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High flow cytometric scores identify adverse prognostic subgroups within the revised international prognostic scoring system for myelodysplastic syndromes

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Summary

The estimation of survival of myelodysplastic syndromes (MDS) and risk of progression into acute myeloid leukaemia is challenging due to the heterogeneous clinical course. The most widely used prognostic scoring system (International Prognostic Scoring System [IPSS]) was recently revised (IPSS-R). The aim of this study was to investigate the prognostic relevance of flow cytometry (FC) in the context of the IPSS-R. Bone marrow aspirates were analysed by FC in 159 patients with MDS. A flow score was calculated by applying the flow cytometric scoring system (FCSS). Patients were assigned to IPSS and IPSS-R risk groups. The FCSS correlated with the World Health Organization classification, IPSS and IPSS-R risk groups. Mild flow cytometric abnormalities were associated with significantly better overall survival (OS) and lower risk of disease evolution. The presence of aberrant myeloid progenitors was associated with transfusion dependency and disease progression. Most importantly, the FCSS identified prognostic subgroups within the IPSS-R cytogenetic good risk and low risk group. Flow cytometric analysis in patients with MDS provides additional prognostic information and is complementary to the IPSS-R. The addition of a flow cytometric score next to the clinical parameters within the IPSS-R is a further refinement of prognostication of patients with MDS.

Keywords: flow cytometry, myelodysplastic syndromes, prognosis, IPSS-R.

The myelodysplastic syndromes (MDS) represent a group of clonal myeloid disorders with heterogeneous clinical presentation and course. The clinical course of patients with MDS is characterized by two scenarios: the development of progressive cytopenia or transformation into an acute myeloid leukaemia (AML). Estimation of survival and/or risk to develop an AML are challenging due to heterogeneity even within subgroups of MDS. The World Health Organization (WHO) classification, although devised for the diagnosis of MDS, also includes prognostic information (Malcovati *et al*, 2005a). The WHO classification already recognized that an increased percentage of blasts and the presence of multi-lineage dysplasia by morphology (i.e. dysplasia in the erythroid, megakaryocytic and myeloid lineage) in the bone marrow (BM) is associated with an adverse prognosis. The International Prognostic Scoring System (IPSS) is based on three components consisting of the percentage of BM blasts, cytogenetics and the number of cytopenias in the peripheral blood, which are scored for in a weighed manner (Greenberg *et al*, 1997). The IPSS recognizes four different risk groups (low, intermediate-1, intermediate-2 and high), characterized by increasing risk of death and transformation to AML. The scoring system provides prognostic information on newly diagnosed patients with MDS, guides treatment decision-making and is helpful in selecting patients for clinical trials. Evidence emerged that the in cytogenetic subgroups, the weight that was assigned to each variable in the score and the cut-off points of the other components of the IPSS should be redefined (Malcovati *et al*, 2011; Schanz *et al*, 2011). Recently a collaborative approach resulted in revision of the IPSS (IPSS-R) (Greenberg *et al*, 2012). The most important adjustments are that in the IPSS-R, five cytogenetic categories are applied instead of three in the IPSS, the cut-off points for the percentage of blasts in the BM are adjusted and the depth of the individual cytopenias are taken into account.

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Although not yet included in the revised IPSS, studies applying molecular analyses and flow cytometry (FC) show that these techniques might add to identification of subgroups and refinement of prognostication of MDS.

In particular, FC has shown advancement for the prognostication of MDS (Wells *et al*, 2003; van de Loosdrecht *et al*, 2008; Scott *et al*, 2008). The application of FC for MDS is based on the concept that subtle disturbances in haematopoiesis that are not recognized by morphology, can be determined by FC (Malcovati *et al*, 2005b; van de Loosdrecht *et al*, 2008). This has consequences for both the diagnosis and prognosis of patients with MDS. A flow cytometric scoring system (FCSS) was developed by Wells *et al* (2003), and showed that patients with severe dyspoiesis by FC have worse prognosis compared with MDS patients with no-to-mild abnormalities (van de Loosdrecht *et al*, 2008; Scott *et al*, 2008). The severity of flow cytometric abnormalities in haematopoiesis, as reflected by a high FCSS, was predictive for post-allogeneic transplantation outcome in MDS (Wells *et al*, 2003; Scott *et al*, 2008). Previous studies have shown that the FCSS correlates with the WHO and IPSS classification and has prognostic value for the clinical behaviour of MDS (Wells *et al*, 2003; van de Loosdrecht *et al*, 2008; Scott *et al*, 2008; Kern *et al*, 2010; Matarraz *et al*, 2010; Chu *et al*, 2011). The feasibility of the implementation of FC for the diagnosis and prognosis of MDS is currently being investigated by an international working party (van de Loosdrecht *et al*, 2009, 2013; Westers *et al*, 2012).

The aim of this study was to investigate whether the FCSS is of prognostic value in the context of the IPSS-R. We here show that the FCSS combined with the IPSS-R was a better predictor for overall survival of patients with MDS than the IPSS-R on its own, which indicates that flow cytometric analysis is instrumental for the refinement of prognostication in MDS.

Materials and methods

Patients

Patients (n = 159; 103 male vs. 56 female) meeting the minimal diagnostic criteria for MDS (Valent *et al*, 2007) were included in the study between 2004 and 2012. The characteristics of the patients with MDS are described in Table I. From this cohort, data from 54 patients were previously reported (van de Loosdrecht *et al*, 2008; Westers *et al*, 2010). Cytomorphology (May-Grünwald-Giemsa and Perl stain for iron) was evaluated by two haematologists who arehighly experienced in the diagnosis of MDS by morphology. The WHO 2001, 2008 and French-American-British (FAB) classifications were applied for the diagnosis (Bennett *et al*, 1982; Jaffe *et al*, 2001; Swerdlow *et al*, 2008). Patients diagnosed with FAB refractory anaemia with excess blasts in transformation (RAEB- t), which is classified as AML with 20–30% blasts in both of the WHO classifications, were also included in the study to render the patient cohort comparable with the IPSS and IPSS-R cohorts. Conventional karyotyping was performed by using the International System for Human Cytogenetic Nomenclature (ISCN) guidelines (Mitelman, 1995). In cases where no metaphases could be analysed, fluorescence *in situ* hybridization (FISH) was executed as recommended (Valent *et al*, 2007). All samples were drawn after informed consent and in conformance with the Declaration of Helsinki. The study was approved by the Medical Ethics Committee of VU University Medical Centre, Amsterdam, Netherlands.

Table I. Patient characteristics.

	n (%)
WHO 2001 classification (Jaffe *et al*, 2001)	159
RA(RS)	20 (13)
RCMD(RS)	71 (45)
RAEB-1	21 (13)
RAEB-2	23 (14)
MDS-U	7 (4)
MDS with del(5q)	1 (1)
Hypoplastic MDS	7 (4)
FAB classification (Bennett *et al*, 1982)	
RAEB-T	9 (6)
IPSS (Greenberg *et al*, 1997)	158*
Low	50 (32)
Intermediate-1	65 (41)
Intermediate-2	29 (18)
High	14 (9)
Cytogenetics known	144
Good	107 (74)
Intermediate	18 (13)
Poor	19 (13)
IPSS-R (Greenberg *et al*, 2012)	155**
Very low	22 (14)
Low	60 (39)
Intermediate	35 (23)
High	27 (17)
Very high	11 (7)
Cytogenetics known	144
Very good	3 (2)
Good	104 (72)
Intermediate	18 (12)
Poor	8 (6)
Very poor	11 (8)

WHO, World Health Organization; RA(RS), refractory anaemia with or without ring sideroblasts; RCMD(RS), refractory cytopenia with multilineage dysplasia with or without ring sideroblasts; RAEB-1 refractory anaemia with excess of blasts (5–10%); RAEB-2 refractory anaemia with excess of blasts (10–20%); MDS-U, myelodysplastic syndrome, unclassifiedFAB French-American-British; RAEB-T refractory anaemia with excess of blasts in transformation; IPSS, International Prognostic Scoring System; IPSS-R revised International Prognostic Scoring System.

*IPSS from 1 patient missing, because of lack of cytogenetic data and peripheral blood values.

**IPSS-R from three patients missing, because of lack of cytogenetic data and/or peripheral blood values.

Patients were assigned to risk groups by applying the IPSS and IPSS-R in four and five subgroups, respectively (Greenberg *et al*, 1997, 2012). The adjustments that were made to the IPSS to create the IPSS-R include definition of new cut-off points for BM blast percentage by morphology, refined categorization of cytogenetic abnormalities, creating five rather than three subgroups for cytogenetic classification and scoring for the depths of the cytopenias. If information on one component of the IPSS and/or IPSS-R was missing, the minimal score was given. In total, 19 out of 159 patients had one parameter missing for the IPSS or IPSS-R. To avoid pitfalls or bias due to missing data, a multiple imputation analysis was performed. The results of the imputation analysis were in agreement with the results from the original data. If more than one component was missing, the patient was not included in the analyses.

Pathological controls and healthy volunteers

As a control group, BM samples of 61 patients with cytopenia and a confirmed diagnosis of a non-myeloid haematological disorder were collected. The composition of the pathological control group followed the guidelines defined by the European LeukaemiaNet (ELN) working party (van de Loosdrecht *et al*, 2009; Westers *et al*, 2012). The diagnoses of patients in the pathological control group are shown in Table S1. As a reference population for normal haematopoiesis, aspirates drawn from healthy controls and individuals undergoing cardiac surgery were collected and analysed concomitantly ($n = 36$). The median age of the pathological controls was 62 years (range 33–89 years), compared with 59 years (range 38–81 years) in the healthy control group and 66 years (range 23–89 years) in the MDS patient cohort. The deviation in age between the groups was not significantly different. All samples were drawn after informed consent.

Definition of transfusion, progression and assessment of response

Patients were defined as transfusion dependent if they had received ≥two units of red blood cells within 8 weeks for at least 4 months at an Hb level of ≤90 g/l. For disease progression or evolution, the definition stated by Cheson *et al* (2006) was used.

Flow cytometric analysis of bone marrow samples

Immunophenotyping of BM cells was performed by using four-colour FC, as recommended by the ELN working party (van de Loosdrecht *et al*, 2009; Westers *et al*, 2012). All samples were processed and analysed within 24 h. Mature erythrocytes were lysed with ammonium chloride in order to perform analysis on total nucleated BM cells. The panel of monoclonal antibodies that were used in this study included fluorescein isothiocyanate (FITC) conjugated: CD5 (clone DK23), CD13 (WM-47), CD16 (DJ130c) from DakoCytomation Glostrup, Denmark; CD15 (MMA), CD34 (8G12) from BD Biosciences (San Jose, CA, USA); CD36 (CLB-IVC7) from Sanquin, Amsterdam, The Netherlands; phycoerythrin (PE) -conjugated: CD7 (M-T701), CD11b (D12), CD13 (L138), CD19 (SJ25C1), CD33 (P67·6), CD56 (My31), CD117 (104D2) and CD123 (9F5) from BD Biosciences; CD10 (SS2/36), CD64 (10·1) from DakoCytomation; peridinin-chlorophyll protein (PerCP) conjugated: CD45 (2D1) from BD Biosciences; allophycocyanin (APC) conjugated: CD11b (D12), CD13 (WM15), CD14 (MoP9), CD33 (P67·6), CD34 (8G12), HLA-DR (L243) from BD Biosciences and CD117 (104D2) from DakoCytomation. A FACSCalibur flow cytometer was used for measurements and data analysis was done by using Cell Quest Pro Software (BD Biosciences).

Cell populations of interest were selected by sideward light scatter (SSC) and CD45 properties after exclusion of (nucleated) red blood cells and debris. Mature myeloid cells were defined as $CD45^{dim}$ and SSC^{high}. Monocytes were identified by $CD45^{bright}$ and $SSC^{intermediate}$ in combination with CD14 or $CD33^{bright}$ expression. Myeloid progenitor cells were defined as $CD45^{dim}$, SSC^{low} in combination with CD34 and/or expression of a myeloid marker, such as CD117 and/or CD13. B cell progenitors were discriminated from myeloid progenitors by lower CD45, lower SSC properties and back gating with CD19 (van de Loosdrecht *et al*, 2009; Westers *et al*, 2012). Aberrant expression of a marker was defined as ≥two standard deviations above or below the mean reference value of the age-matched healthy control group. A minimum number of 250 events within the myeloid progenitor and monocyte compartment was measured to make a valid analysis of abnormalities in this cell compartment. Myeloid progenitors were considered positive for asynchronous or lineage infidelity marker expression (LIM) if ≥20% of cells (clustered together) expressed CD11b, CD5, CD19, CD56 and/or CD25 based on cut-off values in routine immunophenotyping diagnostics of leukaemia (Terwijn *et al*, 2009). Aberrant expression of CD7 was assessed in the context of CD13 expression. In normal haematopoiesis, CD7 can be expressed on $CD34^{pos}$ and $CD13^{dim}$ cells that are differentiating. Therefore, abnormal expression of CD7 on myeloid progenitors can be distinguished by quantifying CD7 expression on $CD13^{bright}$ cells. If CD7 was expressed as a cluster on ≥10% of $CD13^{pos\text{-}bright}$ myeloid progenitors, the myeloid progenitors were regarded aberrantly positive for this marker.

The FCSS was calculated for each subject, by transforming the number of aberrancies in the maturing myelomonocytic compartment and the percentage of myeloid progenitors in a weighed manner, as described in Table II (Wells *et al*, 2003). A maximum of five points can be scored for aberrancies in the differentiation of maturing myelomonocytic cells. In addition, the percentage of myeloid progenitors is scored for in a weighted manner, to a maximum of four points. The FCSS was categorized into normal to mild (0–1 points), moderate (2–3 points) and severe dysplasia (≥4 points) (Wells *et al*, 2003). The modification to the FCSS as

described by Cutler *et al* (2011) was applied. If clearly abnormal myeloid progenitors were present at <5% in the absence of abnormalities in the mature myelomonocytic compartment, two points were scored.

Statistical analysis

The data was checked for Gaussian distribution. If data passed the normality test, the student's *t*-test was applied to compare different groups; otherwise the Mann Whitney U test was used. To investigate correlations between the FCSS, IPSS and IPSS-R, Pearson's (for Gaussian data) or Spearman's rank correlation coefficient (non-Gaussian data) was applied. The Pearson Chi-square test was applied for testing the significance of categorical data in a contingency table. Differences in overall survival (OS) were assessed by Kaplan-Meier analysis and the significance was determined using the log-rank test. A hazard ratio (HR) was calculated with the Cox proportional hazards model to express the degree of hazard of death or disease progression for a subgroup. The OS time was defined as the period from date of diagnosis until death or date of last visit for patients that were still alive at data analysis. The time to disease evolution was defined as the period from date of diagnosis until establishment of progression into at least a RAEB type 1 (RAEB-1) or AML. To determine whether the FCSS is of significant added value beside IPSS(-R) in predicting survival, a likelihood ratio-test was performed in a Cox regression analysis. Statistical calculations were performed by SPSS 20.0 (IBM Corp., Armonk, NY, USA). A *P*-value $\leq 0{\cdot}05$ was regarded as significant.

Results

Comparison of overall survival and disease progression by using the IPSS and IPSS-R

The IPSS could be calculated in158 patients and the IPSS-R in 155 patients (Table I). One component for calculation of the IPSS(-R) was missing in 19 cases (four unsuccessful BM aspirations,14 missing or unsuccessful cytogenetics and one insufficient information on peripheral blood values). The number of patients that received disease modifying treatment (either allogeneic/autologous stem cell transplantation or azacitidine or lenalidomide) during the course of their disease was 35 (22%), 10 (6·3%) and 1, respectively. Transfusions and/or growth factor treatment were regarded as non-intensive treatment modalities.

Survival data of patients diagnosed with MDS between 2004–2008 and between 2009–2012 was not significantly different. OS and evolution to AML based on IPSS and IPSS-R for our cohort were determined and compared with the survival and progression data of the original IPSS and IPSS-R study. Patients in the IPSS intermediate-2 and high-risk groups had worse OS compared with the IPSS low and intermediate-1 risk groups (Figure S1A). Time to disease evolution was shorter for the IPSS intermediate-2 and high-risk groups compared with IPSS low and intermediate-1 risk groups (Figure S1B). Similar to the IPSS, the IPSS-R distinguished subgroups of patients with different OS (Fig 1A-B).

Although the IPSS-R did not separate prognostic subgroups as well as the former IPSS risk groups in the current set of data, prognostic subgroups could be identified. Patients within the IPSS-R very low and low groups have clearly better OS and longer time to disease progression than patients within the intermediate, high and very high risk groups.

The FCSS correlates with the WHO classification

Flow cytometric scores were calculated for all patients and (pathological) controls. The median FCSS of the total MDS cohort was 4 (range 0–8), which was significantly higher than the median FCSS of the pathological controls (median FCSS = 1, range 0–4, $P < 0{\cdot}001$) and healthy controls (median FCSS = 1, range 0–2, $P < 0{\cdot}001$).

Patients with more advanced stages of MDS (RAEB-1 and RAEB-2) had relatively higher FCSS values by FC compared with patients with refractory anaemia (with ringed sideroblasts) (RA[RS]) and refractory cytopenia with multilineage dysplasia (and ringed sideroblasts) (RCMD [RS]) ($n = 144$, Spearman's correlation = 0·55, $P < 0.\ 001$) (Figure S2A). However, within WHO subgroups, the distribution of flow cytometric abnormalities was heterogeneous. For example, the FCSS in the group of patients with RA varied from 2 (mild abnormalities) to 7 (severe abnormalities).

The FCSS correlates with the IPSS and IPSS-R and cytogenetic subgroups

A positive correlation was found between the FCSS, IPSS and IPSS-R ($n = 158$, Spearman's correlation = 0·52, $P < 0{\cdot}001$ and $n = 155$, Pearson = 0·59, $P < 0{\cdot}001$, respectively) (Fig 2 and Figure S2B). Moreover, there was a trend for correlation between the FCSS and IPSS cytogenetic subgroups, Spearman's correlation 0·14, $P = 0{\cdot}08$. The FCSS and IPSS-R cytogenetic subgroups showed a stronger, significant positive correlation (Spearman's correlation 0·19, $P = 0{\cdot}02$) (Fig 2 and Figure S2C). This indicates that patients with more complex cytogenetic abnormalities, as defined by the IPSS-R, have more severe dysplastic features by FC than patients with normal to single cytogenetic abnormalities. Despite the significant correlation between the FCSS and IPSS-R (cytogenetic) subgroups, the distribution of the FCSS remained heterogeneous within the (cytogenetic) subgroups.

High FCSS and aberrant marker expression on myeloid progenitors is associated with progressive disease and transfusion dependency

Transfusion history was known for 111 patients; of these patients, 37·8% ($n = 42$) were transfusion-dependent by

Table II. The components of the flow cytometric scoring system (FCSS).

Points	Monocytic cells
1	<5% myeloid progenitors with aberrancies defined as: Abnormal granularity Abnormal expression of CD45 Abnormal expression of CD34 Abnormal expression of CD117 Abnormal expression of CD13 Abnormal expression of CD33 Abnormal expression of HLA-DR Expression of CD11b Presence of lymphoid antigens CD5, CD7 or CD19
2	i) In case of 5–10% myeloid progenitors, two extra points are scored or ii) In case of <5% abnormal myeloid progenitors with absence of other abnormalities in the maturing myelo/monocytic cells
3	In case of 11–20% myeloid progenitors, three extra points are scored
4	In case of 21–30% myeloid progenitors, four extra points are scored

Points	Maturing myeloid cells
1	One of the following is present: Abnormal granularity Abnormal expression of CD45 Abnormal CD13/CD16 differentiation pattern Abnormal expression of CD33 Abnormal expression of HLA-DR Abnormal expression of CD11b Asynchronous shift to the left Overexpression of CD56 Decreased myeloid to lymphoid ratio (<1): 1 point extra, independent of other abnormalities
2	2–3 of the above abnormalities in either myeloid or monocytic cells in absence of other abnormalities or presence of CD34 or lymphoid antigens (CD5, CD7 or CD19)
3	4 or more of the above abnormalities or 1 or more of the abnormalities plus presence of CD34 or lymphoid antigens
4	Both myeloid cells and monocytes showed 2 or 3 abnormalities

Points	Monocytic cells
1	One of the following is present: Abnormal granularity Abnormal expression of CD45 Lack of CD14 expression Abnormal CD13 or CD16 expression Abnormal expression of CD33 Abnormal expression of HLA-DR Abnormal expression of CD11b Overexpression of CD56 Decreased or increased number relative to lymphocytes
2	2–3 of the above abnormalities in either myeloid or monocytic cells in absence of other abnormalities or presence of CD34 or lymphoid antigens (CD5, CD7 or CD19)
3	4 or more of the above abnormalities or 1 or more of the abnormalities plus presence of CD34 or lymphoid antigens
4	Both myeloid cells and monocytes showed 2 or 3 abnormalities

Adapted from work that was originally published in *Blood*: Wells *et al* (2003) Myeloid and monocytic dyspoiesis as determined by flow cytometric scoring in myelodysplastic syndromes correlates with the IPSS and with outcome after hematopoietic stem cell transplantation. Blood, 102, 394-403.© 2003 American Society of Hematology.

WHO classification-based Prognostic Scoring System (WPSS) criteria. Follow-up with respect to the course of the disease was known for 120 patients; 26·7% ($n = 32$) of patients showed progressive disease (Cheson *et al*, 2006). The median FCSS was significantly lower in transfusion-independent or non-progressive disease patients as compared to transfusion-dependent and progressive disease patients, (median FCSS 3 (range 0–7) vs. median FCSS 5 (range 2–8), $P < 0·001$, respectively).

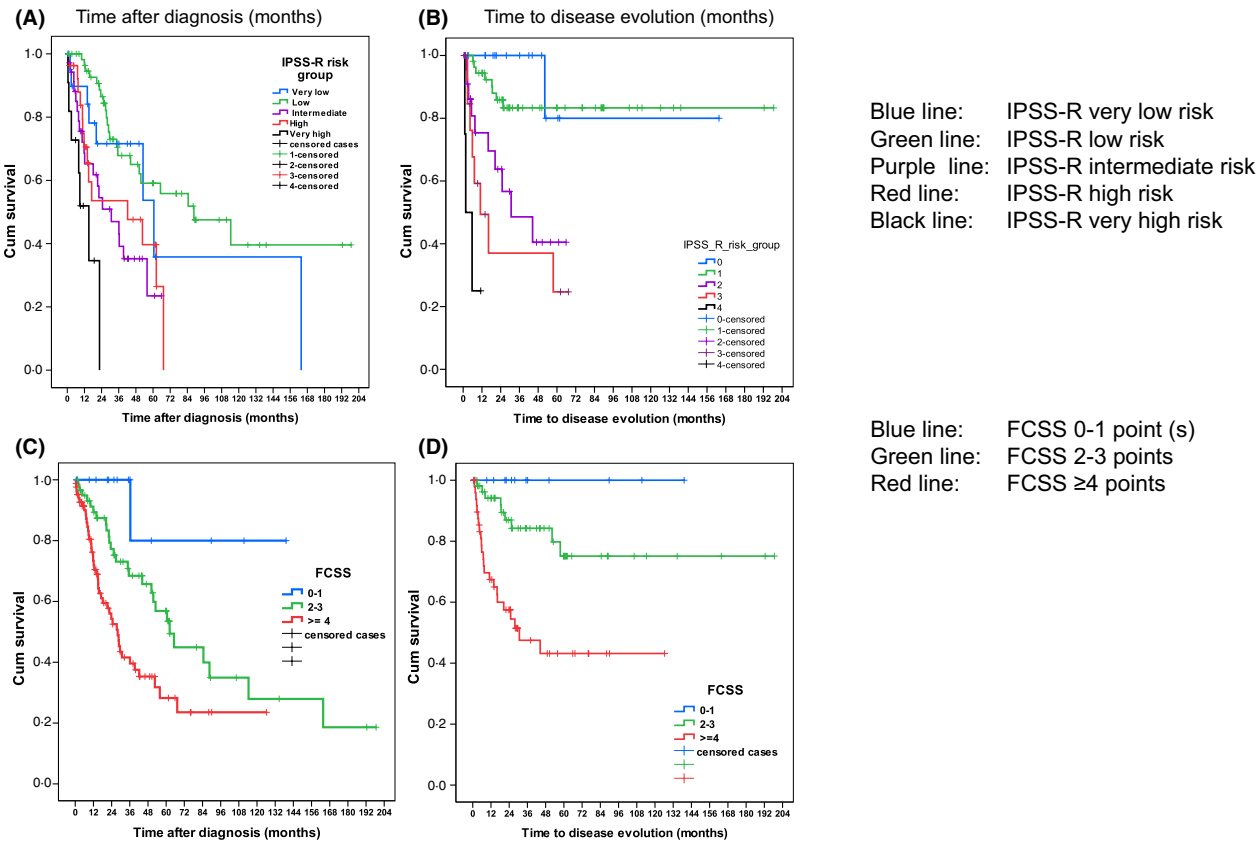
Interestingly, there was a highly significant association of transfusion dependency and/or progressive disease with aberrant marker expression on myeloid progenitors as assessed by FC (Pearson Chi-Square, $P = 0·001$). For this analysis, aberrant myeloid progenitors were defined as positive for LIM expression (CD5, CD7, CD11b, CD19, CD25 and/or CD56).

Furthermore, patients with aberrant myeloid progenitors, as defined above, had a 1·8 times increased risk of death compared with patients without aberrant myeloid progenitors ($P = 0·02$).

Normal to minimal flow cytometric abnormalities are associated with a favourable prognosis in patients with MDS

Three different prognostic subgroups could be distinguished within our cohort by using the defined cut-off points for the FCSS of 0–1 points (normal to minimal flow cytometric abnormalities), 2–3 points (mild flow cytometric abnormalities) and ≥4 points (severe flow cytometric abnormalities) (Wells *et al*, 2003; Cutler *et al*, 2011).

Patients with only mild flow cytometric abnormalities had significantly better OS compared with patients with severe flow cytometric abnormalities (median OS 62·3 months, range 1·3–198·6 months vs. median OS 28 months range 0·5–126·3 months, respectively, $P = 0·01$) (Fig 1C). The difference in OS between patients with no abnormalities



Survival	Median (months, (years))	Range (months)		Median (in months, (years))
IPSS-R study cohort (n = 155)			**IPSS-R reference cohort**	
very low (n = 22)	60·6 (5·1)	0·7-163·7	Very Low	105·6 (8·8)
low (n = 60)	88·6 (7·4)	0·5-198·6	Low	63·6 (5·3)
intermediate (n = 35)	30·8 (2·6)	0·7-65·9	Intermediate	36 (3)
high (n = 27)	42·2 (3·5)	0·9-67·3	High	19·2 (1·6)
very high (n = 11)	15·1 (1·3)	0·5-22·5	Very high	9·6 (0·8)
FCSS (n = 159)				
no to minimal (0-1 point, n = 15)	not reached	0·7-139·2		
mild (2-3 points, n = 60)	62·3 (5·2)	1·3-198·6		
severe (≥4 points, n = 84)	28 (2·3)	0·5-126·3		

Time to disease progression	Median (months, (years))	Range (months)
IPSS-R study cohort (n = 116)		
very low (n = 18)	not reached	0·7-163·7
low (n = 56)	not reached	1·3-198·6
intermediate (n = 24)	30·7 (2·6)	0·7-65·9
high (n = 14)	11 (0·9)	0·.9-67·3
very high (n = 4)	1·6 (0·1)	1·1-11·2
FCSS (n = 120)		
no to minimal (0-1 point, n = 13)	not reached	1·4-111·4
mild (2-3 points, n = 56)	not reached	1·3-198·6
severe (≥4 points, n = 51)	30·7 (2·6)	0·7-126·3

Fig 1. Overall survival and time to disease progression by IPSS-R and FCSS. (A) Patients with higher IPSS-R scores (intermediate (purple line), high (red line) and very high (black line)) had significantly worse overall survival than patients with lower scores [very low (blue line) and low (green line)], $P < 0{\cdot}001$. (B) Evolution to at least RAEB-1 or AML was significantly lower in patients with IPSS-R very low/low risk disease (blue and green line, respectively) compared with patients with IPSS-R intermediate/high/very high risk, (purple, red and black line, respectively) $P < 0{\cdot}001$. (C) Overall survival was significantly better in patients with FCSS 0–1 points (blue line) or 2–3 points (green line), compared with patients with ≥4 points (red line), $P < 0{\cdot}001$. (D) Evolution to at least RAEB-1 or AML was significantly lower in patients with FCSS 0–1 points (blue line) or 2–3 points (red line), compared with patients with ≥4 points (blue line), $P < 0{\cdot}001$. FCSS, flow cytometric scoring system; IPSS-R, International Prognostic Scoring System revised; RAEB-1 refractory anaemia with excess of blasts (5–10%); AML, acute myeloid leukaemia. The survival data of the IPSS-R reference cohort is derived from Greenberg *et al* (2012).

(median OS not reached) compared with patients with mild abnormalities as assessed by FC was statistically not significant. The risk of death was 5·9- and 12·1-fold higher in patients with moderate or severe flow cytometric abnormalities compared with patients with no to minimal flow cytometric abnormalities.

Median time to disease evolution was not reached for patients with 0–1 point and 2–3 points and 30·7 months (range 0·7–126·3 months) for patients with ≥4 points. Similarly, patients with no to minimal flow cytometric abnormalities had significantly longer time to disease progression compared to patients with mild and severe flow cytometric abnormalities, $P < 0{\cdot}001$ (Fig 1D).

The FCSS can identify prognostic subgroups within the IPSS-R good cytogenetic risk group and IPSS-R low risk group

Most refinement of the IPSS-R vs. the IPSS was achieved by applying new cytogenetic risk groups. Therefore, we assessed whether the FCSS was able to differentiate prognostic subgroups within the cytogenetic subgroups of the IPSS-R. The majority of patients with MDS in our cohort were within the IPSS-R cytogenetic good risk (72%, 104/144), consistent with the distribution in the IPSS-R patient cohort (Greenberg *et al*, 2012). Although patients were homogeneous with respect to prognosis based on cytogenetics, by

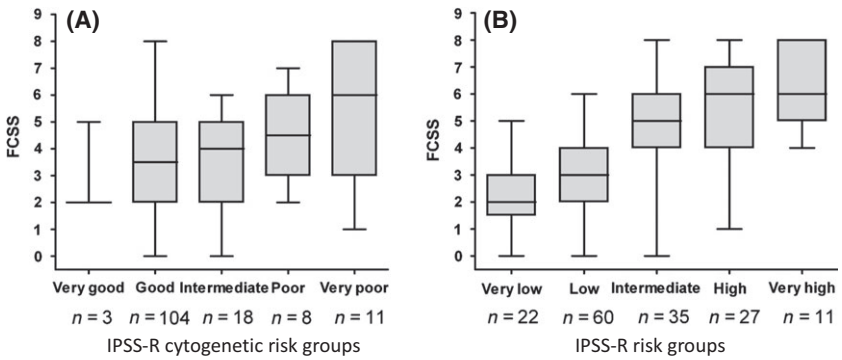


Fig 2. Distribution of FCSS scores over IPSS-R cytogenetic and IPSS-R categories. (A) IPSS-R cytogenetic subgroups are depicted on the *x*-axis, from very good to very poor and the FCSS on the *y*-axis. The FCSS between the IPSS-R cytogenetic subgroups was not significantly different. (B) IPSS-R risk groups are depicted on the *x*-axis, from very low risk to very high risk and the FCSS on the *y*-axis. The FCSS was significantly higher in IPSS-R low vs. intermediate risk patients, $P < 0{\cdot}001$ and borderline significant for IPSS-R intermediate vs. high risk patients, $P = 0{\cdot}07$. FCSS, flow cytometric scoring system; IPSS-R, International Prognostic Scoring System revised.

analysing the degree of dysmyelopoiesis by FC, patients could be identified with good, intermediate and worse prognosis (Fig 3). Furthermore, within the IPSS-R low risk group, the FCSS identified patients with different OS depending on the degree of flow cytometric aberrancies in haematopoiesis (Fig 4A). IPSS-R low risk patients with no to minimal flow cytometric aberrancies (0–1 point) had better OS than low risk patients with mild flow cytometric aberrancies (2–3 points). When the IPSS-R high and very high risk groups were pooled, there was a trend towards worse OS for patients with severe flow cytometric abnormalities compared with mild flow cytometric abnormalities, $P = 0{\cdot}06$.

In contrast to OS, the time to disease progression for IPSS-R low risk patients with mild flow cytometric abnormalities compared to severe abnormalities was not significantly different, $P = 0{\cdot}24$ (Fig 4B). The FCSS was also applied within the IPSS-R very low, intermediate, high and very high subgroups for time to disease progression. However, the size of these subgroups was too small to make any clear statements.

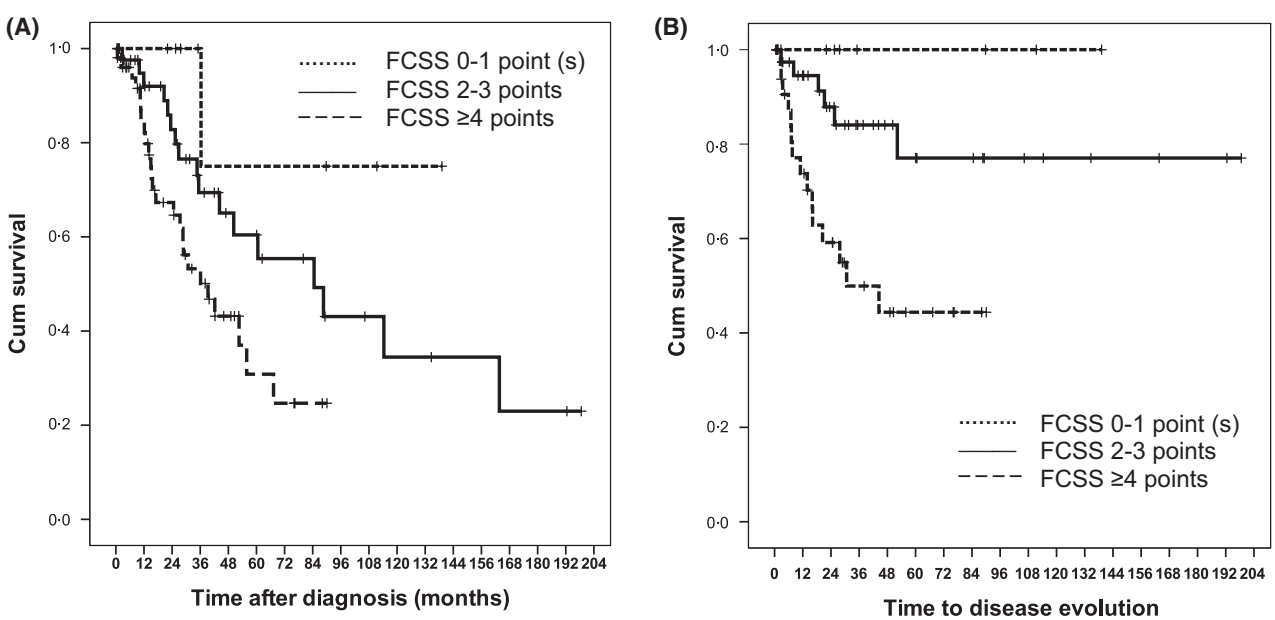


Fig 3. Overall survival and time to disease evolution by FCSS within the IPSS-R cytogenetic good risk subgroup. (A) Overall survival was significantly better in patients with a FCSS of 2–3 points (solid line, $n = 42/104$) compared with patients with ≥4 points (broken line, $n = 52/104$), $P = 0{\cdot}01$. Median overall survival for patients with good risk cytogenetics by IPSS-R and FCSS of 0–1 point (dotted line) was not reached, vs. 84·6 months for patients with FCSS 2–3 points (solid line) and 39·3 months for patients with ≥4 points (striped line). (B) Evolution to at least RAEB-1 or AML was significantly lower in patients with 0–1 points (dotted line, $n = 8/82$) or 2–3 points (solid line, $n = 40/82$), compared with patients with ≥4 points (striped line, $n = 34/82$), $P = 0{\cdot}002$. Median time to disease evolution was not reached for patients with 0–1 point and 2–3 points. Median time to disease evolution for patients with ≥4 points was 30·7 months. FCSS, flow cytometric scoring system; IPSS-R, International Prognostic Scoring System revised; RAEB-1 refractory anaemia with excess of blasts (5–10%); AML, acute myeloid leukaemia.

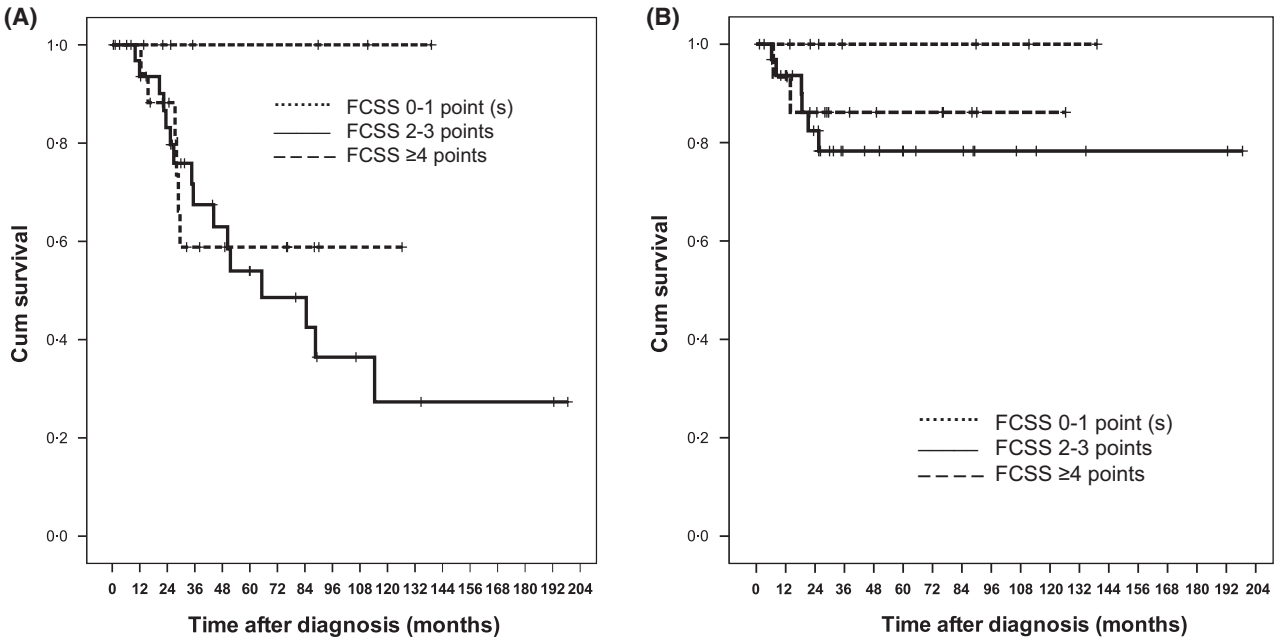


Fig 4. Overall survival and time to disease evolution by FCSS within the IPSS-R low risk group. (A) Overall survival was significantly better in patients with a FCSS of 0–1 point (dotted line, $n = 7/60$) compared with patients with 2–3 points (solid line, $n = 35/60$), $P = 0{\cdot}05$. Median overall survival for patients within the IPSS-R low risk group and FCSS of 0–1 point was not reached, vs. 65·2 months for patients with FCSS 2–3 points and not reached for patients with ≥4 points (striped line). (B) Evolution to at least RAEB-1 or AML was lower in patients with 0–1 points (dotted line, $n = 7/56$) compared with patients with 2–3 points (solid line, $n = 34/56$) and/or patients with ≥4 points (striped line, $n = 15/56$), $P = 0{\cdot}24$. Median time to disease evolution was not reached for all patient groups. FCSS, flow cytometric scoring system; IPSS-R, International Prognostic Scoring System revised; RAEB-1 refractory anaemia with excess of blasts (5–10%); AML, acute myeloid leukaemia.

In a likelihood ratio-test, the combination of FCSS and IPSS-R was statistically better in predicting OS in MDS patients than only IPSS-R as a single model, $P = 0{\cdot}02$. This was also the case for the combination of the IPSS and FCSS, $P = 0{\cdot}01$.

Discussion

The risk stratification by IPSS and, more recently, IPSS-R of patients with MDS is of great value in clinical practice, for estimation of survival and transformation to AML. There is evidence that BM analysis by FC of patients with MDS might add to refinement of prognostication. Therefore, the aim of our study was to investigate whether the FCSS is of prognostic value in the setting of the IPSS and IPSS-R.

We showed that the FCSS correlated with the WHO, IPSS and IPSS-R classifications, thereby confirming previously published data (van de Loosdrecht *et al*, 2008; Chu *et al*, 2011). In our cohort, the distribution of patients over the IPSS and IPSS-R subgroups was comparable with the original IPSS-R cohort (Greenberg *et al*, 1997, 2012. In our cohort, the IPSS risk stratification, probably due to small numbers, provided a better separation of prognostic subgroups than the IPSS-R. In general, OS rates in our MDS cohort tended to be longer than the IPSS reference cohort. This might be explained by different modalities as the majority of our data was obtained after 2004, compared with the IPSS reference cohort for whom the data was collected before 1997. Furthermore, our patient group included patients receiving (intensive) chemotherapy and/or haematopoietic stem cell transplantation, while the IPSS-R reference cohort included patients with MDS who were not treated with disease-modifying therapies during the MDS phase.

The FCSS correlated with the IPSS and IPSS-R cytogenetic categories (van de Loosdrecht *et al*, 2008). The correlation with IPSS-R cytogenetic categories was stronger than the previously defined IPSS cytogenetic subgroups. Higher FCSS scores were found in patients with more complex cytogenetic abnormalities (Cutler *et al*, 2011). This underlines that the degree of genetic disruption is associated with the degree of dyspoiesis as assessed by FC (Cutler *et al*, 2011). In the IPSS-R reference cohort as well as in our patient cohort, a large proportion of patients was in the IPSS-R cytogenetic good risk group. As one of the most important improvements in the IPSS classification is the reclassification of cytogenetic subgroups, we analysed whether the FCSS could differentiate subgroups within this cytogenetic category. The FCSS was able to identify three groups of patients with different OS and time to disease progression in the IPSS-R cytogenetic good risk group. This emphasizes that flow cytometric aberrancies might be of additional prognostic value.

Multilineage dysplasia, as assessed by morphology, is a known prognostic factor that is reflected by the lower survival of patients with RCMD(RS) compared with patients with RA (RS) (Malcovati *et al*, 2005a). An interesting observation from

our study is that in some RA(RS) cases, severe flow cytometric abnormalities in the myeloid lineage could be detected despite the fact that by morphology, these patients had unilineage (erythroid) dysplasia. Thus, the finding of severe flow cytometric abnormalities in myeloid differentiation of some patients with RA(RS) might have a negative impact on their prognosis. For patients with RA(RS) or RCMD(RS) and severe dysplasia as defined by FC, this might imply that their prognosis is similar to patients with RAEB-1 and/or RAEB-2.

In the WPSS, the presence of multilineage dysplasia assessed by morphology was included as a prognostic criterion (Malcovati *et al*, 2007). Given that FC recognizes dysplasia, it could be hypothesized, based on our findings, that the added value of the FCSS to IPSS-R might be due to the detection of multilineage dysplasia. However, the WHO subgroups were evenly distributed over the mild and severe FCSS subgroups, which is in contradiction with multilineage dysplasia being the cause for higher scores in FCSS. Moreover, all patients with no to mild FCSS in our cohort were RCMD(RS) patients, which again contradicts that multilineage dysplasia assessed by FC would be the sole contributor to refinement of IPSS-R risk stratification (Figure S2A).

The FCSS includes analysis of aberrant marker expression of myeloid progenitors. Previously, we have shown that the presence of aberrant myeloid progenitors is of clinical importance. From our current, larger study, it can be concluded that patients with MDS and a severe FCSS and/or aberrant myeloid progenitors have an adverse clinical course and are likely to be(come) transfusion-dependent or develop progressive disease.

Patients within the IPSS-R low risk group with severe flow cytometric abnormalities might have a similar prognosis as IPSS-R high-risk patients. This was supported by the finding that IPSS-R low risk patients with mild abnormalities in haematopoiesis by FC have better survival than low risk patients with severe abnormalities. From these findings it was hypothesized that scoring for flow cytometric abnormalities as part of the IPSS-R might refine risk assessment in MDS patients and give a better estimation of survival. From the likelihood ratio-test it was concluded that FCSS and IPSS-R combined appeared to give the most accurate estimation of survival for patients with MDS in our cohort. The impact of our findings is that FC might aid in selecting seemingly low risk patients who might benefit from more active treatment approaches. It is expected that with the progress made in the field of molecular research, the presence of specific molecular abnormalities will also be included in future prognostic scores (Bejar *et al*, 2012).

In conclusion, flow cytometric analysis of BM of patients with MDS provides additional prognostic information and is complementary to the IPSS-R. The addition of a flow cytometric score next to the clinical parameters within the IPSS-R would be a refinement of prognostication of patients with MDS.

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Authors' contributions

CA drafted the manuscript, performed and analysed experiments. TMW performed and analysed experiments and revised the manuscript and validated the final version of the manuscript. CC performed and analysed experiments. BIW provided statistical advice, revised the manuscript and validated the final version of the manuscript. EMPC and GJO validated the final version of the manuscript. AAL designed the study, provided bone marrow samples, revised the manuscript and validated the final version of the manuscript.

Conflict of interest

The authors report no potential conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Overall survival and time to disease progression by IPSS.

Fig S2. The distribution of FCSS over the WHO, IPSS cytogenetic and IPSS subgroups.

Table S1. Pathologic controls.

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