD34+ cell clusters	29/60 (49%)	20/149 (11%)	<0.001
Intrasinusoidal hematopoiesis	1/63 (2%)	69/153 (45%)	<0.001
Hypolobulated, multinucleated megakaryocytes, micromegakaryocytes	47/64 (73%)	15/153 (10%)	<0.001
'Cloudlike' or 'balloon-shaped' megakaryocytic nuclei	15/64 (23%)	136/153 (89%)	<0.001
Megakaryocyte clusters	52/64 (81%)	135/153 (88%)	NS

Abbreviations: MDS-F, myelodysplastic syndromes with bone marrow fibrosis; NS, not significant; PMF, primary myelofibrosis.

Cytomorphological evaluation of granulocytic dysplasia

Granulocytic dysplasia according to the WHO 2008 criteria was detected in 209 out of 314 MDS patients (67%) and in 83% of cases was associated with the presence of neutropenia ($P<0.001$). Median number of dysplastic granulocytic cells was 19% (range 10–100%). Multiple morphological abnormalities were present in 90% of MDS patients with evidence of granulocytic dysplasia.

As a first step we analysed the prevalence of single morphological abnormalities in both MDS patients and controls (Supplementary Table 2). Patients with non-clonal cytopenia showed morphological abnormalities in granulocytic lineage in 109 cases (54%). In 24 subjects (22%) the number of abnormal cells was $\geqslant$10%. Multiple cytological abnormalities were detected in 22 patients (20%). No significant association between morphological granulocytic abnormalities and specific clinical conditions was found.

Six non-cytopenic controls showed morphological abnormalities in granulocytic lineage (8%). In all cases a single morphological abnormality was present. In one case the number of abnormal cells was >10%.

Increased myeloblasts, Auer rods, nuclear/cytoplasmic asynchronism, abnormal nuclear shape, nuclear extrusions, pseudo Pelger–Hüet anomaly and neutrophil degranulation were more frequently observed in MDS than in controls (*P* from 0.024 to <0.001, Supplementary Table 2).

A morphological score to define minimal criteria for granulocytic dysplasia was developed (Table 2 and Supplementary Figure 2). *K*-coefficient for inter-observer reproducibility in recognition of granulocytic abnormalities included in the score ranged from 0.81 to 0.92. Focusing on blast cells, there was a strong agreement in the percentage of marrow blast when considered as a continuous variable (*K*-test 0.92). When stratifying the blast percentage according to the WHO thresholds, the concordance was not completely satisfactory in cases with blast count <5% (*K*-test 0.71).

Overall, 192/209 MDS patients received a correct diagnosis of granulocytic dysplasia (sensitivity 92%). Score sensitivity ranged from 86% in subjects with refractory cytopenia with multilineage dysplasia to 100% in subjects with refractory anemia with excess blasts type 2.

Table 1C. Patients with peripheral blood cytopenia and marrow hypocellularity

	Hypo-MDS	*AA*	P-*value*
Number of patients	34	66	
Median age (years)	67 (39–78)	38 (21–67)	<0.001
Sex (male/female)	21/13 (62%/38%)	28/38 (42%/58%)	0.06
Clinicobiological features			
Absolute neutrophil count ($\times 10^9$/l)	1.26 (0.07–3.4)	1.17 (0.02–3.7)	NS
Hemoglobin (g/dl)	9.5 (6.1–11.5)	10 (6.3–11.4)	NS
Platelet count ($\times 10^9$/l)	91 (6–202)	63 (2–108)	0.006
PNH clone			NS
Patients studied	22	28	
Positive patients	2 (9%)	7 (25%)	
Cytogenetics			<0.001
Patients studied	34	52	
Failed/normal/abnormal	4 (12%)/16 (47%)/14 (41%)	10 (19%)/38 (73%)/4 (8%)	
Cytological parameters			
Erythroid dysplasia			<0.001
Evaluable patients	22	31	
Positive patients	16 (71%)	3(10%)	
Granulocytic dysplasia			<0.001
Evaluable patients	25	39	
Positive patients	14 (56%)	5 (12%)	
Megakaryocytic dysplasia			—
Evaluable patients	4	5	
Positive patients	1	—	
Histological parameters			
CD34+ progenitor cells (%)	6% (1–19)	0 (0–2)	<0.001
CD34+ cell clusters	14/34 (41%)	0	<0.001
Hypolobulated, multinucleated megakaryocytes			
Patients studied	34	52	
Not evaluable/absent/present	15 (44%)/6 (18%)/13 (38%)	25 (48%)/26 (50%)/1 (2%)	<0.001

Abbreviations: AA, aplastic anemia; Hypo-MDS, hypoplastic myelodysplastic syndrome; NS, not significant.

Granulocytic dysplasia was incorrectly detected in 6 control subjects (including 3 patients with immune cytopenia and 3 patients with chemotherapy-induced neutropenia) as well as in 15 MDS patients previously diagnosed with a pure erythroid disorder (refractory cytopenia with unilineage dysplasia) by applying the WHO 2008 criteria. Overall, specificity was 95%. Based on the score cutoff value, the positive predictive value and negative predictive value were 90% and 96%, respectively.

There was a significant positive correlation between the score value and the number of granulocytic dysplastic cells ($r = 0.69$, $P < 0.001$), while a negative association was noticed between the score value and absolute neutrophil count ($r = -0.43$, $P < 0.001$). Patents with poor/very poor risk according to MDS cytogenetic scoring system, presented higher score values with respect to those with intermediate or good/very good cytogenetic risk ($P = 0.001$). A significant correlation was observed between the granulocytic score value and the number of somatic mutations ($r = 0.63$, $P < 0.001$) and a high score value was significantly associated with the presence of *ASXL1*, *RUNX1*, *TP53* and *SRSF2* gene mutations (*P* from 0.03 to 0.001).

Cytomorphological evaluation of megakaryocytic dysplasia

Thirty or more megakaryocytes were evaluable in 573 patients (97%). Megakaryocytic dysplasia according to the WHO 2008 criteria was detected in 149/306 MDS patients (49%) and in 88% of cases was associated with the presence of thrombocytopenia ($P < 0.001$). In MDS with megakaryocytic dysplasia, median number of dysplastic megakaryocytes was 53% (range 12–100%). Multiple morphological abnormalities were present in 87% of MDS patients with megakaryocytic dysplasia.

As a first step we analysed the prevalence of single morphological abnormalities in both MDS patients and controls (Supplementary Table 3). Patients with non-clonal cytopenia showed morphological abnormalities in megakaryocytic lineage in 91 cases (34%). In 22 cases (24%) the number of abnormal cells was $\geqslant$10%. Multiple cytological abnormalities were detected in 23 patients (25%). No significant association between morphological megakaryocytic abnormalities and specific clinical conditions was found.

Non-cytopenic controls showed morphological abnormalities in megakaryocytic lineage in three cases, including vacuolated, monolobulated and hypolobulated megakaryocytes, respectively. In one case the number of abnormal cells was >10%.

Micromegakaryocytes, small binucleated forms, megakaryocytes with multiple separated nuclei and hypolobulated/monolobulated megakaryocytes were more frequently detected in MDS with respect to controls ($P < 0.001$, Supplementary Table 3).

A score to define minimal morphological criteria for megakaryocytic dysplasia was developed (Table 2 and Supplementary Figure 2). *K*-coefficient for inter-observer reproducibility in recognition of megakaryocytic abnormalities ranged from 0.81 to 0.88.

Overall, 137/149 MDS patients received a correct diagnosis of megakaryocytic dysplasia (sensitivity 92%). Megakaryocytic dysplasia was incorrectly detected in 12 controls (3 patients with immune cytopenia, 5 with cytopenia induced by chemotherapy and 4 with infective cytopenia) and in 26 MDS patients (3 refractory cytopenia with unilineage dysplasia, 13 refractory cytopenia with multilineage dysplasia and 10 refractory anemia with excess blasts) previously diagnosed without megakaryocytic dysplasia by applying the WHO 2008 criteria. Overall, specificity was 91%. Based on the score cutoff value, the positive predictive value and negative predictive value were 78% and 97%, respectively.

Table 2. Calculation of the morphological score for the definition of bone marrow dysplasia

Morphological abnormalities[a]	*Cutoff values*[b]	*AUC*	*Cohen's K-coefficient (inter-observer agreement)*[c]	*Variable weighted score*[d]
Erythroid lineage				
Megaloblastoid changes	>5%	0.814, $P<0.001$	0.83	2
Bi- or multinuclearity	>3%	0.679, $P<0.001$	0.87	1
	>5%	0.698, $P<0.001$		2
Nuclear lobulation or irregular contours	>3%	0.674, $P<0.001$	0.84	1
Pyknosis	>5%	0.677, $P<0.001$	0.81	1
Cytoplasmic fraying	⩾7%	0.602, $P<0.001$	0.82	1
Ring sideroblasts	>5%	0.650, $P<0.001$	0.95	2
	⩾15%	0.719, $P<0.001$		3
Ferritin sideroblasts	⩾30%	0.670, $P<0.001$	0.92	1
Granulocytic lineage				
Myeloblasts	>3%	0.777, $P<0.001$	0.92	1
	>5%	0.723, $P<0.001$		3
Auer rods	⩾1%	0.524, $P=0.001$	0.90	3
Pseudo Pelger–Hüet anomaly	>3%	0.714, $P<0.001$	0.87	1
	>5%	0.814, $P<0.001$		2
Abnormal nuclear shape	⩾7%	0.700, $P<0.001$	0.86	1
Neutrophil hypogranulation	>3%	0.791, $P<0.001$	0.81	1
	>5%	0.821, $P<0.001$		2
Megakaryocytic lineage				
Micromegakaryocytes	>5%	0.916, $P<0.001$	0.88	3
Small binucleated megakaryocytes	>5%	0.845, $P=0.001$	0.81	1
Megakaryocytes with multiple separated nuclei	>5%	0.750, $P<0.001$	0.84	2
Hypolobated or monolobar megakaryocytes	>5%	0.646, $P<0.001$	0.86	2

Abbreviation: AUC, area under the receiver operating curve. [a]Standardized definition of morphological abnormalities and representative pictures are reported in Supplementary File 2 and Supplementary Figure 2, respectively. [b]Percentage of hematopoietic cells carrying the specific morphological abnormality. [c]Inter-observer agreement between two morphologist expert panels was evaluated in bone marrow samples from 203 patients. [d]Erythroid dysplasia was defined in the presence of a score value ⩾3 (a minimum of 10% of dysplastic erythroid cells is required to reach a score value ⩾3); Granulocytic dysplasia was defined in the presence of a score value ⩾3 (a minimum of 10% of dysplastic granulocytic cells is required to reach a score value ⩾3, with the exception of cases with >5% blasts or with the presence of Auer rods); Megakaryocytic dysplasia was defined in the presence of a score value ⩾3; (a minimum of 10% of dysplastic megakaryocytes is required to reach a score value ⩾3, with the exception of cases with >5% micromegakaryocytes).

There was a significant positive correlation between the megakaryocytic score value and number of dysplastic megakaryocytes ($r=0.76$, $P<0.001$), while a negative correlation was noticed between the score value and platelet count ($r=-0.32$, $P=0.001$). No significant correlation was noticed between the score value and cytogenetic risk according to MDS cytogenetic scoring system. A borderline association was noticed between the score value and the number of somatic mutations as detected by next-generation sequencing ($P=0.057$).

Validation of the diagnostic utility of the morphological score

The diagnostic value and the inter-observer reproducibility of the morphological score were tested in an independent cohort of 203 patients, including 160 subjects with a conclusive diagnosis of MDS and 43 affected with non-clonal cytopenia. (Table 1A) Erythroid, granulocytic and megakaryocytic dysplasia according to the WHO criteria were present in 153 (97%), 104 (66%) and 56 (35%) cases, respectively. Samples were independently reviewed by the two panels of expert morphologists. The results of the diagnostic validation of the morphological score were reported in the Table 3. Overall, *K*-coefficient for agreement in the WHO category attribution between the two panels was 0.82. Focusing on patients without excess blasts, *K*-coefficient was 0.75, while considering patients with excess blasts, *K*-coefficient was 0.87.

Prognostic effect of the evaluation of marrow dysplasia by the morphological score

We performed these analyses on 478 MDS patients belonging to both learning and validation cohort. Erythroid morphological score value did not significantly affect patient survival (Figure 2a), while the presence of both granulocytic and megakaryocytic dysplasia as assessed by morphological score had a significant prognostic effect both in univariable analysis ($P<0.001$, Figure 2b) as in a multivariable Cox model considering demographic factors, hemoglobin level, neutrophil and platelet count, percentage of marrow blasts and MDS cytogenetic scoring system as covariates (hazard ratio: 3.26; $P<0.001$ and hazard ratio: 2.21, $P<0.001$, respectively).

In order to identify the best threshold of dysplastic granulocytes and megakaryocytes in capturing prognostic information in MDS patients, we fitted multiple multivariable models considering different cutoff values (⩾10, ⩾30 and ⩾60) and compared them by Akaike criterion to assess the better trade-off between fit and complexity among these different models. Focusing on granulocytic dysplasia, Akaike values for models including as cut off ⩾10, ⩾30 and ⩾60% of dysplastic cells were 428, 434 and 430, respectively, thus indicating that current WHO criteria are more likely to capture prognostic information in MDS patients. Focusing on megakaryocytic dysplasia, Akaike values for models including as cutoff ⩾10, ⩾30 and ⩾60% dysplastic cells were 421, 411 and 418, respectively, thus indicating that cutoff ⩾30% is more likely to capture prognostic information in MDS patients.

Differential diagnosis of myeloid neoplasms with grade 2–3 marrow fibrosis

To define cytomorphological and histological criteria for the differential diagnosis of myeloid neoplasms with fibrosis, we

Table 3. Independent diagnostic validation and inter-observer reproducibility of the morphological score

	Morphologist panel 1	*Morphologist panel 2*	*Concordance between panel 1 and 2 (K-test)*
Erythroid dysplasia			
Patients with dysplasia correctly detected	141/153	137/153	0.83
Sensitivity	92%	87%	
Patients without dysplasia correctly detected	44/48	42/48	
Specificity	92%	88%	
Granulocytic dysplasia			
Patients with dysplasia correctly detected	93/104	94/104	0.82
Sensitivity	89%	90%	
Patients without dysplasia correctly detected	95/97	85/97	
Specificity	98%	88%	
Megakaryocytic dysplasia			
Patients with dysplasia correctly detected	50/56	48/56	0.86
Sensitivity	89%	86%	
Patients without dysplasia correctly detected	143/145	136/145	
Specificity	99%	94%	

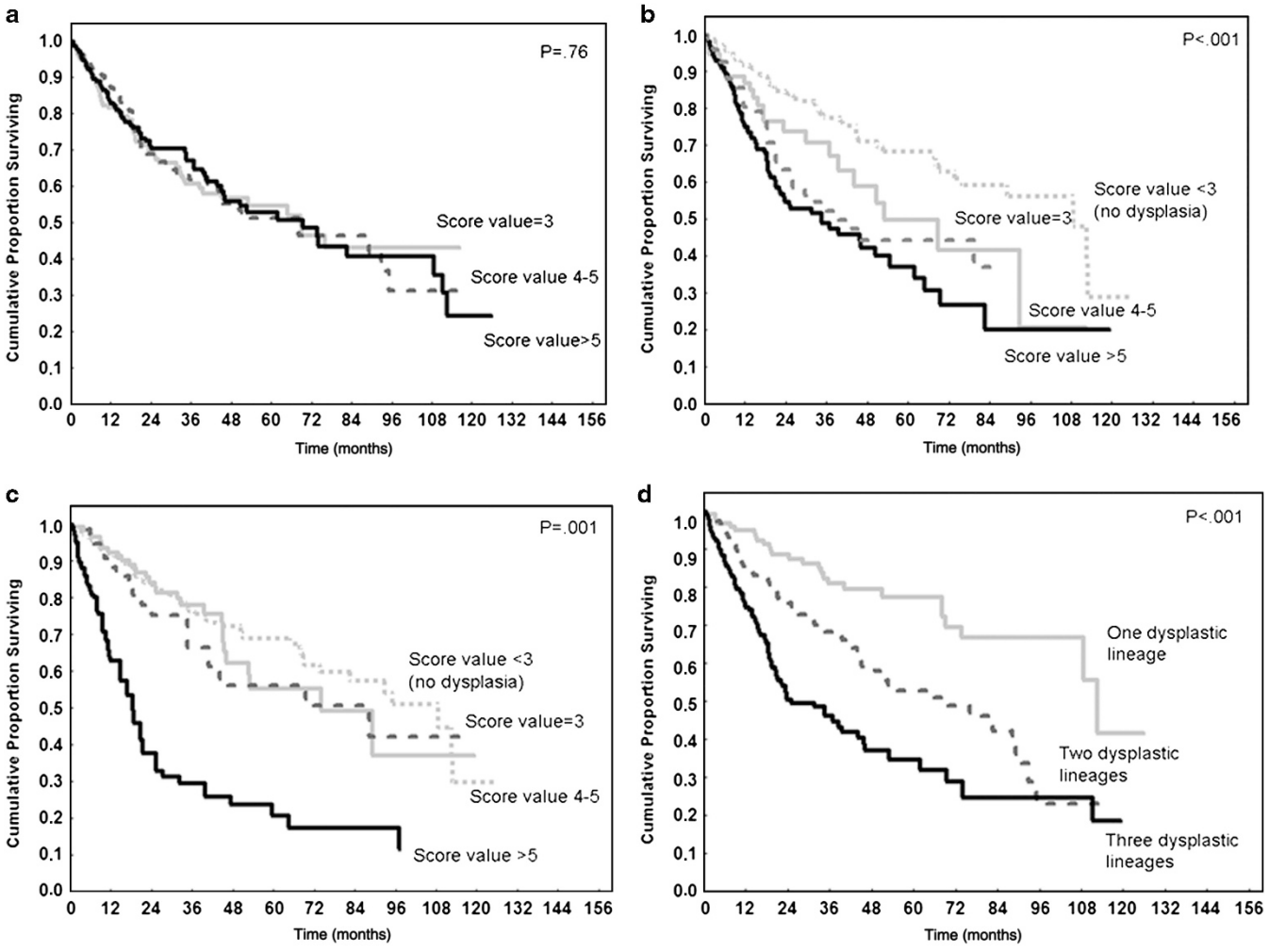


Figure 2. (**a**–**c**) Overall survival according to erythroid, granulocytic and megakaryocytic morphological score value; (**d**) overall survival according to the number of dysplastic hematopoietic lineages as defined by applying morphological scores.

evaluated patients with peripheral blood cytopenia and grade 2–3 bone marrow fibrosis seen at the Department of Hematology Oncology, Pavia, from 2001 and 2012. Overall, 242 patients matched selection criteria. A review of histologic parameters as well as an evaluation of marrow dysplasia by using the above-defined morphological score was performed (Figure 1).

We compared clinicopathological characteristics of the 64 patients definitely classified as MDS with bone marrow fibrosis (MDS-F) with those of the 153 patients with primary myelofibrosis (PMF) (Table 1B and Supplementary Figure 3). Patients with MDS-F had more profound cytopenia and lower circulating CD34+ cell count ($P<0.001$). Leukoerythroblastic blood smear and splenomegaly were less common in patients with MDS-F ($P<0.001$), and only four of them carried *JAK 2*, *MPL* or *CALR* gene mutations ($P<0.001$).

Cytological erythroid dysplasia was evaluable in 95 cases (44%). Erythroid dysplasia was found in 90% of evaluable MDS-F patients and in 34% of PMF patients (more common morphological abnormalities in PMF included megaloblastic changes and sideroblasts). Granulocytic dysplasia was evaluable in 89 patients (41%), while blast count according to the WHO criteria was evaluable in 57 cases (26%). Granulocytic dysplasia was found in 77% of evaluable MDS-F patients and in 6% of PMF patients. In both groups, megakaryocytic dysplasia was evaluable only in a minority of cases (21 patients, 9%).

According to histological evaluation, patients with MDS-F had more frequently erythroid hyperplasia, hypolobulated megakaryocytes or megakaryocytes with multiple separated nuclei ($P<0.001$), increased CD34+ progenitors and CD34+ cell clusters ($P<0.001$). By contrast, patients with PMF more frequently presented increased leukoerythroblastic ratio ($P<0.001$), 'cloudlike' or 'balloon-shaped' megakaryocytic nuclei ($P<0.001$) and intrasinusoidal hematopoiesis ($P<0.001$).

A diagnostic score for myeloid neoplasms with fibrosis was developed, including both cytological and histological parameters (Table 4). *K*-coefficient for inter-observer reproducibility of score parameters ranged from 0.84 to 0.95.

Two hundred and seventeen patients entered the analysis. In 21 cases (10%) a conclusive diagnosis was not reached by applying the score. We then focused on 196 evaluable patients; a correct diagnosis of MDS-F was performed in 61 out of 64 patients (sensitivity 95%), while 9 cases affected with PMF were incorrectly classified as MDS-F (95% specificity). Positive and negative predictive values were 87% and 98%, respectively.

Differential diagnosis of myeloid disorders with marrow hypocellularity

To define criteria for the differential diagnosis of myeloid disorders with reduced marrow cellularity, we evaluated patients with peripheral blood cytopenia and bone marrow hypocellularity seen at the Department of Hematology Oncology, Pavia, from 2001 and 2012. Overall, 104 subjects matched selection criteria. A review of histological parameters as well as an evaluation of marrow dysplasia by using the above-defined morphological score was performed (Figure 1).

We then compared clinicopathological characteristics of the 34 patients definitely classified as hypoplastic-MDS (Hypo-MDS) with those of the 66 patients with aplastic anemia (Table 1C and Supplementary Figure 4).

Cytological erythroid dysplasia was evaluable in 53 cases (53%). Erythroid dysplasia was found in 71% of evaluable Hypo-MDS patients and in 10% of patients with aplastic anemia ($P<0.001$). Granulocytic dysplasia was evaluable in 64 patients (64%), and was found in 56% of evaluable Hypo-MDS patients and in 12% of patients with aplastic anemia ($P<0.001$). Megakaryocytic dysplasia was evaluable only in a little proportion of patients (9%).

According to histological evaluation, patients with Hypo-MDS present increased CD34+ hematopoietic progenitors and CD34+ cell clusters ($P<0.001$) with respect to patients with aplastic anemia. Finally, MDS patients present more frequently abnormal karyotype ($P<0.001$).

A diagnostic score for the diagnosis of Hypo-MDS was developed (Table 5). *K*-coefficient for inter-observer reproducibility of score parameters ranged from 0.81 to 0.90.

According to this model, a correct diagnosis of MDS was performed in 28/34 patients (sensitivity 82%). None of the patients affected with marrow aplasia was incorrectly classified as MDS (100% specificity). Positive and negative predictive values were 100% and 92%, respectively.

DISCUSSION

According to the WHO classification, dysplasia and ineffective hematopoiesis in one or more of the major myeloid cell lines, defined as a percentage of cells manifesting morphological abnormalities $\geqslant$10%, are distinctive characteristics of MDS.[1] The characteristics of dysplasia are relevant when distinguishing between the various types of MDS and may be important in predicting disease biology and outcome.[17,18,21,22]

Major limitations in the diagnostic approach to MDS lay in the scarce reproducibility of morphological analysis of dysplasia and in the poor specificity of several dysplastic changes that make difficult differentiating MDS from other non-clonal disorders.[8,11,12,23] According to previous observations, in our study morphological abnormalities involving 10% or more cells (mostly in erythroid lineage) were detected in a significant proportion of control patients affected with non-clonal cytopenia, and in some non-cytopenic controls.[6,9,24,25]

Taking advantage of a systematic morphological review of a large patient population, we herein analysed the frequency and

Table 4. Calculation of the score for the differential diagnosis of myeloid neoplasm with bone marrow fibrosis

Variable	*Cohen's* K-*coefficient (inter-operator variability)*	*Variable weighted score*[a]
Cytological parameters		
Morphological erythroid and/or granulocytic dysplasia (according to morphological score)	0.86	1
Histological parameters		
Leukoerythroblastic ratio >2:1	0.88	−1
Hypolobulated, multinucleated megakaryocytes	0.84	1
'Cloudlike' or 'balloon-shaped' megakaryocytic nuclei	0.90	−1
CD34+ progenitor cells <1%	0.91	−1
CD34+ progenitor cells $\geqslant$5%	0.93	2
Presence of CD34+ cell clusters	0.92	1
Intrasinusoidal hematopoiesis	0.95	−1

Abbreviations: MDS-F, myelodysplastic syndromes with bone marrow fibrosis; PMF, primary myelofibrosis. Most relevant histological features in MDS-F and PMF patients are reported in Supplementary Figure 3. [a]A score value >0 is suggestive for a diagnosis of MDS-F, while a negative score value is suggestive for a diagnosis of PMF. In case of score value of 0, no conclusive diagnosis can be made.

Table 5. Calculation of the score for the differential diagnosis of myeloid disorders associated with reduced marrow cellularity

Variable	*Cohen's K-coefficient (inter-operator variability)*	*Score value*[a]
Cytological parameters		
Morphological erythroid dysplasia (according to morphological score)	0.81	1
Morphological granulocytic dysplasia (according to morphological score)	0.83	1
Histological parameters		
Hypolobulated, multinucleated megakaryocytes	0.82	1
CD34+ progenitor cells $\geqslant$5%	0.91	2
Presence of CD34+ cell clusters	0.90	1
Molecular features		
Abnormal karyotype (excluding trisomy 8)	—	2

Abbreviations: MDS-Hypo, myelodysplastic syndromes with marrow hypocellularity. Most relevant histological features in patients affected with MDS-Hypo are reported in Supplementary Figure 4. [a]A score value $\geqslant$3 is suggestive for a diagnosis of MDS.

the impact of cytomorphological bone marrow abnormalities in patients with peripheral blood cytopenias, and we combined them into a score, which allows us to define minimal criteria associated to the presence of marrow dysplasia. The morphological score showed a high sensitivity and specificity (>90%), even in patients with early-stage disease that frequently lack specific markers of dysplasia and abnormal karyotype. These results were confirmed in an independent patient population. None of the subjects without peripheral blood cytopenia (including healthy subjects) was incorrectly classified. Importantly, by applying standardized criteria the inter-observer reproducibility for the definition of each morphological variable associated with marrow dysplasia was satisfactory.

Among morphological parameters considered by the WHO classification, two have relevant prognostic implications, that is, multilineage dysplasia and the percentage of bone marrow blasts.[1,26–29] We observed that granulocytic and megakaryocytic dysplasia as assessed by morphological score significantly affected the probability of survival. The threshold of 10% of granulocytic dysplastic cells was the best cutoff to capture adverse prognosis, while a threshold of 30% of dysplastic megakaryocytes appeared more appropriate to detect patients with reduced survival.[2,23,24,27]

The percentage of blast cells in peripheral blood and bone marrow is closely associated with the risk of leukemic evolution and its evaluation is included in all currently available prognostic scores.[26,28,29] By applying standardized criteria for recognizing blast cells[13] we obtained an adequate inter-observer agreement for cases with $\geqslant$5% bone marrow blasts, while it was not as good when the blast count was <5%. These findings may have some clinical implication, as a cutoff of 2% blast in bone marrow was introduced in the revised International Prognostic Scoring System to define early-stage patients with increased risk of disease progression.[29]

Important steps have recently been made in characterizing the molecular basis of MDS, and acquired somatic mutations have been detected in several genes (encoding for components of RNA spliceosome, DNA methylation, chromatin modification, transcription regulation, DNA repair, signal transduction, cohesin complex and others).[17,18,30–32]

Although the spread of massive genotyping methods will soon make it possible for clinicians to detect a broad range of genetic aberrations at a reasonable cost, morphology will likely continue to have clinical relevance in MDS. In fact, a large genomic heterogeneity is present within MDS patient population (with only few genes consistently mutated in >10% of cases) and the driver genes whose mutations are responsible for MDS are frequently mutated in other myeloid neoplasms.[17,29–32] In addition, the implementation of standardized morphological criteria to define marrow dysplasia is essential to define specific associations between genotype and disease phenotypes, and then recognize disease entities based on distinctive genetic profiles.[17,18] In this context, we confirmed a close relationship between the presence of ring sideroblasts and SF3B1 mutations,[31] and observed an association between severe granulocytic dysplasia as detected by morphological score and mutations of *ASXL1*, *RUNX1*, *TP53* and *SRSF2* genes, that were reported to increase the risk of leukemic evolution.[32]

As 15%–20% of MDS present marrow fibrosis or hypocellularity, we addressed the issue of differential diagnosis among either myeloid neoplasms with fibrosis and myeloid disorders with marrow hypoplasia. In both cases the cytological WHO criteria for detecting marrow dysplasia are difficult to apply due to the presence of a low number of cell in marrow specimens, and the integration between cytological and histological evaluation is even more important to reach a correct diagnosis.[14,15]

MDS-F are characterized by severe marrow failure and high risk of leukemic evolution.[14,33] The most common scenario in clinical practice is the differential diagnosis between MDS-F and PMF. The presence of multilineage dysplasia of hypolobulated/multinucleated megakaryocytes and increased CD34+ marrow progenitor cell percentage were the parameter most significantly associated with a myelodysplastic phenotype, whereas patients with PMF had more frequently granulocytic hyperplasia, 'cloudlike' or 'balloon-shaped' megakaryocytic nuclei and intrasinusoidal hematopoiesis. We showed that the combination of these parameters into a score allows a correct classification of the great majority of cases with satisfactory inter-observer agreement.

Mutations in genes of JAK/STAT pathway are found in ~60–70% of patients with PMF and predicts the risk of major clinical events. Recently, CALR mutations were described in most JAK2- and MPL-negative patients.[19,34] By contrast, these mutations are rarely observed in MDS-F.[14]

Among myeloid disorders with marrow hypoplasia we found that the presence of clear morphological dysplasia (especially in granulocytic/megakaryocytic lineage), increased percentage of CD34+ marrow progenitors and/or CD34+ cell clusters are consistent with a myelodysplastic phenotype. Isolated erythroid dysplasia may be present also in cases with a definitive diagnosis of aplastic anemia.[35,36] Cytogenetics abnormalities are more frequently (although not exclusively) found in MDS cases. It must be emphasized, however, that these markedly fatty marrows are frequently cytogenetic failure. By integrating cytological and histological evaluation, a correct classification was obtained in >80% of cases.

In conclusion, the proposed morphological score for detecting marrow dysplasia may represent a useful tool that can be implemented in the work-up of patients with suspected MDS. The integration of cytological and histological parameters significantly improves the identification of MDS cases among myeloid disorders with fibrosis and hypocellularity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by the Fondazione Berlucchi, Brescia, Fondazione Veronesi, Milan, Fondazione IRCCS Policlinico San Matteo, Pavia, and Fondazione Cariplo/Regione Lombardia, Milan, Italy, to MGDP and by the Associazione Italiana per la Ricerca sul Cancro, Milan, and Fondazione Cariplo, Milan, to MC.

AUTHOR CONTRIBUTIONS

MGDP, ET, EB, RI and MC conceived this study. ET, EB, MP, LM, GMR, UG, RM, IA, CE, MU, GC, FQ, RB, AC, EM and AO collected data. EP, DP and PJC performed molecular analyses. CP and VF analyzed the data. MGDP, ET, EB, MP, AO, RI and MC wrote the manuscript, which was approved by all authors.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)