Flow Cytometry in Myelodysplastic Syndromes: Evolving from Laboratory Practice to Clinical Implementation

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Printing of this thesis was financially supported by Amgen, Astellas, BD Biosciences and Celgene

ISBN: 978-90-6464-940-0

Lay out: Ferdinand van Nispen, Citroenvlinder-dtp.nl, *my*-thesis.nl, Bilthoven, The Netherlands
Cover by: www.facebook.com/wewengkang.nl
Printed by: GVO drukkers & vormgevers B.V. | Ponsen & Looijen, Ede, The Netherlands

VRIJE UNIVERSITEIT

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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. F.A. van der Duyn Schouten,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Geneeskunde
op vrijdag 18 december 2015 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

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geboren te Velsen

promotoren: prof.dr. A.A. van de Loosdrecht
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copromotor: dr. T.M. Westers

Adaequatio intellectus et rei

Voor Süleyman Akcaova

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Adapted from

Alhan C, Westers TM, Ossenkoppele GJ, van de Loosdrecht AA. Chapter 8 Flow cytometry in myelodysplastic syndromes. The Myelodysplastic Syndromes. Springer International, 2011

Della Porta MG, Ogata K, Picone C et al. Multicentric validation of a reproducible flow cytometric score for the diagnosis of low-grade myelodysplastic syndromes: results of a European LeukemiaNet study. Haematologica 2012; 97: 1209-1217

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Introduction

This thesis encompasses the results of research of the laboratory and clinical application of flow cytometry (FC) for myelodysplastic syndromes (MDS). Myelodysplastic syndromes were presumably described in literature for the first time in 1900 by Leube (a macrocytic anemia progressing to acute leukemia; BerlKlinWochenschr. 1900;37:851). The author used the term 'leukanamie' to define a macrocytic anemia that transformed to acute leukemia. Until the 1970s the term 'pre-leukemia' was used to diagnose patients with macrocytic anemia who developed acute leukemia after several years. However, a proportion of patients did not develop leukemia but succumbed to the consequences of cytopenia. 'Pre-leukemia' did not properly describe the malady and myelodysplastic syndromes or MDS became widely accepted to describe this bone marrow (BM) disorder.

The incidence of MDS in Western-European countries is between 3.7 to 6.1 per 100,000 individuals per year. Men are more frequently affected than women; incidence 5.5 per 100,000 men and 4.4 per 100,000 women, respectively. (1,2) There is an association with older age; after the age of 60, the incidence increases up to 50 per 100,000 individuals a year. (3)

Myelodysplastic syndromes is characterized by ineffective hematopoiesis in the BM that can result in one or more cytopenia(s), affecting the erythroidlineage (anemia), and/or white blood cell lineage (neutropenia) and/or megakaryocytic lineage (thrombocytopenia). Approximately 25% of the patients will develop acute myeloid leukemia (AML). The remainder of the patients can remain stable for years or become erythrocyte transfusion dependent due to refractory anemia. Other complications that can arise are related to the neutropenia (infections) and/or thrombocytopenia (bleeding). Myelodysplastic syndromes are diagnosed by performing morphologic examination of a BM aspirate to evaluate dysplasia together with a peripheral blood smear, BM biopsy and cytogenetic analysis. (4,5) Furthermore, exclusion of other causes as an explanation for the cytopenia(s) is required.

Part I Technical aspects

Flow cytometry as a diagnostic tool in MDS

The current standard for the diagnosis of MDS is the detection of dysplastic features in the erythroid, megakaryocytic and/or myeloid cell lineages in the bone marrow (BM) by cytomorphology. Dysplasia can be difficult to assess and in combination with a normal karyotype, the diagnosis of MDS can be challenging. Therefore, efforts have been made to improve diagnostic tools, such as applying FC for MDS. Flow cytometry is based on intrinsic physical properties of the cells such as size, corresponding with forward light scatter (FSC) and cytoplasmic granularity, corresponding with sideward light scatter (SSC). Furthermore, by labelling cells with fluorochrome-conjugated monoclonal antibodies it is possible to detect surface, cytoplasmic or nuclear antigens. The application of FC as a diagnostic tool is based on the concept that maturation and differentiation of hematopoietic cells is a tightly controlled process, leading to highly conserved levels of antigen expression at different stages of development. (6) In MDS, precursor cell formation is affected resulting in deviation from the normal level of antigen expression in the (im)maturemyelo-monocytic, erythroid and megakaryocytic cell lineages. (7-11) Aberrancies in hematopoiesis that can be observed in MDS are: the expression of lymphoid antigens on myeloid cells, over or under expression or loss of antigen expression, the expression of immature antigens on mature cells and vice versa (asynchronous antigen expression), and abnormal differentiation patterns between antigens. Knowledge of the immunophenotype of BM cells from healthy individuals and changes during hematopoietic differentiation is essential in order to understand abnormalities in differentiation that occur in MDS.

Normal myeloid progenitor cells by flow cytometry

Flow cytometry can be used to identify distinct subpopulations of myeloid progenitor cells based on antigen expression levels. CD45 is a leukocyte common antigen and is expressed on all leukocytes. In combination with SSC, CD45 is a powerful means to delineate lymphocytes, monocytes, maturing myeloid cells and myeloid progenitors in normal BM. Normal myeloid progenitors are identified by diminished CD45 (CD45dim) expression, low to intermediate SSC, heterogeneous expression of CD34 and CD117, CD13, CD33, HLA-DR and absence of CD11b. CD34 is expressed on the most immature myeloid, lymphoid and erythroid cells and is rapidly lost at the early stage of differentiation. CD117 is expressed on immature myeloid

and erythroid progenitors and is lost during myeloid and erythroid differentiation. CD13 is expressed at the myeloid progenitor stage. CD33 is a marker that is expressed on all myeloid cells. HLA-DR is lost at an early stage during myeloid differentiation.

Normal maturing myeloid cells by flow cytometry

In normal BM, neutrophils can be discriminated by intermediate CD45 expression and high SSC properties as compared to lymphocytes. Neutrophils can be distinguished from monocytes that have higher CD45 expression and lower SSC properties compared with neutrophils. (6) By morphology, five stages of neutrophil differentiation can be discerned. The myeloblastsdifferentiate subsequently via promyelocytes, myelocytes, metamyelocytes, towards band and segmented neutrophils. By FC, these neutrophil maturation stages can be distinguished by different expression levels of CD11b, CD13 and CD16. Normal neutrophil differentiation patterns are shown in figure 1a. Myeloblasts express CD34, CD117 and CD33 and high levels of CD13 and lack CD11b and CD16 expression. CD11b is absent on promyelocytes, defined by CD117 expression and CD34 absence, and is initially expressed at low levels on myelocytes and is high at the metamyelocyte and segmented neutrophil stage. CD16 follows a similar pattern, but has intermediate expression from the metamyelocyte stage and is highly expressed on mature neutrophils. In contrast, CD13 is present at high levels just before the promyelocyte stage, decreases with differentiation to the myelocyte stage and increases again at the metamyelocyte stage to become highly expressed on segmented neutrophils. The graphic representation of CD11b and CD16, CD11b and CD13, CD16 and CD13 forms three highly conserved patterns for neutrophil differentiation in normal hematopoiesis. A disturbance in differentiation of neutrophils is reflected by aberrancies in the patterns of myeloid antigens: CD13, CD16 and CD11b, as shown in figure 1b and 1c. Maturing myeloid cells have highest CD33 intensity compared with immature myeloid progenitor cells and most mature myeloid cells.

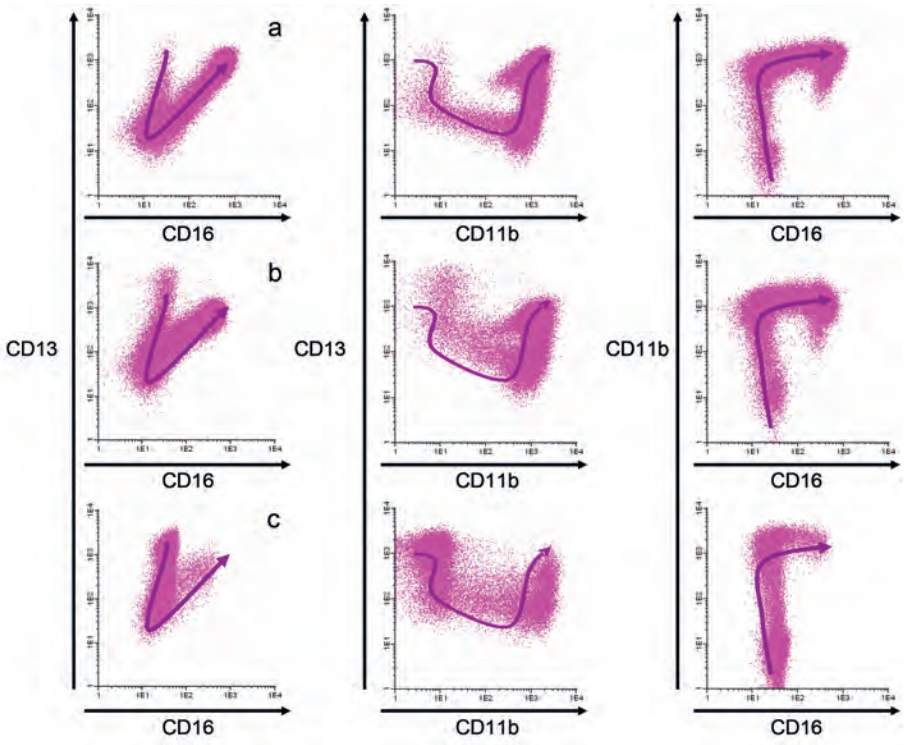

Figure 1 Neutrophil differentiation in MDS

To analyze neutrophil differentiation patterns it is recommended to use the antibody combination CD11b/CD13/CD45/CD16. Panel ashows neutrophils differentiation in the BM of an healthy individual. The arrows indicate the direction of differentiation of the maturing myeloid cells. Panel b shows neutrophil differentiation in the BM of a low risk MDS patient. As can be seen from CD11b/CD13 pattern, in this case CD13 expression is increased on the immature myeloid cells. The CD13/CD16 pattern is more condensed compared with the pattern of the healthy individual in panel a. Panel c shows neutrophil differentiation in a high risk MDS. The CD13/CD16 pattern is compact and the number of immature myeloid cells is increased (leftshift). In the CD11b/CD13 plot there is a proportional decrease in CD11bdim cells and mature CD11b+ CD13+ cells. CD11b is also overexpressed as can be seen from the CD11b/CD13 and CD11b/CD16 patterns.

Normal monocytic lineage by flow cytometry

The added value of FC in monocytic lineage analysis can be substantial since dyspoiesis in the monocytic lineage is difficult to identify by morphology on the BM level because dysplastic features of monocytes are not as distinct as other cell populations. By morphology, monocytic cells can be identified as myelo/monoblasts, promonocytes and monocytes. Within tissues the monocytes may further differentiate into macrophages. Differentiation from monoblasts to promonocytes

is marked by an increase in CD33 expression and loss of CD34 expression with an intermediate expression of CD45. Subsequently, CD45 expression is increased and CD14 and CD11b are gained at the monocytic stage. Furthermore, monocytes retain HLA-DR expression upon differentiation in contrast to the loss of HLA-DR expression during neutrophil maturation. (6)
Combinations of the antibodies CD11b and HLA-DR, CD14, or CD300e(IREM2) with CD36 and/or CD64, enable the discrimination of immature and mature monocytes. Analysis of CD14 alone can give an underestimation of the proportion of monocytes in the BM because it is only expressed on mature monocytes.

Normal erythroid and megakaryocytic lineage by flow cytometry

The detection of erythroid dysplasia by FC in MDS is limited due to lack of markers. Erythroid cells can be identified by the absence of CD45 and low scatter properties. The markers that are mainly investigated in the context of dyserythropoiesis in MDS are CD235a and CD71. CD235a or glycophorin A is expressed early upon differentiation at the erythroblast stage of erythroid development and is maintained throughout terminal red cell differentiation. (12) CD71 is the antibody recognizing the transferrin receptor. The transferrin receptor is needed for uptake of iron. Nucleated erythroid cells express CD71 but anucleated erythrocytes lack CD71 expression. Erythroid precursor cells express CD117 and upon maturation to terminally differentiated erythroid cells CD117 expression is lost. (6,12) CD105 (endoglin) is a receptor for members of the transforming growth factor beta superfamily (TGF-ß) and is specifically expressed on erythroblasts. Using the combination of CD36, CD105 and CD117 the immature erythropoietic cell compartment can be analyzed. (13) Other markers that are associated with erythroid dysplasia in MDS are the intracellular ferritin subunits, H-ferritin and L-ferritin and mitochondrial ferritin (MtF). In MDS with ring sideroblasts (RS), erythroid precursor cells express increased iron receptors at the cell surface compared with patients without RS, which is associated with iron storage in the mitochondria. Flow cytometric analyses of proteins of iron metabolism in MDS patients are an indirect way of analyzing erythroid dysplasia. Patients with RS had higher levels of MtF and CD105 than patients without RS. Furthermore, MtF expression is related to presence of RS in BM by morphology. (14,15)
Analysis of dyspoiesis in the megakaryocytic lineage by FC is limited due to technical aspects. The number of megakaryocytes is low compared to the other cell lineages in the BM so analysis by FC requires extensive enrichment. Furthermore, binding of platelets to non-megakaryocytic cells may result in false positivity for platelet-

associated antigens, such as CD36. Since platelets are derived from megakaryocytes in the BM, feasibility of immunophenotyping of platelets in PB is currently under investigation. (16)

Diagnostic application of flow cytometry in MDS

The diagnostic utility of multi-parameter FC of the myeloid, erythroid and megakaryocytic lineage in the BM for MDS was first described by Stetler-Stevenson et al. (9) This study showed that FC could be instrumental in the detection of immunophenotypic aberrancies in cytopenic patients with non-diagnostic BM morphology and cytogenetics.

Several studies have shown that flow cytometric analyses correlate with established diagnostic classification systems, such as French-American-British (FAB) and World Health Organization (WHO). (17-21) To display the results of flow cytometric data, a numerical flow cytometric scoring system (FCSS) was developed and validated. (17,22,23) The components of the FCSS and other flow cytometric abnormalities that can be found in BM of patients with MDS will be extensively discussed in the chapters of this thesis.

To widely apply FC in laboratories it is necessary to make a diagnostic test that is simple and reproducible, with a high sensitivity and specificity. In a collaborative study between Japan and Italy, a flow cytometric test to diagnose low risk MDS patients was designed based on four cardinal parameters. (24) These included the percentage of myeloid progenitors, B cell progenitors, CD45 expression of myeloid progenitors and neutrophil SSC. The results of this study were validated in an international multicentre study. (25)

This FCSS showed a good correlation with WHO 2001 classification for MDS. (21) However, flow scores were heterogeneous within WHO subgroups. (26) This indicates that FC might identify subgroups within existing classification systems and offer a more refined classification with potential prognostic impact. Interestingly, FC identified patients with MDS RA(RS) that already had multi-lineage involvement according to FC but with only erythroid dysplasia by morphology. (27) Previously, multilineage dysplasia as determined by morphology was associated with adverse clinical outcome. (28) A study from our group also showed that aberrancies in the myelo-monocytic lineage could be detected in the majority of patients with MDS and unilineage erythroid dysplasia according to morphology and WHO classification. (21) Flow cytometric analyses of BM of cytopenic patients needs to be further developed and validated, also in new disease categories such as idiopathic cytopenia of unknown significance.

Standardization of flow cytometry in MDS: the Dutch working party, International and European LeukemiaNet working party for flow cytometry in MDS

The growing interest in the application of FC for MDS in the laboratory as well as in the clinic initiated the establishment of a consortium within the Netherlands and the international and European LeukemiaNet (IMDS-Flow) to provide guidance. The aim of the Dutch, European and international partners was to standardize FC for MDS. The fruitful collaboration resulted in several publications providing technical guidance for those interested in FC in MDS and in multi-center trials to validate flow cytometric data. In **chapter** two, the results of the participationand collaboration with IMDS-Flow are summarized. Variations in sample handling and processing were described in a report from the ELNet working conference for the standardization of FC in MDS. (28) Basic requirements such as adequate standardization of flow cytometric instruments and procedures should be accomplished. Furthermore, knowledge of what may cause changes in antigen expression relationships in hematopoiesis that are not attributable to dyspoiesis in MDS and the application of multiparameter analysis can help to overcome some of these pitfalls. **Chapter** three describes the efforts of the members of the Dutch Working Party on Flow Cytometry in MDS to standardize flow cytometric analysis of BM samples in the Netherlands. **Chapter** four provides an overview of technical considerations and illustrates pitfalls when performing flow cytometric analyses of patients with MDS. Peripheral blood contamination is regarded as a pitfall in flow cytometric analysis of BM samples of patients with MDS. However, when only peripheral blood is available, the results of flow cytometric analysis might still be of value for the diagnosis, prognosis and treatment monitoring for MDS. This is discussed in **chapter** five.

Part II Flow cytometric prognostic scoring systems

The subgroups in the FAB, WHO 2001 and 2008 classification systems provide prognostic information. MDS patients in FAB RAEB/RAEB-T, WHO RAEB-1 and RAEB-2 have higher risk of progression to an AML and shorter overall survival compared with MDS FAB RA(RS) and WHO 2001/2008 RA(RS)/RCMD(RS). As described above flow cytometric evaluation of BM of MDS patients is statistically correlated with the established classification systems FAB and WHO 2001. Within the low risk categories, multi lineage dysplasia is of prognostic relevance. The IPSS and WPSS provide a prognostic scoring system integrating not only morphologic information but also karyotyping and transfusion need, respectively. Several studies show that flow cytometric aberrancies in BM of MDS patients correlate with the prognostic scoring systems IPSS and WPSS. (19,20,21,27,29-31)
In studies that quantified the number of flow cytometric aberrancies in BM of MDS patients, high numbers of aberrancies were associated with (high risk) MDS categories and an adverse clinical outcome. (20,21,32) Wells et al. were one of the pioneering groups with regard to flow cytometric prognostic scoring systems for MDS and designed a flow cytometric scoring system (FCSS) based on aberrancies in the (im)maturemyelo-monocytic compartments. The FCSS comprised the categories normal-mild (0-1 points), moderate (2-3 points) and severe (4 points or more) degrees of dysplasia. The FCSS correlated with post transplantation outcome independent of the IPSS score in a group of patients with MDS that were treated with allogeneic hematopoietic stem cell transplantation. **Chapter** six describes the results from a study in which the FCSS provides additional prognostic information within the newly revised IPSS (IPSS-R) risk groups. (33) To calculate the FCSS from flow cytometric data, a large number of variables need to be measured and analysed. Widespread implementation of flow cytometric methods for MDS is still hampered by the complexity of the analysis. The aim of the study, which is described in **chapter** seven was to construct an MDS flow cytometric scoring system (MFS) that could be applied in any laboratory. The statistical analysis resulted in a prognostic flow cytometric score that consists of only three parameters providing information on overall survival of patients with MDS even within the currently used IPSS-R. These findings underscore the importance of adding flow cytometric analysis of BM to the (diagnostic) work-up of patients with MDS to further refine prognostication.

Part III Prediction of response to treatment by flow cytometry

An appropriate and active approach to the treatment of MDS patients is important, not only for improvement of the quality of life, but also for better overall survival. (34) The first line of treatment in IPSS low and intermediate-1 risk MDS patients is supportive therapy consisting of transfusions and growth factors such as erythropoietin (Epo) and human recombinant granulocyte-CSF (G-CSF). Response to treatment can be predicted by a model that includes pre-treatment endogenous serum Epo levels and transfusion requirements which distinguishes three patient groups: MDS patients that are likely to respond to growth factor treatment (74%), with intermediate probability (23%) and poor response to treatment (7%). (35, 36) **Chapter** eight describes a study of 46 patients with IPSS low and intermediate-1 risk MDS who were treated with Epo and G-CSF and evaluated by FC, the presence of immunophenotypically aberrant myeloid progenitors was instrumental in predicting response to growth factor treatment. (37) In **chapter** nine we show that aberrant myeloid progenitors were also predictive for response to treatment with azacitidine in intermediate-2 and high risk patients with MDS. (38)
The findings described in this part of the thesis show the potential of FC to aid in treatment decision making for patients with MDS.

Conclusion

In summary, in this thesis we aimed to describe the technical aspects and recommendations for FC in MDS. Furthermore, we investigated the implications of flow cytometric findings for the prognosis and treatment monitoring of patients with MDS. These results contribute to the further implementation of FC in MDS, both in the laboratory as well as for future prognostic models for MDS that will be used in the clinic and for treatment decision making.

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Technical aspects of flow cytometry in myelodysplastic syndromes

2

Standardization of flow cytometry in myelodysplastic syndromes

Adapted from

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Standardization of flow cytometry in myelodysplastic syndromes within the international consortium and the European LeukemiaNet Working Group

In March 2008, representatives from 18 international and European institutes (IMDS-Flow working group) participated in a European LeukemiaNet (ELN) workshop held in Amsterdam as a first step towards standardization of flow cytometry in myelodysplastic syndromes (MDS).

In the reports published by IMDS-Flow, minimal requirements to analyze dysplasia in the bone marrow of patients with (a suspicion of) MDS were refined. Consensus was reached regarding standard methods for cell sampling, handling and processing. The group also defined minimal combinations of antibodies to analyze aberrant immunophenotypes and thus dysplasia. (Table 1) Examples are altered numbers of myeloid progenitor cells, aberrant expression of markers on myeloid progenitor cells, maturing myeloid cells, monocytes or erythroid precursors and the expression of lineage infidelity markers. The proposed core markers that are described in table 2 should enable a categorization of flow cytometric results in cytopenic patients as 'normal', 'suggestive of', or 'diagnostic of' MDS. A flow cytometric report should include a description of validated flow cytometric abnormalities such as aberrant marker expression on myeloid progenitors and, furthermore, dysgranulopoiesis and/or dysmonocytopoiesis, if at least two abnormalities are evidenced. Figure 1 shows an example of flow cytometric analysis of maturing myeloid cells derived from a bone marrow sample of a patient with MDS as compared with myeloid maturation in a normal bone marrow sample.

Finally, coincidence of MDS with other disorders should be indicated, such as clonal lymphoproliferative disease, mastocytosis, autoimmune diseases and paroxysmal nocturnal hemoglobinuria-associated glycosyl-phospatidyl-inositol-deficient cells.

It should be stressed that flow cytometry in MDS can only be used as a part of an integrated diagnosis. Repeated flow cytometric assessments are highly recommended, not only in inconclusive cases, but also to monitor the course of the disease in untreated, mainly low-risk MDS patients, and during treatment with current available drugs.

Members of IMDS-Flow initiated studies either individually or as a group aiming to standardize and facilitate the implementation of flow cytometry in laboratory and clinical practice of MDS. The results of the collaboration within the Dutch Working Party on Flow Cytometry in MDS is outlined in chapter 3. Technical issues and pitfalls

that might hamper flow cytometric analysis that were addressed by IMDS-Flow will bediscussed in the next chapters of this thesis.

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

Bone marrow subset	Recommended analyses
Erythroid compartment*	% of nucleated erythroid cells
	relation CD71 and CD235a
	expression of CD71
	expression of CD36
	expression of CD117
Myeloid and monocytic progenitors	% of cells in nucleated cell fraction**
	expression of CD45
	expression of CD34
	expression of CD117
	expression of HLA-DR
	asynchronous expression of CD11b, CD15
	expression of CD5, CD7, CD19, CD56***
Maturing neutrophils	% of cells as ratio to lymphocytes
	SSC as ratio vs. SSC of lymphocytes
	relation of CD13 and CD11b
	relation of CD13 and CD16
	relation CD15 and CD10
Monocytes	% of cells as ratio to lymphocytes
	relation of HLA-DR and CD11b
	relation of CD36 and CD14
	expression of CD13 and CD33
	expression of CD56***
Progenitor B cells	enumeration as fraction of total CD34+

*under evaluation
**discrepancies in counts between several definitions indicate aberrancies
***don't overcall, be aware of normal cut-off values (also in stressed marrow)

Table 2. Proposed core markers in the analysis of dysplasia by flow cytometry

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

Note: CD2 and CD25 can be added to analyze aberrant CD117+ mast cells

Multi-color density plots of a normal bone marrow sample and a case of MDS are illustrated in panel A and B, respectively (CD45 (X-axis) vs. SSC (Y-axis)). Cell populations displayed are erythroid cells (CD45-SSClow, indicated in red), blast cells (CD45dimSSClow-int, blue), lymphocytes (CD45brightSSClow, green), monocytes (CD45int-brightSSCint, orange) and neutrophils (CD45dimSSCint-high,pink). Granularity (presented as SSC) of MDS neutrophils in panel B is aberrantly decreased as compared to that of the neutrophils in a normal control (panel A), indicating hypogranularity. In panel C and D maturation patterns of the selected neutrophil subpopulations are shown in CD16 (X-axis) vs. CD13 (Y-axis) plots. In panel C, the normal maturation from CD13+CD16- immature neutrophils, via a CD13dim interphase towards CD13+CD16+ mature neutrophils is displayed; panel D demonstrates an example of an aberrant maturation profile of MDS neutrophils. In panel E and F, maturation patterns of monocytes are shown in a CD36 (X-axis) vs. CD14 (Y-axis) plot. Panel E presents the normal maturation from CD36+CD14- immature monocytes towards CD36+CD14+ mature monocytes. In the example of an MDS case shown in panel F, either aberrantly increased numbers of immature monocytes are present or CD14 expression is aberrantly low or lost on mature monocytes. In panel G and H, expression patterns of CD56 on monocytes (orange) are shown in a CD2 (X-axis) vs. CD56 (Y-axis) plot, as an internal reference CD2+ lymphocytes are shown in green. In a normal bone marrow no to weak expression of CD56 is observed (panel G); in the displayed example of an MDS case, monocytes aberrantly express CD56 (>1 log, panel H).

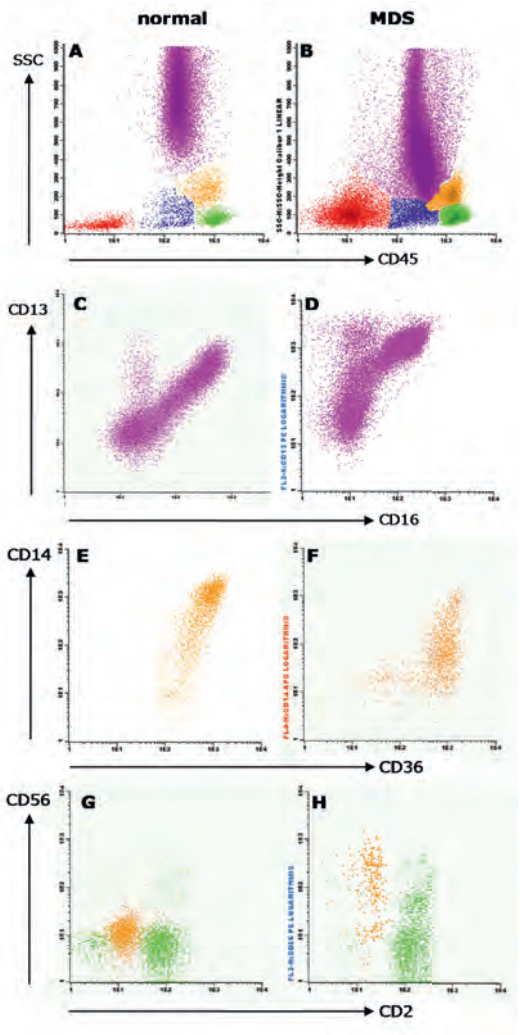

Figure 1 Immunophenotypic patterns in the maturing myeloid-monocytic population of an MDS bone marrow sample as compared to a normal control

3

Implementation of flow cytometry in the diagnostic work-up of myelodysplastic syndromes in a multicenter approach

Report from the Dutch Working Party on Flow Cytometry in MDS

Westers TM, van der Velden VH, Alhan C, Bekkema R, Bijkerk A, Brooimans RA, Cali C, Dräger AM, de Haas V, Homburg C, de Jong A, Kuiper-Kramer PE, Leenders M, Lommerse I, te Marvelde JG, van der Molen-Sinke JK, Moshaver B, Mulder AB, Preijers FW, Schindhelm RK, van der Sluijs A, van Wering ER, Westra AH, van de Loosdrecht AA: Working Party on Flow Cytometry in MDS of Dutch Society of Cytometry (NVC).

Leukemia Research 2012; 36: 422-430

Abstract

Flow cytometry (FC) is recognized as an important tool in the diagnosis of myelodysplastic syndromes (MDS) especially when standard criteria fail. A working group within the Dutch Society of Cytometry aimed to implement FC in the diagnostic work-up of MDS. Hereto, guidelines for data acquisition, analysis and interpretation were formulated. Based on discussions on analyses of list mode data files and fresh MDS bone marrow samples and recent literature, the guidelines were modified. Over the years (2005–2011), the concordance between the participating centers increased indicating that the proposed guidelines contributed to a more objective, standardized FC analysis, thereby ratifying the implementation of FC in MDS.

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders of the bone marrow characterized by peripheral cytopenia in one or more cell lineages. In addition to improving treatment protocols for higher risk MDS, new treatment options are available for low and intermediate-I risk MDS. This underscores the importance of correct classification. Several studies have shown that flow cytometric (FC) analysis allows identification of abnormal erythroid, myeloid and monocytic differentiation thereby contributing to the diagnosis of MDS [1], [2] and [3]. Hence, FC analysis of bone marrow cells has been introduced as an important co-criterion, particularly in cases in which cytomorphology and/or cytogenetics are inconclusive [4]. In order to apply flow cytometry (FC) in MDS, standardization of the experimental procedure as well as data interpretation are crucial. Therefore, a working group of the Dutch Society of Cytometry was established in 2005 aiming at the implementation of FC in the diagnostic work-up of MDS in the Netherlands. Several meetings were convened to educate participants and to discuss ways to standardize analysis and, more importantly, interpretation of data in multiple centers. Meanwhile, participation within the MDS working group of the EuropeanLeukemiaNet (ELNet) accelerated agreements on standardization of procedures and analysis [5]. This report describes the process of implementing FC in the diagnostic work-up of MDS in the Netherlands; in addition, suggestions for further improvement are made.

Materials and methods

Review of literature on FC in MDS and introduction to pattern recognition

Since knowledge of normal maturation patterns and expression levels of certain antigens is of utmost importance, a series of publications were reviewed in the first meeting of the working group [6], [7], [8], [9],[10] and [11]. In order to train participants in pattern recognition, printed dot plots of neutrophil and monocyte maturation from 10 MDS samples and 5 normal bone marrow samples were sent to every center. Patients were classified according to WHO2001 as RA (n = 1), RA-RS (n = 1), RCMD (n = 3), RCMD-RS (n = 1), RAEB-I (n = 3) and MDS-U (n = 1). Results were reported to the coordinating center (VU University Medical Center) and discussed in subsequent meetings.

Design of an antibody panel

Data from literature combined with experience from the centers that already gained experience with FC in MDS and experience from the Dutch working group on the analysis of minimal residual disease in AML (Dutch Society of Cytometry) was used to compose a 4-color panel of antibody combinations (Table 1)[7] and [12]. This panel was evaluated by comparison of the results from the different centers and adjusted accordingly.

Table 1 Proposed 4-color antibody combinations for flow cytometric analysis of bone marrow samples in myelodysplastic syndromes.

Tube					Lineage
1	CD45	PBS	PBS	PBS	
2	CD45	CD16	CD13	CD11b	Myelomonocytic
3	CD45	CD71	CD235a	CD117	Erythroid
4	CD45	CD36	CD33	CD14	Myelomonocytic
5	CD45	CD15	HLA-DR	CD11b	Myelomonocytic
6	CD45	CD34	HLA-DR	CD123	Immature/basophilic/DC
7	CD45	CD34	CD13+33	CD117	Myelomonocytic
8	CD45	CD34	CD7	CD56	Immature/lymphocytes
9	CD45	CD34	CD5	CD19	Immature/lymphocytes
10[a]	CD45	CD34	CD15	CD11b	Myelomonocytic

Abbreviations: PBS: phosphate buffered saline; DC: dendritic cells.
aThe proposed panel (2005) was extended in 2007. No restriction to clones and fluorescent conjugates was laid on.

Definition of various subpopulations and gating strategy

Since strict definitions of distinct subpopulations are essential to analyze aberrancies on those subpopulations properly; definitions for the various subpopulations are depicted in Table 2. The accuracy of these definitions was tested by comparing FC differentials of several bone marrow samples distributed as list mode data files (from 2007 onwards). It was agreed on that every subpopulation should be expressed as percentage of all nucleated cells as denominator (white blood cells including nucleated red cells). To that end, analysis should be performed according to the following terms: (i) exclusion of debris using the forward vs. sideward light scatter (FSC/SSC) plot, (ii) selection of white blood cells and subpopulations using the CD45/SSC plot; (iii) fine-tuning of selection of subpopulations by specific markers such as CD34 for blast cells, $CD14^{+}$ and $CD33^{bright}$ for monocytes and $CD33^{dim}$ for neutrophils and (iv) backgating of defined subpopulations in CD45/SSC and FSC/SSC plots (Fig. 1). Of note, throughout this document the word "blast" is used to indicate immature progenitor cells as defined by FC.

Table 2 Definitions for identification of subpopulations in bone marrow by flow cytometry.

Subpopulation	Definition 2006	Definition adjustments in 2007, 2008 or 2011
Nucleated red cells	$CD45^{-}CD235a^{+}CD71^{+}$	$CD45^{-}CD235a^{+}CD71^{+}$ SSC^{low}
Myeloblasts	$CD45^{dim}$ SSC^{low} $CD34^{+}$, $CD117^{+}$check for $CD34^{-}$ blasts: $CD45^{dim}CD13/33$	$CD45^{dim}$ SSC^{low} $CD34^{+}$ $CD117^{+}$ in combination with a myeloid marker (CD13, CD33) or $CD45^{dim}$ SSC^{low} $CD117^{+}$ $HLA\text{-}DR^{+}CD11b^{-}$(2008)
Maturingneutrophils	$CD15^{+}$ back gated in CD45/SSC plot	$CD33^{dim}$ back gated in CD45/SSC plot
Monocytes	$CD14^{+}$ back gated in CD45/SSC plot	$CD64^{bright}$ and $CD33^{bright}$ back gated in CD45/SSC plot; $CD14^{+}$ on mature monocytes (2011)
Lymphocytes	$CD45^{bright}$ SSC^{low} $CD5^{+}$ or $CD19^{+}$	$CD45^{bright}$ SSC^{low}
Precursor B cells	$CD45^{dim}$ SSC^{low} $CD34^{+}$ $CD19^{+}$	Conform
Plasmacytoid DC	$CD123^{+}$ $HLA\text{-}DR^{++}$	$CD123^{+}$ $HLA\text{-}DR^{++}$ regardless of $CD34^{+}$
Basophils	$CD123^{+}$ $HLA\text{-}DR^{-}$	$CD123^{+}$ $HLA\text{-}DR^{-}$ SSC^{low}
Promyelocytes	–	$CD34^{-}CD117^{+}$ $CD34^{-}CD117^{+}$ SSC^{high} (2008)

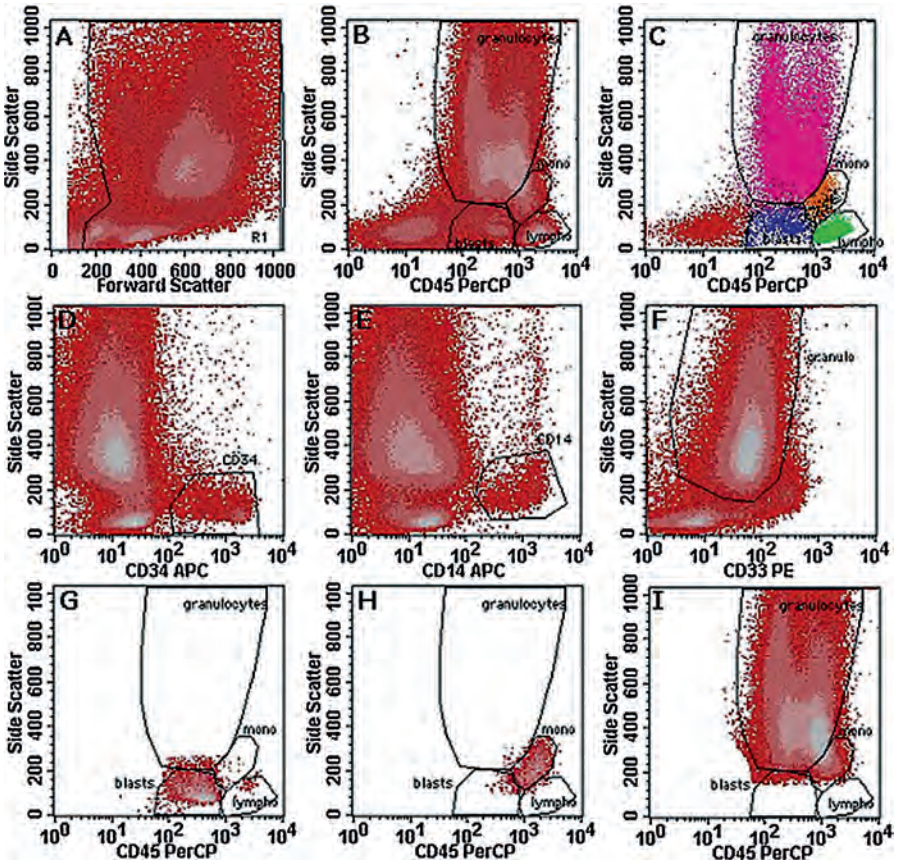

Fig. 1. Gating strategy for the flow cytometric analysis of defined subpopulations in a MDS bone marrow sample.

Scatter properties (FSC vs. SSC plot) are used to remove debris (selection of cells via region R1). CD45 is present in every combination of antibodies to allow primary gating of subpopulations based on CD45 expression and SSC properties. A density plot and the corresponding multi-colored dot plot are depicted in panel B and C, respectively. Cell populations recognized in this way are erythroid cells ($CD45^{-}SSC^{low}$, red colored cells in panel C), blast cells ($CD45^{dim}SSC^{low\text{-}int}$, blue colored cells), lymphocytes ($CD45^{bright}SSC^{low}$, green colored cells), monocytes ($CD45^{int\text{-}bright}SSC^{int}$ orange colored cells) and neutrophils ($CD45^{dim}SSC^{int\text{-}high}$ pink colored cells). Similar colors were used for every plotted subpopulation in the analyses to facilitate comparison between centers. CD34, CD14 and $CD33^{dim}$ positive populations (panels D, E and F, respectively) were back gated in CD45/SSC plots (panel G, H and I, respectively) and FSC/SSC plots (data not shown) to determine whether the analysis gates were appropriate; in this way for instance $CD45^{-}$ blasts or overlap in granulocyte and monocyte populations might be recognized (panel I). Further information on the application of CD33 in the recognition of subpopulations is displayed in Fig. 3.

Analysis of list mode data files

List mode data files were supplied by the VU University Medical Center, Amsterdam. These concerned analyses of bone marrow samples of cytopenic patients suspected for having MDS (median age 68, range 41–89) and two bone marrow samples from healthy volunteers (age: 41 and 54) for reference; samples were processed according to ELNet guidelines [5]. All samples were drawn after informed consent; patients and controls were anonymous except to the distributing center. According to WHO2001, patients were classified as RCMD (n = 6), RCMD-RS (n = 2), RAEB-I (n = 2), RAEB-II (n = 1), CMML-1 (n = 1) and MDS-U (n = 1), three patients remained inconclusive and one patient appeared to have complete remission of CML. Cytogenetic anomalies were detected in only two patients (Supplementary Table 1). Of note, participants were unaware of the patients' classification until discussion on results was closed. For all of the supplied data files, centers were requested to identify proportions of each subpopulation. To compare the data between the separate centers the interlaboratory variation, defined as 100% × (SD/mean), was calculated. In addition, aberrancies in any of the defined subpopulations, i.e. erythroid cells, myeloid blasts, maturing neutrophils and monocytes, should be reported. To evaluate improvement in analysis and interpretation, some of the list mode data files were sent out twice without prior knowledge of the participants (Supplementary Table 1, patients #3, 4 and 9).

Analysis of fresh bone marrow samples

Fresh heparinized bone marrow samples of patients that were referred to the VU University Medical Center were distributed to compare FC procedures in different centers; all samples were drawn after informed consent. These patients were suspected for having MDS (n = 3, median age: 77, range 73–89); these patients were classified as RCMD-RS, CMML-1 and idiopathic cytpenia of unknown significance (ICUS). For standardization purposes all centers kept samples overnight at room temperature before analysis by a lyse-stain procedure according to ELNet recommendations. (5) Samples were analyzed on flow cytometers from BD Biosciences (FACS Calibur or FACS Canto II; seven centers) and Beckman Coulter (FC500; one center). It was agreed to acquire at least a minimum of 100,000 events for each tube; at least 250 blast cells should be acquired.

Results

Review of literature and pattern recognition

Potential aberrancies as demonstrated in several studies were cataloged per subpopulation, i.e. erythroid subpopulation, myeloid blasts, maturing neutrophils and monocytes (summary in Supplementary Table 2)[8], [9], [10] and [11]. Since many aberrancies concern altered relations between antigens (e.g. HLA-DR vs. CD11b, CD16 vs. CD13), training in pattern recognition was regarded necessary. Hereto, distributed dotplots of 15 samples, ten MDS cases and five normal controls, were discussed (CD16/CD13/CD11b and CD33/CD14/CD36 for neutrophil and monocyte maturation, respectively). In six out of ten patients results were similar: either normal or aberrant maturation profiles were reported. Most discussion was raised in analyzing plots from the RA(RS) patients, while every center reported normal profiles in the MDS-U patient (solely dysmegakaryopoiesis according to cytomorphology). These results indicate that pattern recognition can be performed reliably; however, experience and normal references or even overlay reference images are essential to conclude on normal and aberrant profiles.

Validation of antibody combinations for FC in MDS

The proposed panel of 4-color antibody combinations (Table 1) covered all currently known requirements for proper analysis of maturation patterns in the defined erythroid and myelomonocytic subpopulations and aberrant marker expression in the myelomonocytic lineage. According to the proposed combinations every center compiled an antibody panel (either 4-, 5- or 6-color) by using antibody clones and fluorochromes available in that particular center. Each center run a set of controls and potential MDS cases and presented their data in subsequent meetings. The differences in interpretation of maturation patterns between centers were mainly caused by differences in the number of acquired events and the amount of events displayed in the plots. Therefore, we agreed on the acquisition of 100,000 leukocytes per tube and most importantly, similar amounts of events should be displayed per subpopulation, i.e. 20,000 events for neutrophils and the erythroid lineage; 1000 events for monocytes, for proper comparison of maturation patterns between patients and controls, and between centers. Moreover, every center presented their data using similar markers on either X-axis or Y-axis. These agreements resulted in reference patterns for each center that, despite differences in antibody clones,

fluorochromes, and instrument set-up, could relatively easily be interpreted by other centers.

Experience with the proposed panel revealed that it did not allow accurate analysis of CD11b and CD15 expression on myeloid blasts. Since both markers have been reported to be important for prognosis of MDS patients [9] and [13], these markers were combined with CD34 in a supplementary staining (adjustment agreed on in 2007). Of note, the proposed panel was adopted by the ELNet working group on standardization of FC in MDS (Amsterdam, 2008) [5].

Validation of the definition of various subpopulations

The accuracy of the subpopulations' definitions (Table 2) was tested by comparing results for several bone marrow samples distributed as list mode data files. Overall, seven sets of data concerning 19 samples were distributed from 2007 onto 2009. The centers reported the proportions of every myelomonocytic, erythroid and lymphoid subpopulation in each sample according to the defined gating strategy (Fig. 1). The interlaboratory variation for the percentage of a specific subpopulation correlated significantly to the size of the population ($p < 0.001$, Spearman's rho −0.719, Fig. 2A). Ongoing experience, discussion on discrepancies in the FC differentials and subsequent reanalysis led to refined definitions (Table 2). Reanalysis of the data demonstrated the learning curve, the interlaboratory variation decreased upon reanalysis (Fig. 2B). Thus, discussions, adjustments and increasing experience contributed to standardization.

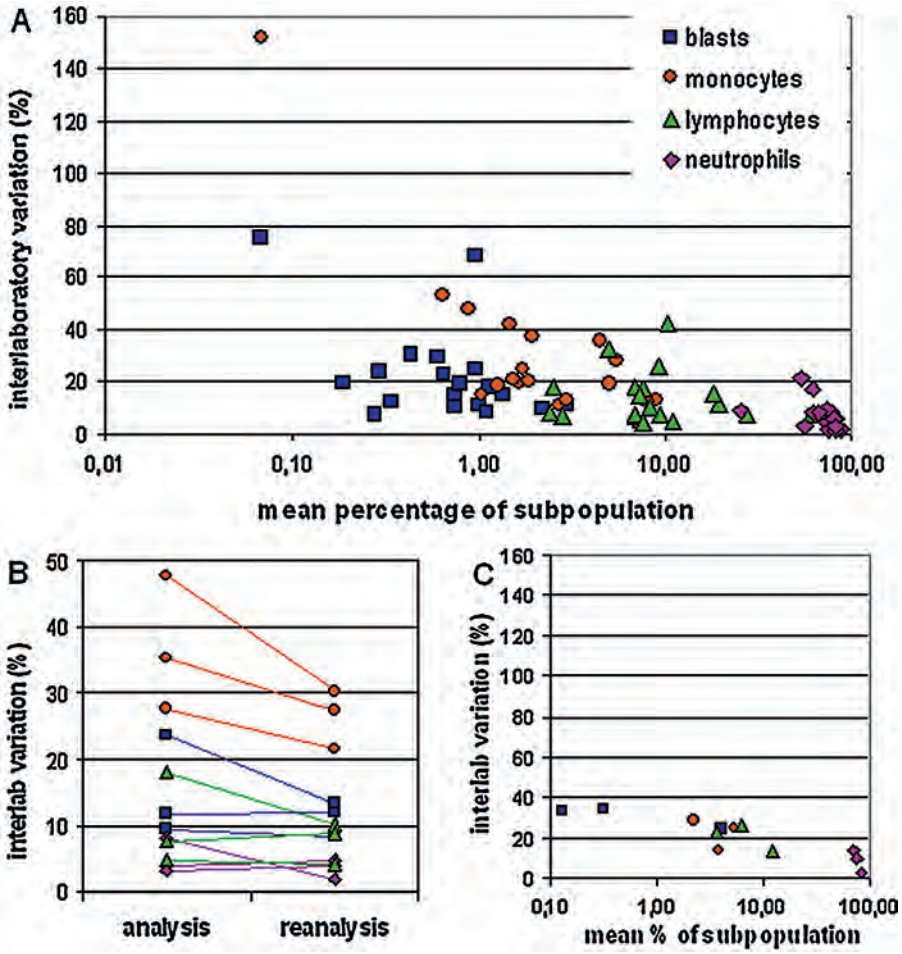

Fig. 2. Interlaboratory variation in analysis of the differential of MDS bone marrow samples.

Panel A depicts the relation of the interlaboratory variation (Y-axis, 100 × (SD of all centers' results/mean of all centers' results)) to the size of the defined subpopulations after analysis of the list mode data files (X-axis, n = 19). Neutrophils, monocytes, blasts and lymphocytes are displayed in pink squares, orange circles, blue squares and green triangles, respectively. Reanalysis of list mode data files 1.5–2 years after initial analysis demonstrates the learning curve as depicted in panel B. Panel C depicts the relation of the interlaboratory variation to the size of the defined subpopulations in three freshly processed bone marrow samples.

Subjects of discussion and adjustments are listed below:

- Large differences were encountered when a lot of debris was present in the sample. The results improved by applying the same white blood cell gate (i.e. CD45) for analysis of each tube of a particular bone marrow sample.

- Discrepancies in the erythroid lineage were caused by aggregation of erythroid cells due to the antibodies used (CD71 and CD235a). Higher consistency between centers was obtained by excluding these aggregates (i.e. erythroid cells with high SSC).
- CD15 appeared to be unsuitable for defining maturing neutrophils since the IgM isotype of the antibody used within the working group led to aggregation of cells. Consequently, lower percentages of neutrophils were observed as compared to other tubes without CD15. Although CD15 of IgG_3isotype performed well, this antibody reacted more strongly with monocytes (data not shown); therefore, it was not regarded appropriate to separate monocytes and granulocytes. Thus, neutrophils were redefined based on SSC^{high}, $CD45^{dim}$ and $CD33^{dim}$ expression (2007). This definition could even easily distinguish monocytes and neutrophils in patients with hypogranulated neutrophils and a low CD33 expression due to a polymorphism (Fig. 3) [10] and [14].
- Largest variation between centers was seen in the size of the monocytic subpopulation (Fig. 3A and B), despite the adjustments that were made to gate and back gate monocytes. Maturing monocytes can be recognized in the CD45/SSC plot ($CD45^{dim/bright}$ and SSC^{int}); in case of severe hypogranularity of neutrophils CD14 is mandatory for defining lineage restriction; alternatively, CD33 can be used (Fig. 3). Recently, CD64 was explored as an additional marker to define and enumerate monocytes more accurately. The application of CD64 in combination with CD33, CD14 and CD45 revealed that identifying monocytes by CD14 and SSC alone underestimates the amount of monocytic cells within a sample. Promonocytes (and monoblasts) already show bright expression of CD64 and CD33, while CD14 is absent to dimly expressed. Thus, it is advised to use CD64 (or CD36) and CD33 in combination with SSC to define monocytoid cells; CD14 informs about the distribution of immature and mature monocytoid cells in this fraction.
- Varying percentages of monocytes were also caused by including or excluding cells with higher SSC (potential doublets due to adherence to neutrophils) and with lower FSC, probably representing cells undergoing apoptosis (Fig. 3); consensus was reached to analyze all $CD14^+$ monocytes despite their FSC.

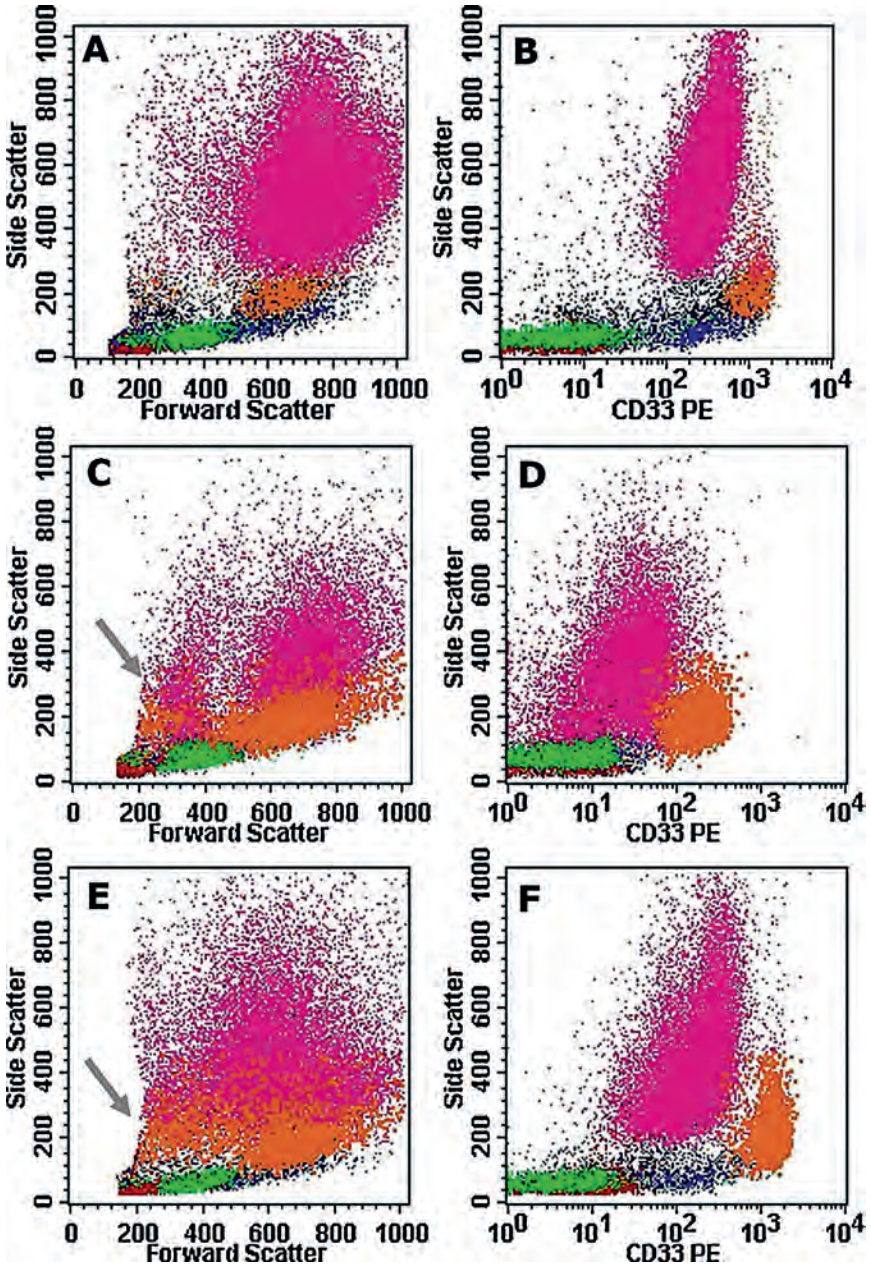

Fig. 3. CD33 is helpful in distinguishing neutrophils and monocytes in hypogranular bone marrow samples. Forward scatter vs. sideward scatter and CD33 vs. sideward scatter plots are depicted from a normal bone marrow sample (panels A and B) and two low/int-1 MDS patients (panels C and D, panels E and F). Neutrophils, monocytes blasts and lymphocytes are displayed in pink, orange, blue and green, respectively. The grey arrows in panels C and E indicate possibly apoptotic monocytes and neutrophils with lower forward scatter properties.

- The initial definition of promyelocytes ($CD34^{-}CD117^{+}$) raised problems in dissecting these cells from CD34-negative blasts and early erythroid precursors; identification in combination with SSC was recommended. A better definition might be $CD34^{-}CD117^{+}HLA\text{-}DR^{-}CD15^{+}$, though this combination is unsuitable for 4-color FC in combination with CD45.
- The first definition of lymphocytes excluded Natural Killer cells. It was agreed to enumerate lymphocytes based on $CD45^{bright}SSC^{low}$ expression in the tube that contained solely CD45.
- Aberrant CD19 expression on myeloid blasts and the presence of lymphoid progenitors might be confused. Although, progenitor B cells are often low or missing in MDS cases [2], [15] and [16], strict gating strategies based on scatter properties or other cell lineage specific markers are recommended.

Analysis of immunophenotypic aberrancies in MDS patients (list mode data files)
In addition to the differential, the centers reported the observed aberrancies in the myelomonocytic and erythroid subpopulation (Supplementary Table 2) in each data set of a particular sample. Based on discussions on the results, several pitfalls in reporting and analysis of data were identified; these are summarized below.

Pitfalls in data acquisition: instrument set-up and quality control
Only a stable, tightly controlled analytic process enables comparison of results between different samples and appropriate analysis of differentiation patterns. Notably, the performance and sensitivity of the fluorescence detectors of the flow cytometer should be set and monitored using fluorescent calibration beads at a regular, preferably daily, basis. Furthermore, fixed regions for lymphocytes, neutrophils and monocytes during data acquisition could illustrate correct sample handling and instrument set-up.

Pitfalls in data reporting
- Overestimation of the number of aberrancies can occur. For instance, abnormal CD13 expression can cause several aberrancies in differentiation patterns; it was agreed to report these abnormalities as one.
- Altered CD33 expression can be due to a polymorphism [10] and [14] resulting in altered expression in every myelomonocytic subpopulation

(myeloid blasts, neutrophils and monocytes) rather than in a single subpopulation; this should be noted but not regarded aberrant.
- Over interpretation of results in small subpopulations, e.g. in case of very low blast counts or severe monocytopenia. In such cases more events should be acquired (>250 per subpopulation), otherwise data in this subpopulation should be considered not evaluable.
- CD56 expression can be overcalled as aberrant; it is also expressed on activated neutrophils and monocytes; CD56 should only be considered aberrantly expressed when it is 1 log decade above activated cells (example in Fig. 4D).

Pitfalls in data analysis

In general, some of the failures to recognize certain aberrancies in the supplied data sets of MDS patients were due to an insufficient amount of normal reference samples supplied for comparison of the results.

Pitfalls in analysis of erythroid cells

Discordant results were obtained for decreased expression of CD235a on erythroid precursors. Part of the laboratories scored the latter as aberrant, whereas others concluded that low expression was due to abundance of non-lysed erythrocytes; it was agreed that in these cases it should not be considered aberrant.

Pitfalls in analysis of myeloid blasts

Regarding analysis of myeloblasts failure to recognize aberrant homogeneous expression of CD117 as compared to normal heterogeneous CD117 expression (examples in Fig. 4A), HLA-DR over or under expression or even lack of HLA-DR, and CD13 over or underexpressionwere most common. Since CD13 expression was not analyzed in combination with CD34, the interpretation of CD13 expression on myeloid blasts was less accurate due to contamination of the $CD45^{dim}$ region with maturing cells. Inclusion of CD34 in the tubes might solve this problem; though this requires additional tubes or more than 4-color analysis.

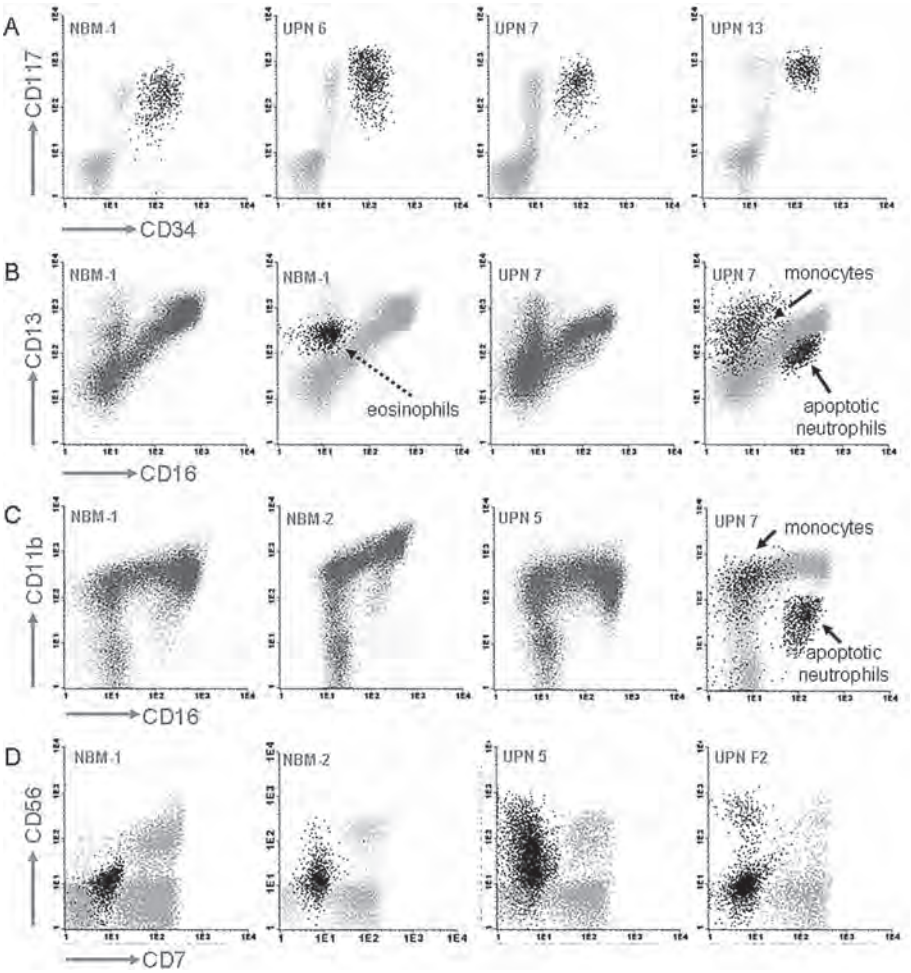

Fig. 4. Examples of interpretation of flow cytometric profiles of the immature and maturing myelomonocytic lineage with respect to analysis of dysplasia. In panel A, CD34 is plotted on the X-axis and CD117 on the Y-axis. Myeloid cells were selected based on CD45 expression and light scatter properties as described in Fig. 1. Immature myeloid progenitors (highlighted in black, $CD34^{+}CD117^{+}$) mature towards promyelocytes (in grey, $CD34^{-}CD117^{+}$) and further on to myelocytes, metamyelocytes, bands and segmented neutrophils (in grey, $CD34^{-}CD117^{-}$). In normal bone marrow, CD117 is rather heterogeneously expressed on the immature myeloid progenitors (utmost graph on the left hand side, NBM-1). In patient 6 (graph middle left), CD117 expression is heterogeneous on immature cells, but might be overexpressed in a part of the population (>0.5 log). Regarding patient 7, it was discussed whether the myeloid progenitors demonstrated homogenous CD117 expression, as is undoubtfully the case in patient 13 (utmost right graph, homogenous CD117 overexpression). Calculation of the coefficient of variance in CD117 expression (available in CellQuestPro Software) might be helpful in this respect. A low variance as compared to normal controls indicates homogeneity; this was not the case in patient 7. In panel B, myeloid cells were selected based on CD45 expression and light scatter properties (see also Fig. 1); CD16 is plotted on the X-axis, CD13 on the Y-axis. Myeloid cells mature from $CD13^{+}CD16^{-}$ towards $CD13^{+/-}CD16^{-}$ promyelocytes, then CD13 and CD16 gradually increase towards the bands and segmented neutrophils (both $CD13^{+}CD16^{+}$, graph utmost left).

Eosinophils can interfere in this pattern as is demonstrated in the graph middle left (highlighted in black, dashed arrow). The expression profile of CD16 and CD13 in patient 7 was considered aberrant (graph middle right). Part of this was caused by contamination of the hypogranular neutrophil fraction by monocytes (graph utmost right, $CD13^{+}CD16^{-}$ highlighted in black), and moreover, by the presence of a population of apoptotic neutrophils ($CD13^{dim}CD16^{+/dim}$). The latter populations are also illustrated in panel C (graph utmost right, CD16 vs. CD11b). The CD16 vs. CD13 profile in patient 7 was considered aberrant: a concave in stead of a convex shape and low CD16 expression (>0.5 log) on the most mature cells. Patient 5 was discussed for a potentially low expression of CD11b on the more mature neutrophils as illustrated in panel C (graph middle right, CD16 on X-axis and CD11b on Y-axis). As a reference, two normal bone marrow profiles were included (graph utmost left and graph middle left). Comparing UPN 5 to NBM-1, a normal CD11b might be concluded while comparison with NBM-2 (that demonstrates higher CD16 expression) might indicate an aberrant profile. This stresses that data should always be compared to a set of controls (minimum of 10, preferably age-matched). In panel D, monocytes (in black) and lymphocytes as a reference (in grey) are depicted; CD7 is plotted on the X-axis, CD56 on the Y-axis. Upon activation CD56 expression can be observed on monocytes; therefore, CD56 expression should be at least >1 log different from normal to be called aberrant. In patient F2 (graph utmost right) a clear subpopulation of $CD56^{+}CD7^{-}$ monocytes is shown, while in patient 5 (graph middle right) monocytes show dim to bright expression of CD56. This population was called aberrant since over 20% of the monocytes had >1 log increase in CD56 expression.

Expression of CD5, CD7 or CD56 on myeloid blasts was sometimes overestimated due to inappropriate comparison with background fluorescence of blast cells. Moreover, adherence of T cells to myeloid blasts might impede interpretation of results; therefore, back gating strategies must always be employed and doublets must be excluded. Cut off values of aberrant marker expression such as CD7 were recurrently discussed; expression levels of these markers in normal bone marrow samples have to be available for accurate interpretation. In order to regard a marker as being aberrantly expressed, it was agreed that at least a dense population of 10% of blasts cells should be positive for this marker. Furthermore, the marker should have at least half a log difference in expression as compared to the negative control.

Pitfalls in analysis of myeloid cells

Often missed aberrancies in the maturing neutrophil fraction concerned low SSC, altered intensity of CD15, aberrant patterns of CD16 vs. CD13 and CD11b vs. CD13 (examples in Fig. 4B and C). Additional analysis by annexin V and 7-AAD revealed that the presence of CD16dim and/or CD11bdim neutrophils must be noted as representation of apoptosis due to sample handling rather than aberrancy [17] and [18] Pattern recognition is hard to standardize and requires intensive training and experience. Furthermore, over interpretation of CD56dim expression as aberrancy occurred rather frequently.

Pitfalls in analysis of monocytic cells

Aberrant HLA-DR vs. CD11b pattern and aberrant expression of CD13 (homogenously high or low or heterogeneous) were often neglected aberrancies in monocytes. CD56^{dim} expression was regularly described as aberrantly high. Moreover, hypogranularity of neutrophils hampered the analysis of aberrancies in the monocytic subpopulation as discussed above.

Pitfall in all subpopulations

Technical irregularities can result in altered SSC and CD45 expression. Therefore, SSC and CD45 expression of a subpopulation must be reported as ratio to SSC and CD45 of lymphocytes as internal reference.

Improvement in data interpretation

Three list mode data files were distributed twice to the centers to evaluate whether adjustments and increased experience resulted in more concordant results. Indeed, during the years the degree of concordance in a single sample increased and, hence, variation between centers decreased significantly over time (Fig. 2 and Fig. 5).

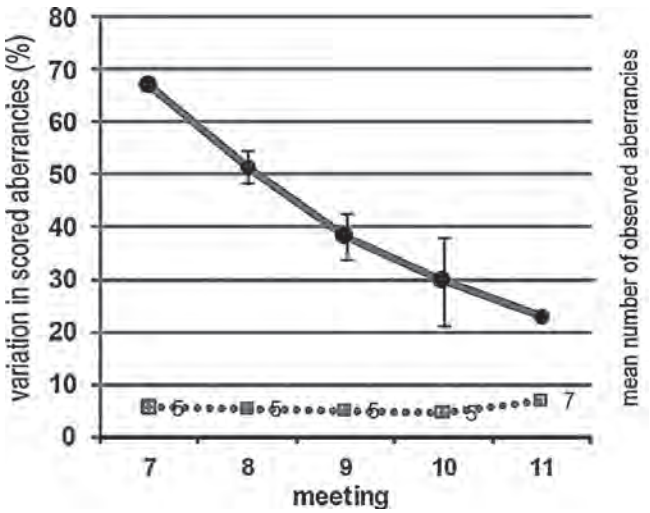

Fig. 5. Interlaboratory variation in scoring of aberrancies in the erythroid lineage and (im)mature myeloid lineage during time. Observed aberrancies in the flow cytometric analysis of the (im)mature myeloid lineage were enumerated per patient and for every center. Per patient sample, variation in scoring for aberrancies between the centers was calculated (100% × (SD/mean), solid line). Mean variation in aberrancies of all samples discussed in a particular meeting is depicted on the Y-axis, consecutive working group meetings are displayed along the X-axis (2007–2008). Different data sets were analyzed for every meeting (2–4 patients per set, re-analyses of previously analyzed samples were excluded from this graph). Per data set the average amount of observed aberrancies was rather similar (means indicated along a dashed line), therefore we concluded that during time the degree of concordance between centers increased.

Multi-center analysis of fresh MDS bone marrow samples

Before participating centers could continue to simultaneously analyze fresh bone marrow samples, it was urged that every center should have analyzed at least 10 normal (age-matched) bone marrow samples with their own antibody panel. In the individual centers, control samples were obtained, after informed consent, from patients that underwent cardiac surgery or staging of lymphoma and some healthy volunteers. After this criterion was met, bone marrow samples from three patients suspected for having MDS were distributed (2008).

Analysis of the differential in fresh bone marrow samples

In the three distributed samples the variation in the differential was comparable to that observed for the list mode data files (Fig. 2C and A, respectively). Of note, blast percentages in two of the samples were below 0.5%, explaining the relatively large interlaboratory variation. These data indicates that potential differences in the experimental procedures in the different centers have only minor impact on the final results.

Analysis of aberrancies in fresh bone marrow samples

The number of aberrancies in the distributed MDS samples was low. Main discordance was seen in the interpretation of expression of CD7 and CD15 on myeloid blasts, and interpretation CD56 expression on monocytes, all related to inappropriate interpretation of background staining as discussed above.

Overall, no discrepancies between centers were observed with respect to 4- or more than 4-color analysis, despite that more stringent gating strategies could be applied in >4-color analysis. This might further improve concordance between laboratories with respect to the immunological differential. Of note, the aberrancies detected within the subpopulations might be of higher importance than the size of the subpopulation itself. This will be evaluated in future meetings.

Discussion

Cases of myelodysplastic syndromes (MDS) will benefit from a multiparametric diagnostic approach. It has already been demonstrated that flow cytometry (FC) can add to the diagnosis of MDS [1], [2], [3], [8], [11],[16], [19], [20], [21], [22] and [23]. The Dutch working group on FC in MDS proposed guidelines for analysis of MDS samples in accordance with those from the European LeukemiaNet (ELNet) working group on standardization of FC in MDS. [5] In eight centers, we studied whether implementation of these guidelines provided a good platform for MDS analysis. Discussions and experience increased concordance between centers; moreover, we demonstrated that minor differences in experimental procedures between the centers had no effect on final results. Our effort may be a first step, yet more standardized (i.e. similar clones and fluorochromes) or even simpler, less expensive panels may be better in view of implementation in large scale routine diagnostics. The proposed antibody combinations are thought to be achievable in all centers; though, expanding the antibody combinations to a >4-color combination will enable more stringent gating strategies to define subpopulations and hence, evaluation of aberrancies; this will be explored in future meetings.

The proposed marker set should enable a categorization of the results of FC analysis as normal or as (possibly) in agreement with MDS. A FC report should include a description of validated FC abnormalities such as aberrant marker expression on myeloid progenitor cells (e.g. lineage infidelity marker expression), decreased side ward light scatter of granulocytes and CD56 over expression on monocytes. In a current study from the Dutch Hematology-Oncology Group (HOVON89, www.HOVON.nl) on the treatment of patients with low and intermediate-1 risk MDS, FC of bone marrow samples is incorporated to prospectively validate current described aberrancies and to analyze its value in monitoring response to treatment. All samples will be analyzed centrally by one of the participating centers while analysis by another working group member in parallel enables comparison of results, validation of the FC procedures and data interpretation.

Translation of results of FC analysis into numerical scores would highly facilitate implementation of FC into clinical practice. Many of the reports on FC in MDS make an effort to design such diagnostic scores, though multi-center validation of flow-scores for the diagnosis and/or prognosis of MDS is scarce (examples of validated flow-scores in our set of patients in Supplementary Table 1) [2] and [10].

Besides the importance of technical aspects such as flow cytometer calibration, longitudinal quality control and reagent validation, we experienced that training is at least as important. Especially pattern recognition is hard to standardize. Since differentiation patterns depend on instrument set-up, antibody clones and conjugates, and the antibody combinations, it is essential that results of analysis of MDS bone marrow samples obtained within a particular center are always compared to results for samples from age-matched healthy individuals analyzed in the same center. The development of new software tools especially in combination with standardized 8-color panels may significantly facilitate MDS analysis in future [24] and [25].

Upon expanding experience the working group discussed whether centers in the Netherlands that are yet not involved can be trained. Importantly, these centers should have extensive experience in the field of FC and need to be involved in (inter)national treatment protocols (e.g. EORTC and HOVON studies). To guide the introduction of FC in MDS in these centers, a standard operating procedure for analysis of MDS bone marrow samples by FC has been written and an informative meeting has been convened (February 2011, www.cytometrie.nl).

In summary, the proposed guidelines and discussions on FC in MDS contributed to a more objective, standardized analysis and data interpretation. This implies that implementation of FC in the diagnostic work-up of MDS is feasible; though before centers participate in FC analysis of MDS, training and experience is of utmost importance.

To optimize standardization of FC in MDS, discussions on FC criteria for diagnosis of MDS (a scoring system) and validation of all aberrancies against a cohort of age-matched controls are regarded essential. Hereto, experience from the working group on FC in MDS of the Dutch Society of Cytometry was already incorporated into the ELNet meetings on standardization of FC in MDS. Members of the Dutch working group will continue their participation within future ELNet meetings to extend their knowledge in order to contribute to the diagnosis and prognostication of MDS.

Supplementary data

Supplementary Table 1. Characteristics of patients analyzed by flow cytometry in consecutive meetings

UPN	morphology	cytogenetics	sample	FCSS	diagnostic score*
NBM-1	normal	nd	list mode data	1	0
NBM-2	normal	nd	list mode data	2	1
1	RCMD	normal (good)	list mode data	5	2
2	inconclusive	normal (good)	list mode data	na**	na**
3	MDS-U	normal (good)	list mode data	3	2
4	inconclusive	normal (good)	list mode data	5	1
5	RCMD	na	list mode data	5	2
6	RCMD-RS	normal (good)	list mode data	5	2
7	RAEB-1	na	list mode data	2	2
8	RCMD	na	list mode data	0	0
9	RCMD-RS	normal (good)	list mode data	5	3
10	RAEB-1	normal (good)	list mode data	6	3
11	CML in CR	abnormal (int)	list mode data	3	0
12	inconclusive	na	list mode data	2	0
13	RAEB-2	normal (good)	list mode data	na**	na**
14	CMML-1	normal (good)	list mode data	4	3
15	RCMD	normal (good)	list mode data	2	2
16	RCMD	na	list mode data	3	3
17	RCMD	abnormal (good)	list mode data	2	3
F1	RCMD-RS	normal (good)	fresh bone marrow	2	2
F2	CMML-1	normal (good)	fresh bone marrow	5	3
F3	ICUS	na	fresh bone marrow	3	2

Note: Morphology is reported according to WHO2001 (except for patient F3). Results of cytogenetic analysis are reported as normal or abnormal, cytogenetic risk groups are depicted between brackets. Abbreviations: UPN: unique patient number; NBM: normal bone marrow; RCMD: refractory cytopenia with multilineage dysplasia; RS: ring sideroblasts; RAEB refractory anemia with excess of blasts; MDS-U myelodysplastic syndrome unclassified; CMML: chronic myelomonocytic leukemia; CML: chronic myeloid leukemia; CR: complete remission; ICUS: idiopathic cytpenia of unknown significance; na: not available; nd: not done; FCSS: flow cytometric score of dysplasia in the myeloid-monocytic lineage according to Wells et al., 0-1: normal, 2-3: mild dysplasia, 4-9: severe dysplasia [10]; *: a four-parameter diagnostic score according to the model designed by Ogata and Della Porta et al. was calculated; parameters taken into account were increased percentages of CD34+ cells, in- or decreased expression of CD45 on CD34+ myeloid progenitors, decreased percentage of B cell progenitors within the CD34+ population and decreased sideward light scatter of neutrophils; scores ≥2 indicate MDS with a specificity of approximately 90% and a sensitivity of 70% [2];**: no reliable scores could be calculated because of hemodilution of the bone marrow sample due to dry-tap. List mode datafiles were supplied by the VU University Medical Center (Amsterdam). Patients F1-F3 that were referred to the VU University Medical Center, gave informed consent for distribution and analysis of their bone marrow among participating centers. Sanquin Research (Amsterdam) provided the distribution of the samples.

Supplementary Table 2. Flow cytometric aberrancies listed per subpopulation (adapted from references [5, 8-11]

erythroid cells	myeloid blasts
increased percentage per nucleated cells	increased percentage
increased percentage of CD117+ precursors	abnormal granularity*
decreased/increased expression of CD71	abnormal expression* of CD45
asynchronous relation of CD71 vs CD235a	abnormal expression of CD34
	abnormal expression of CD117
	abnormal expression of CD13
	abnormal expression of CD33
	abnormal expression of HLA-DR
	expression of CD11b
	expression of CD15
lymphoid blasts	
decreased percentage per $CD34^+$ population	expression of lineage infidelity markers CD5, CD7, CD19 or CD56

granulocytes (maturing myeloid cells)	monocytes
decreased myeloid/lymphoid ratio (<1)	decreased/increased number as compared to lymphocytes
abnormal granularity*	abnormal granularity*
abnormal expression of CD45*	abnormal expression of CD45*
abnormal CD11b/CD13 pattern	abnormal expression of CD14
abnormal CD16/CD13 pattern	abnormal CD11b/HLA-DR pattern
abnormal expression of CD15	abnormal expression of CD13
abnormal expression of CD33	abnormal expression of CD33
expression of HLA-DR	abnormal expression of CD36
expression of CD34	abnormal expression of HLA-DR
asynchronous shift to the left	expression of CD34
expression of lineage infidelity markers CD5, CD7, CD19	expression of lineage infidelity markers CD5, CD7, CD19
over expression of CD56	over expression of CD56

* expressed as ratio to lymphocytes; all differences from normal should be analyzed versus normal controls and a defined set of pathologic controls (5)

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4

Application of flow cytometry for myelodysplastic syndromes: pitfalls and technical considerations

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Cytometry B Clinical Cytometry 2015; in press

Abstract

The application of flow cytometry (FC) is recommended as part of the diagnostic approach for MDS. The complexity of flow cytometric analysis of bone marrow cells in MDS has been an obstacle for general application. However, in the past years several studies showed practical flow cytometric approaches for the diagnosis and prognosis of MDS. In this report we discuss technical considerations and highlight issues that require special attention when handling and analyzing bone marrow samples of patients with cytopenia and suspicion of MDS.

Table 1. Keypoints for pitfalls and technical recommendations

Sample handling
Lysing procedure
Erythroid cells might be affected by the lysing procedure
Lyse-stain-wash is recommended to prevent suboptimal antibody to (erythroid) cell ratio
Increasing the volume of the lysing solution rather than the duration of lysing improves lyses of erythrocytes
Time to processing
Bone marrow samples should be analyzed within 24 hours
Fixation, time-delay after staining and storage at 4oC influences flow cytometric analysis
Keep conditions for sample handling and processing as constant as possible
Sample quality: peripheral blood contamination
At least 250 cells of each cell population of interest should be measured
Bone marrow subpopulation specific
Myeloid and B cell progenitor cells
The combination of CD11b, CD13, CD117 and/or HLA-DR in combination with CD34 and CD45 is recommended for analysis of myeloid progenitor cells
B cell progenitor cells can be differentiated by using CD19 in combination with SSC properties and CD45
Maturing myeloid cells
Eosinophils, paroxysmal nocturnal hemoglobulinuria and apoptic myeloid cells can interfere with analysis
Monocytes
Hypogranular and/or aberrant myeloid cells, apoptotic monocytes can interfere with analysis

Introduction

In the past years, the number of publications investigating the diagnostic and prognostic value of FC for MDS has increased (1-13). The application of flow cytometry (FC) for myelodysplastic syndromes (MDS) is based on the concept that altered hematopoiesis can be studied by measuring antigen expression levels during differentiation. Knowledge of antigen expression in hematopoietic cells in healthy individuals is obligatory in order to identify abnormal hematopoiesis. Knowledge of phenotypic abnormalities in the bone marrow (BM) that are caused by other disorders is of relevance in order to differentiate from MDS. Especially since flow cytometry is regarded as a recommended technique for the diagnosis of MDS, next to morphologic examination and/or cytogenetics (14,15).

Furthermore, several studies have shown that flow cytometric analysis of BM of patients with MDS provides prognostic information, in addition to currently used prognostic models such as the Revised International Prognostic Scoring System (IPSS-R) (12).

An international consortium, together with the European LeukemiaNet working group for FC in MDS (IMDS-Flow) formulated recommendations for standardization of flow cytometric analysis of bone marrow (BM) aspirates from patients with MDS (16-20). The first meeting was held in 2008 and recommendations were adjusted parallel to developments in the field of FC, such as transition from four-color to eight-color FC. So far, flow cytometric analyses for MDS have been regarded as complicated procedures requiring specific expertise. The purpose of this paper is to give technical considerations, address potential pitfalls and to indicate points that require special attention when analyzing BM samples of patients with cytopenia and suspicion of MDS. Issues are addressed by examples of flow cytometric analyses of healthy subjects and patients with confirmed MDS. Pitfalls and technical issues in flow cytometric analysis of BM analyses are supported by figures with examples from our laboratory and table 1 provides an overview of keypoints. In previously published studies by our group, the methods that were used to process and stain BM samples are extensively described (2,6,12,13).

Pitfalls due to sample handling for flow cytometric analyses

Lysing procedure

The majority of studies that investigate the application of FC for MDS, focus on the analysis of differentiating myelomonocytic cells in the BM. To prevent hindrance of red blood cells in the analysis, lysis of mature red blood cells is performed by using lysing procedures, preferably with ammonium chloride. Differences between laboratories in lysing procedure are common and vary from the kind of lysing solution that is used, lysing at room temperature, on ice or 37°C, for 5 minutes or up to 15 minutes. There is no empiric evidence for what is the best red blood cell lysing procedure for the preparation of a BM sample from patients with MDS (21). The lysing procedure might affect nucleated erythroid cells that remain in the sample. Previously, it was described that red blood cell lysis affects the nucleated erythroid cells by reducing cytoplasm or reducing the cells to naked nuclei, which might influence the analysis of surface antigen expression on maturing erythrocytes (21). In contrast to a more recently published investigation, reporting that the lysing procedure only affected light scatter properties of erythrocytes, but not antigen expression in a qualitative manner (20). It was proposed that $CD105^{pos}$ erythroid cells are resistant to lysis due to lack of the enzyme carbonic anhydrase required for ammonium chloride lysis.
Furthermore, it is recommended to perform a lyse-stain-wash procedure instead of stain-lyse-wash. The ratio of antibody to maturing erythroid cells is not optimal in excess of mature erythrocytes when the stain-lyse-wash procedure is used, especially when analyzing the erythroid lineage. Experiments from our center indicated that increasing the lysing solution to sample volume ratio improves the lysis procedure of mature erythroid cells, rather than increasing the duration of the lysing procedure or repeating the lysing step. The latter would not be recommended since it may result in additional cell loss. Increasing the lysing solution volume would be of particular interest in samples with low white blood cell count. In these cases it is necessary to use a larger volume of the sample to obtain sufficient cells for flow cytometric analysis. Interestingly, erythroid cells of patients with MDS tend to be partly resistant to lysis, resulting in an increased number of erythroid cells remaining in the sample (Fig. 1).

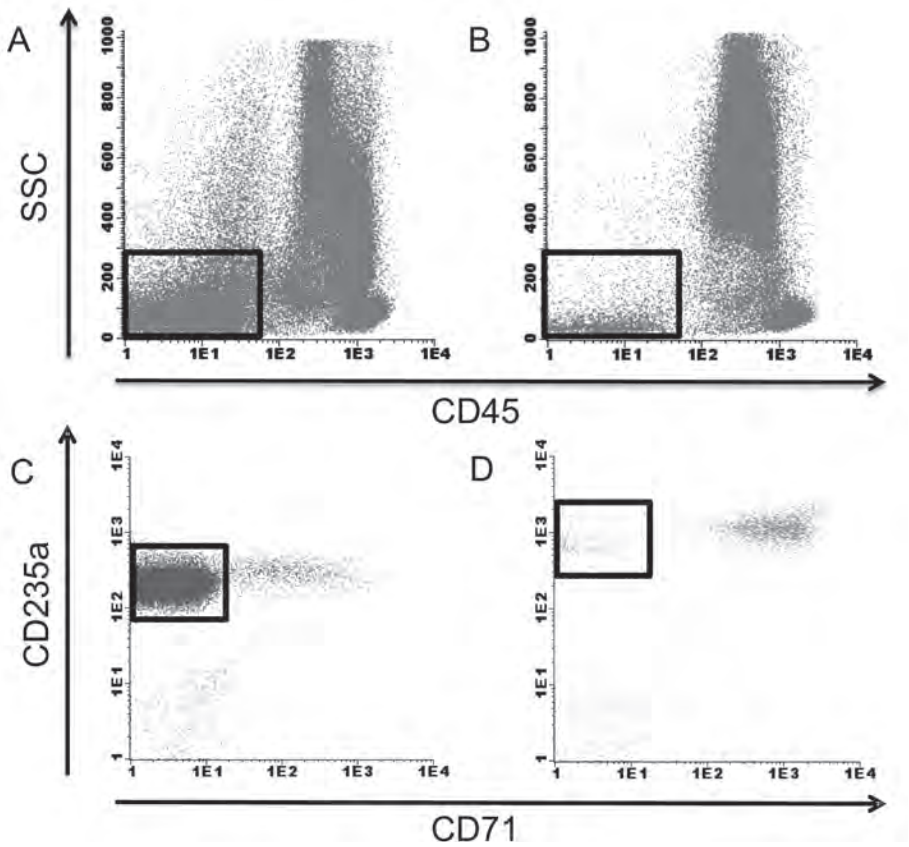

Figure 1. Red blood cell lysis in a MDS bone marrow aspirate compared with that of a healthy volunteer Erythroid cells are indicated by squares in figure 1A and 1B. (A)Flow cytometric analysis of the BM of a patient with MDS refractory anemia with ring sideroblasts that appeared to be partly resistant to red blood cell lysis by ammonium chloride; this is illustrated by an increased number of SSCdim and CD45dim-neg cells with expression CD235a. (B) Representative flow cytometric analysis of the BM of a healthy volunteer with normal response of red blood cells to the lysing procedure. (C) Excess of mature CD71negCD235pos erythroid cells (indicated by the square) after lysis. Consequently, CD235a expression is lower because of a suboptimal antibody to mature erythroid cells ratio. (D) In comparison, representative erythroid analysis of the BM of a healthy volunteer with normal response of red blood cells to lysis and normal levels of CD71 and CD235a expression.

An increased number of erythroid cells remaining in the sample after lysis might create a potential pitfall for analysis of antigen expression on erythrocytes. CD235a levels might be falsely interpreted as decreased due to an increased ratio between erythrocytes and the amount of available antibody to stain. The degree of resistance to lysing procedures can be measured by quantifying the remaining number of erythroid cells in the sample. However, it was agreed on by members of IMDS-Flow that CD235a is not recommended to use as a single marker for flow cytometric analysis of MDS due to pitfalls associated with the application of CD235a.
Assessment of erythrocyte differentiation by FC in BM for MDS is currently under investigation by members of the IMDS-Flow consortium. The first results indicate that CD36, CD71, CD105 and CD117 are of interest for the differentiating MDS from non-MDS cases (21-23). CD235a can still be applied in combination with CD71 to estimate abnormalities in the differentiation pattern of erythrocytes but is not recommended as a single marker due to artefacts associated with lysing procedures. Heterogeneity vs. homogeneity of CD36 and CD71 on erythroid cells which is indicated by the coefficient of variation by FC might be of interest to differentiate MDS from non-MDS (21).

Time to processing

It is recommended by IMDS-Flow to process a BM sample within 24 hours after it is drawn. However, this might not be feasible for every laboratory, for example due the time that is needed to transport the sample. In addition, after staining, samples should be measured as soon as possible, and preferably after fixation. Therefore, we investigated the effect of delay in time to analysis by FC after incubation with antibodies and whether fixation and/or storage at 4°C might prevent time-related changes in antigen expression levels. A delay in the time between staining and analysis results in an increase in sideward scatter (SSC) of neutrophils (Fig. 2). The SSC of neutrophils was calculated as a ratio to the SSC of lymphocytes as internal reference. Moreover, fixation and time delay after fixation enhances this increase in SSC ratio. Storing of stained samples at 4°C further enhanced this effect. Evaluation of the SSC, which is an indication of granularity of neutrophils by FC is an important parameter included in diagnostic and prognostic tools for MDS (1, 4). Time-related and fixative related changes might result in an inadequate interpretation of neutrophil SSC unless procedures are standardized. Other effects of time-delay, fixation and storage at 4°C included an underestimation of the number of $CD14^{pos}$ monocytes, caused by formation of duplets and an increased SSC (Fig 3). Subsequently, the duplets

of monocytes cannot be adequately gated and are lost for further analysis due to increased SSC and interference of neutrophils.

A positive effect of using fixative is the conservation of antigen expression levels. This especially relates to expression levels of CD11b and CD14, which seemed to be most sensitive for time-related decline in our experience.

In summary, awareness of the effects of variables on flow cytometric analysis such as time delay, fixative use or the type of anticoagulant in which the sample is drawn (for example EDTA can affect CD11b expression in contrast to heparin) is the most important. So, the procedures in sample processing and analysis should be as constant as possible. Each laboratory should have its own reference values. Any change that is made to sample handling procedures, processing and analysis should be tested for effects on sample quality and antigen expression levels.

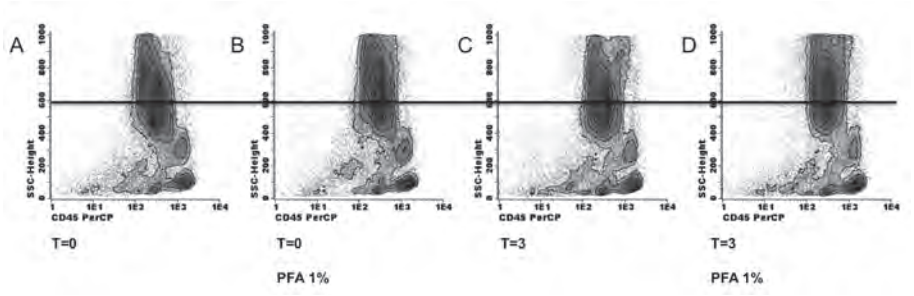

Figure 2. Time and fixative use related changes to the SSC of myeloid cells in a BM sample Maturing myeloid cells are defined by SSChigh and CD45intermediate. The SSC of maturing myeloid cells is calculated as a ratio to lymphocytic cells. Tested in 3 patients with MDS, with consistently increased SSC of neutrophils upon time delay and/or fixative use. The range of increase in SSC was between 0.77 and 2.77 points when fixative was applied and measured without time delay. Time delay further increased the SSC. (A) In this pathologic control, the SSC is normal compared with the reference value of the healthy volunteer cohort. The BM sample is measured at time point 0, without time delay and use of paraformaldehyde 1% solution (PFA) as a fixative. (B) Flow cytometric analysis of the same BM sample, with the use of PFA 1% as the only variation compared with the analysis as depicted in figure 2A. The SSC of myeloid cells is increased by 0.77 points. (C) Analysis of the same BM sample with a three hour time delay between completing the staining procedure and measurement. Compared with time point 0 with or without fixation, the SSC is increased. (D) A time delay of three hours in combination with the use of PFA 1% solution as a fixative results in a further increase of the SSC ratio of myeloid cells.

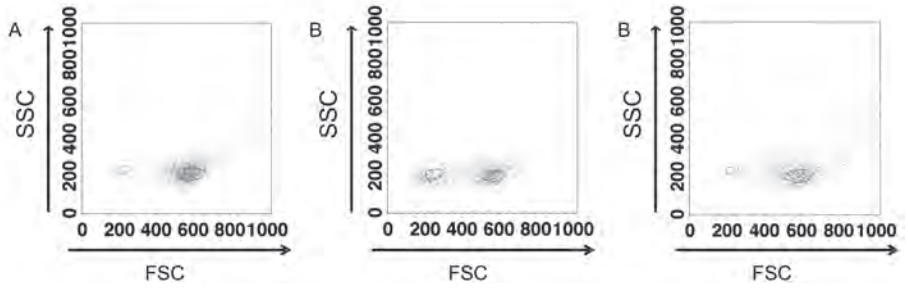

Figure 3. The effect of time-delay and fixation with paraformaldehyde solution on the analysis of monocytes Monocytes were gated by foreward scatter (FSC), sideward scatter (SSC), CD14, CD33 and CD45 properties and then backgated in the FSC vs. SSC plot. (A) At time-point 0 'viable' monocytes are located within the large gate. Few monocytic cells are within the smaller 'non-viable' cell gate. (B) After three hours, the number of 'non-viable' monocytes is increased (small gate) as compared with plot A. 'Viable' monocytes are gated by the larger gate. (C) Fixation with paraformaldehyde 1% solution and after 3 hours of time delay prevents monocytic cells from becoming FSC low or 'non-viable'.

Choice of fluorochromes and antibody combinations

The IMDS-Flow made recommendations for flow cytometric parameters that should be minimally analysed (16,18). Definitions are given for each cell population of interest to detect abnormalities of subpopulations in the BM. The working party did not strive for complete uniformity in antibody combinations and fluorochromes because it is still under investigation which flow cytometric parameters are most relevant for the clinical application, either diagnostic or prognostic, in MDS. Since the development of the first MDS FC proposal, the number of fluorochromes and hence antibody combinations that can be used has rapidly increased. Together with the increase in possibilities, the amount of output increased and the analysis became more and more elaborate. Furthermore, the use of more than four colour FC requires extra attention with regard to optimal instrument settings and for stability and spectral overlap of fluorochromes. The EuroFlow consortium published protocols and instrument settings for eight colour FC for the diagnostic work up of haematological malignancies; specifically AML and lymphoid malignancies (24). This panel enables interchangeability and standardization between laboratories. Their AML panel shows overlap with the antibody combinations that are recommended by IMDS-Flow (16, 18). HLA-DR, CD34, CD117 and CD45 are used as a backbone marker in the EuroFlow panel. Adding CD11b, CD13 and CD16 to the backbone markers provides analysis of maturing myeloid, whereas the second antibody combination CD14, CD64, CD300e and CD35 can be applied for analysis of monocytic differentiation. CD33 in the third combination of CD36, CD71 and

CD105 can serve as an additional method in differentiating between monocytes and neutrophils as recommended by the IMDS-flow working party. Analysis of terminal deoxynucleotidyl transferase (TdT), necessary in analysis of acute leukemia, is not recommended for the analysis of MDS. Adding TdT to a panel requires the use of fixation and permeabilization solutions, which affect forward scatter (FSC) and SSC properties of cells. Furthermore, assessing the SSC of (maturing) myeloid cells is a diagnostic and prognostic flow cytometric criteria for MDS (4,11). Lineage infidelity marker expression on myeloid progenitors should be analyzed according to IMDS guidelines. Most are accounted for in the Euroflow panel; however, CD5 is excluded. CD5 is found in a minority of patients with MDS, approximately 7% in our cohort of patients with MDS, mainly patients with high risk MDS (unpublished data). Therefore, a critical appraisal of antibody combinations is required taking into account the BM disorders for which its use is intended. Moreover, changing panels and fluorochromes affects the appearance of differentiation patterns and requires acquisition of new reference values. Further remarks on antibody combinations will be made in the sections below.

Pitfalls due to sample quality: Peripheral blood contamination

Peripheral blood contamination in the BM is frequently encountered in laboratory practice e.g. due to BM fibrosis. As a result, acquisition of sufficient number of cells for flow cytometric analysis can be challenging, especially regarding progenitor cells. If less than 250 events of a cell population of interest are measured, the analysis is regarded as less reliable to make any definite conclusions on aberrant marker expression. The minimum amount of events is set at 25, to consider a population of cells as aberrant by marker expression measured by FC. Moreover, contamination with peripheral blood can influence CD45 and SSC properties of neutrophils. The most mature neutrophils are higher in CD45 expression and lower in SSC compared with more immature neutrophils. Furthermore, the lack of more immature myeloid cells may also hampers proper interpretation of differentiation patterns. Noteworthy, by morphology, hypogranularity is estimated on peripheral blood smears rather than in the BM because it is most pronounced in mature neutrophils. This suggests that by FC, the SSC should be evaluated in the most mature myeloid cells in the BM. Yet, the contrary was anticipated by Ogata et al. who analysed SSC of $CD10^{neg}$ neutrophils as part of a diagnostic flow cytometric score for MDS (4). This enabled correction for hemodilution; CD10 is expressed on the most mature neutrophils that are most prominent in peripheral blood. However, in a later study, this approach

was abandoned (10). In our experience, overall, gating on $CD10^{pos}$ or $CD10^{neg}$ neutrophils to calculate the SSC did not yield significantly different results compared with gating on all the neutrophils within a sample.

Pitfalls in the analysis of myeloid and B cell progenitors

There is a correlation between the blast count by morphology and the number of myeloid progenitors by FC but discrepancies are common. The flow cytometric definition of a 'blast' or preferably myeloid progenitor cell is not necessarily the same as the morphologic definition of a blast. The choice of antibody combinations to denote myeloid progenitors can influence the quantification of myeloid progenitors by FC. Myeloid progenitors can be differentiated from other subpopulations of cells in the BM by flow cytometric $SSC^{intermediate}$, $CD34^{pos}$ and $CD45^{dim}$ properties. However, in MDS, abnormalities in SSC properties and/or surface marker expression are frequently observed. The joint interpretation of CD11b, CD13, CD117 and/or HLA-DR in combination with CD34 and CD45 is recommended to avoid leaving out myeloid progenitors with abnormal antigen expression from analysis (16, 18). With six or more color FC it is possible to combine all of the aforementioned markers in one tube to analyze the myeloid progenitor cell compartment. Figure 4 shows the flow cytometric analysis of $CD34^{pos}$ myeloid progenitors with abnormal lack of CD45 on a subpopulation. In this case, quantifying myeloid progenitors by only $SSC^{intermediate}$ and $CD45^{dim}$ properties would give an underestimation of the percentage of myeloid progenitors. Furthermore, the absence of CD45 is an indication of the presence of aberrant myeloid progenitors. Caution is warranted; macro platelets can dimly express CD34, but can be differentiated from $CD45^{neg}$ myeloid progenitors by expression of CD36 and lack of other immature myeloid markers.

Figure 5, illustrates that (back) gating with CD34 is insufficient to detect all the myeloid progenitors in the BM. In this patient, aberrant $CD34^{neg}$ myeloid progenitors are present that cannot be differentiated by CD34 and/or CD45 properties alone. Adding CD117 to the combination of antibodies is useful to include all the myeloid progenitors in the analysis.

Furthermore, quantification of myeloid progenitors by FC is not the most important application of FC for MDS. Qualitative abnormalities such as aberrant, over or under, homogeneous or heterogeneous expression of a marker to assess abnormalities in hematopoiesis are the most relevant contributions for the diagnosis and prognosis of MDS. Figure 6 depicts the myeloid progenitor cell analysis of a patient with MDS

and aberrant and homogeneous expression of CD117, compared with myeloid progenitors from BM of a healthy volunteer.

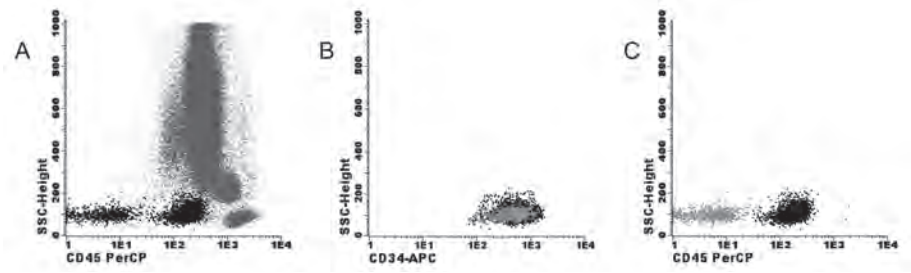

Figure 4. Presence of myeloid progenitors with lack of CD45 in a patient with MDS (A) Flow cytometric analysis of BM cells of a patient with MDS with myeloproliferative features. Myeloid progenitors are defined as $SSC^{intermediate}$ and $CD45^{dim}$ (black dots). The myeloid progenitors were backgated by $CD34^{pos}$ properties. Notably, the $CD34^{pos}$ myeloid progenitors are partially negative for CD45. (B) The $CD45^{dim}$ and $CD45^{neg}$ myeloid progenitors are both $CD34^{pos}$. The $CD45^{neg}$ myeloid progenitors are depicted in gray. (C) The myeloid progenitor population consisting of $CD45^{dim}$ (black dots) and $CD45^{neg}$ (gray dots) myeloid progenitors are depicted.

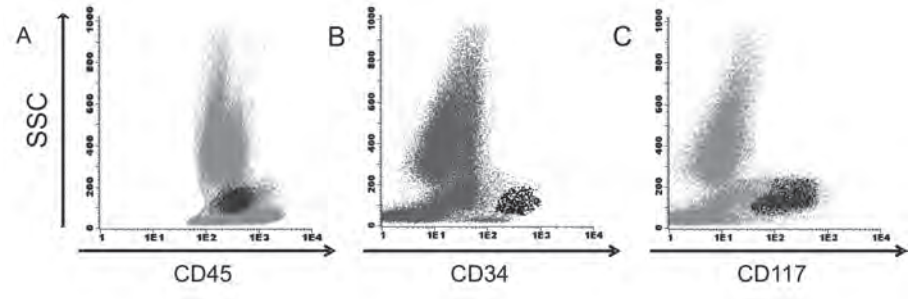

Figure 5. Abnormal $CD34^{neg}$ myeloid progenitors in a patient with MDS **(A) Flow** cytometric analysis of a BM sample of a patient with MDS refractory anemia with excess of blasts (5-10%). Myeloid progenitors are defined as $SSC^{intermediate}$ and $CD45^{dim}$ (black dots). (B) The myeloid progenitors were back gated in the SSC vs. CD45 plot by gating $CD34^{pos}$ cells. Notably, there is a large population of cells with $SSC^{intermediate}$ and $CD45^{dim}$ properties that are not $CD34^{pos}$. (C) The use of CD117 aids in identifying the aberrantly CD34neg myeloid progenitors. The myeloid progenitors are $CD117^{pos}$, $SSC^{intermediate}$ and $CD45^{dim}$ (black dots) but $CD34^{neg}$ as described above.

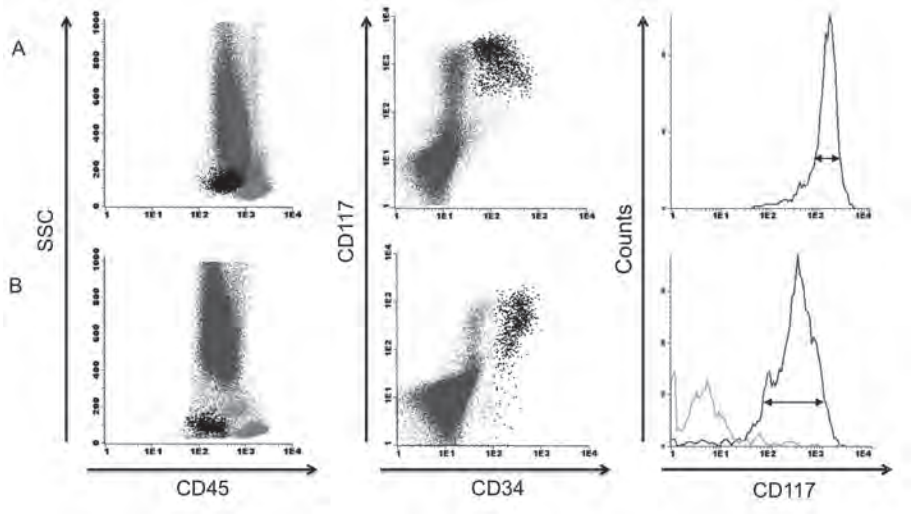

Figure 6. Aberrantly increased and homogeneous expression of CD117 expression on myeloid progenitors in a patient with MDS (A) Myeloid progenitors are defined as SSCintermediate, CD45dim, CD34pos and CD117pos (black dots). The myeloid progenitors of this patient with MDS refractory anemia have abnormal homogeneous and increased expression of CD117. Of note, the myeloid cells lose CD34 expression and eventually also CD117 expression during differentiation. By using the histogram and coefficient of variation of CD117 expression on myeloid progenitors, the degree of heterogeneity or homogeneity is calculated. (B) Representative example of the analysis of myeloid progenitors in the BM of a healthy volunteer. A normal and heterogeneous (histogram) CD117 expression level is depicted.

Within the $SSC^{intermediate}CD45^{dim}$ gate that myeloid progenitors reside in, other cells can be found which may be identified by additional markers. B cell progenitors, for example, can be relatively easily differentiated from myeloid progenitors by using CD19 and/or CD117. Yet, CD19 can be aberrantly expressed on myeloid progenitors. Therefore, adding CD117 is helpful in distinguishing aberrant myeloid progenitor cells from B cell progenitors. With six or more color FC, distinguishing cell populations of interest is less problematic compared with four color FC. Quantification of B cell progenitors is part of a flow cytometric diagnostic score for MDS (4). In contrast to healthy subjects and pathologic controls, patients with MDS have a deceased number of B cell progenitors in the BM.

Basophils have similar SSC and CD45 properties as myeloid progenitors and can interfere with evaluation of the number of myeloid progenitors. Contamination of the myeloid progenitor cell gate by basophils can be identified by slightly higher CD45 expression of basophils and by using CD123 and HLA-DR. Basophils are CD123 positive and HLA-DR negative. Myeloid progenitors can also express

CD123, however, the expression levels are not as high as that of basophils, moreover, (normal) myeloid progenitors express HLA-DR. The cases illustrated in figure 5 and 6 show the importance of adding more markers such as CD117 and/or HLA-DR to the antibody combination because abnormalities can occur in the 'backbone' markers CD34 and/or CD45.

The choice of fluorochromes might be the most important for detection of lineage infidelity markers on myeloid progenitors such as CD7 which is normally expressed by T cells. The rule of thumb is to select a fluorochrome taking into account the expected expression level of the antigen on the cell of interest. For expected low antigen expression levels, it is advised to use an antibody bound to a fluorochrome with strong emission qualities.

Antigen expression levels on cell populations of interest should be analysed by using a reference population. This should be either cells stained with isotype controls which is ideal or, which is more feasible, unstained cells of the cell population of interest. Autofluorescence can vary between cell populations, therefore, it is discouraged to use for example unstained lymphocytes as a reference or control population for myeloid progenitors and the maturing myelomonocytic compartment.

Pitfalls in the analysis of maturing myeloid cells

Identification of subpopulations of myeloid cells that compose the maturation plots is important to differentiate normal from abnormal hematopoiesis (25). Maturation of myeloid cells can be followed by combining the markers CD11b, CD13 and CD16. Antigen expression levels of these (and other) markers fluctuate during differentiation and result in characteristic patterns of differentiation. Figure 7 shows the differentiation pattern of myeloid cells in the BM of a healthy volunteer. Fluorescence activated sorting (FACS) experiments show that subpopulations of myeloid cells defined by antigen expression levels of CD11b and CD13 correspond with morphological stages of differentiation (Fig. 7). Some important issues need to be addressed for the evaluation of abnormalities in myeloid maturation. Some pitfalls in the analysis of SSC were already mentioned above. For calculation of SSC, which corresponds with granularity of myeloid cells, it is of importance to exclude the eosinophils. Eosinophils are the most granular of neutrophils and might lead to the assumption of normal granularity of neutrophils if not excluded, especially in cases with increased numbers of eosinophils (26). Furthermore, it is of relevance to differentiate eosinophils and apoptotic neutrophils in the maturation patterns for the interpretation of myeloid cell differentiation as shown in figure 8. This figure

describes an experiment in which maturing neutrophils were FACS sorted, and later stained with May Grünwald Giemsa and Sudan-Blackfor confirmation that a subpopulation of these cells was apoptotic and from myeloid origin. CD10 expression on mature neutophils also decreases due to apoptosis. The presence of a paroxysmal nocturnal hemoglobulinuria (PNH) clone with abnormal loss of CD16 can be difficult to distinguish from neutrophils with abnormal loss of CD16 as part of dysplastic hematopoiesis in MDS. For confirmation of the presence of a PNH clone, analysis of peripheral blood is required as recommended by guidelines (15). If there are more indications of loss of glycosylphosphatidylinositol-linked proteins, for example loss of CD14 on a subpopulation of mature monocytes, additional flow cytometric tests should be performed to assess the presence of PNH.

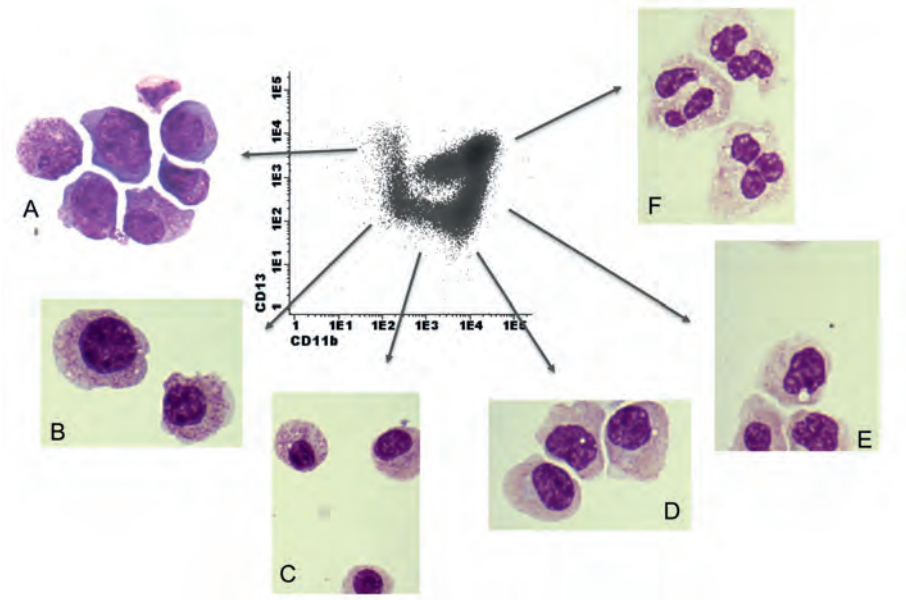

Figure 7. Fluorescence activated cell sorting (FACS) of subpopulations of differentiating myeloid cells in the BM of a healthy individual Flow cytometric analysis based on antigen expression levels of differentiating myeloid cells in the BM corresponds with morphologic stages of maturing neutrophils. The maturing myeloid cells as analyzed by FC were initially gated by SSChigh and CD45dim properties. Myeloid cells differentiate from CD11bnegCD13pos cells towards CD11bposCD13pos. After sorting, all cells were stained with Giemsa for visualization. (A) CD11bnegCD13pos cells were sorted and correspond with myeloblasts and promyelocytes by morphology. (B) CD11bnegCD13neg myeloid cells correspond with promyelocytes and immature myelocytes by morphology. (C) CD11bdimCD13neg myeloid cells correspond with myelocytes by morphology. (D) CD11bposCD13neg cells correspond with myelocytes by morphology. (E) CD11bposCD13dim cells are consistent with myelocytic and metamyelocytic stage of neutrophil differentiation by morphology. (F) CD11bposCD13pos corresponds with the most mature stage of neutrophil differentiation by morphology. Sorting experiments showed segmented neutrophils.

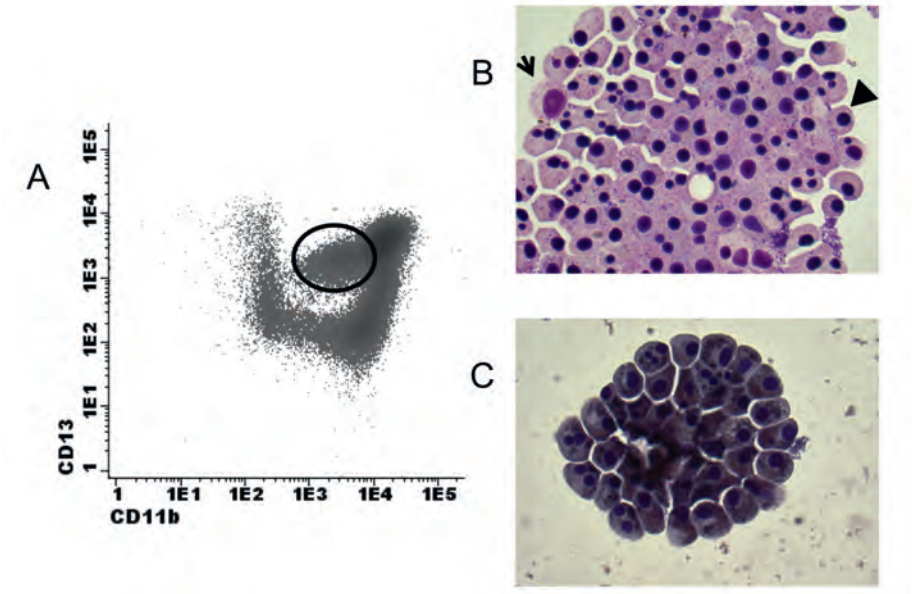

Figure 8. Fluorescence activated cell sorting (FACS) of an apoptotic subpopulation of myeloid cells in the BM of a healthy individual (A) The maturing myeloid cells as analyzed by flow cytometry were initially gated by SSChigh and CD45dim properties. Myeloid cells differentiate from CD11bnegCD13pos cells towards CD11bposCD13pos. The circle in the plot indicates CD11bdimCD13pos myeloid cells that were sorted. (B) After sorting, cells were stained with Giemsa for visualization. Apart from an occasional viable myeloid cell (indicated by the arrow), the majority of cells are clustered apoptotic cells, with pyknotic nuclei (indicated by the arrowhead. (C) Sudan-Black staining of the subpopulation of interest after cell sorting. The cells are positive for Sudan-Black, indicating that the cells are of myeloid origin. Therefore, it was concluded that the CD11bdimCD13pos cells are apoptotic myeloid cells.

Pitfalls in the analysis of monocytes

Monocytes can be identified by $CD33^{bright}$ and $HLA\text{-}DR^{pos}$ expression and backgating by $SSC^{intermediate}$ and $CD45^{bright}$ properties. The more mature monocyte markers CD11b and CD14 can further help to identify mature monocytes. CD36 and CD64 can serve as complementary markers present throughout differentiation. By combining CD14 with CD33 and/or HLA-DR, monocytes can be differentiated from subpopulations of neutrophils with aberrant CD14 expression. Subpopulations of neutrophils can have (abnormal or reactive) HLA-DR expression, giving rise to possible overlap with monocytes. The addition of CD33 in combination with myelomonocytic markers can also aid in separating monocytes from (hypogranular) neutrophils. Of note, the level of CD33 expression is subject to polymorphisms. Therefore, abnormal expression of CD33 on monocytes, neutrophils and myeloid progenitor cells should be assessed in relation to each other.

In some BM samples, the number of monocytes as measured by FC may be underestimated. For instance, apoptosis of monocytes, defined as $SSC^{intermediate}$, $CD45^{bright}$ and $CD14^{pos}$may be reflected by a change in FSC properties (Fig. 3). In cases with FSC^{low} monocytes, caution is warranted when gating based on FSC and SSC plots. To avoid a misinterpretation of the number of mature monocytes in the BM, the FSC^{low} monocytes should be included in the quantification. This can be achieved by adjustment of the debris gate to include these monocytes for analysis.
Yet, analysis of qualitative aberrancies in monocytes may be more important than quantification of this subset. Aberrant expression of CD56 is seen in MDS and described to be associated with chronic myelomonocytic leukemia (27). Activated (normal) monocytes may also express CD56. To ascertain that CD56 is aberrantly expressed, ≥20% of the cells should be one log above normal expression levels of CD56 on monocytes. This is half a log above the level of CD56 expression on activated normal monocytes.
Furthermore, aberrant expression of lymphocytic markers such as CD5 or CD7 can be differentiated from monocytes adhering to T cells or contamination in the gate with T cells by checking whether all T cell markers are expressed or only single. In the case of aberrant expression, only single T cell markers are present. Of note, this is also the case for the analysis of aberrant marker expression on myeloid progenitor cells.

Conclusion

Although there are a considerable number of technical pitfalls for FC in MDS, the majority of problems can be tackled by getting acquainted with normal hematopoiesis and the study of (pathologic non-) MDS hematopoiesis. Moreover, standardization of in-house procedures is of utmost importance to evaluate results reliably. Before any conclusions should made on a flow cytometric analysis of a BM aspirate of a patient with the suspicion of MDS, a critical review of the findings is obligatory. Furthermore, FC should not be interpreted as a single technique, but together with BM morphology, cytogenetics and molecular abnormalities (20). New developments in flow cytometric software with extensive statistical analysis programs may help to objectify flow cytometric results and quantify the distance from normal. Moreover, the lack of markers to follow megakaryocytic differentiation together with the technical limitations to visualize megakaryocytes by FC has prevented its application for MDS. The most recent study investigates abnormalities in surface expression markers of platelets in peripheral blood of patients with MDS (28). Studies are ongoing to determine the true value of analyzing platelet abnormalities by FC for MDS.

In conclusion, FC is a valuable technique that is becoming part of the daily work up of patients with (suspected) MDS, besides morphology, cytogenetics and molecular biology (15). However, technical issues as described in this paper and in the work published by IMDS-Flow should be taken into consideration when implementing FC for MDS.

Conflict of interest

All authors declare there are no conflicts of interest.

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5

Do peripheral blasts count in myelodysplastic syndromes?

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Leukemia Research 2009; 33: 209-211

Myelodysplastic syndromes (MDS) encompasses a heterogeneous group of hematopoietic disorders characterized by bone marrow (BM) failure, peripheral cytopenias and an increased risk of transformation to acute myeloid leukaemia (AML). Patient groups as defined by French-AmericanBritish (FAB) classification are heterogeneous in terms of morphologic features and prognosis. In the current classifi- cation system as defined by the World Health Organization (WHO) uni- and multi-lineage involvement is taken into account [1,2]. Regarding the difference in overall survival (OS) the determination of uni-lineage versus multi-lineage dysplasia is important. By morphology it can be difficult to detect dysplastic changes in the erythroid, megakaryocytic and myelomonocytic lineages. Recently, definitions and standards in the diagnosis of MDS were updated based on current insights [3,4]. In cases with a limited degree of dysplasia where morphology and cytogenetics fail to come up with a conclusive diagnosis, flow cytometric analysis of bone marrow cells might help to establish the diagnosis of MDS. Therefore, flow cytometric analysis of bone marrow cells is included as a co-criterion in the minimal diagnostic criteria for MDS [3]. Prognostication of MDS is an even bigger challenge because of lack of markers or prognostic scoring systems that fully comprise the heterogeneity of MDS. The International Prognostic Scoring System (IPSS) is a prognostic scoring system based on cytogenetics, blast percentage and the amount of cytopenias [5]. The prognostic value is validated only at the diagnosis of MDS. The development of additional prognostic tools is warranted especially since the introduction of new promising drugs in MDS. As a refinement of the WHO classification a scoring system is proposed for the number of peripheral cytopenias and dysplasia in one or more lineage [6]. To provide information on OS and evolution to leukemia at any time during the course of the disease a WHO based time-dependent prognostic scoring system (WPSS) was designed [7]. The WPSS is a prognostic scoring system based on WHO classification, cytogenetics and transfusion requirements. Its application could be limited by subjective criteria like transfusion dependence [8]. By splitting each IPSS group into one with a normal LDH and an elevated LDH level, it was possible to identify MDS patients with poor prognosis within IPSS lower risk groups [9]. Recently, Haase et al. proposed a new categorization of cytogenetic abnormalities which might influence prognostication and therapeutic intervention in near future [10]. Flow cytometry might contribute to identify additional prognostic subgroups in MDS. Moreover, it was shown that flow cytometry identifies low-risk MDS patients with an adverse clinical course by the presence of aberrant myeloid blasts [11–13]. Flow cytometry was even able to identify patients at risk for transfusion dependency

or progressive disease independent of known risk groups. Furthermore, flow cytometry contributed in predicting survival and relapse after stem cell transplantation of MDS patients independent of the IPSS [14]. Using a flow cytrometric scoring system Wells et al. were able to identify patients with a higher probability of relapse within the IPSS INT-1 patient group. In this issue of Leukemia Research, Cesana et al. studied the prognostic value of circulating CD34+ cells (CCD34+) in the peripheral blood as assessed by flow cytometry in 96 patients with MDS at diagnosis and in 35 patients during follow-up [15]. A cut-off value of 10 CCD34+ cells/l was set as optimal to differentiate between prognostic subgroups. High levels of CCD34+ cells were correlated with INT-2/high risk MDS, poor outcome karyotype group, 2–3 cytopenias group, increased numbers of bone marrow blasts and shorter leukemia free survival independently of WHO classification but not of IPSS. Four out of seven patients that evolved to leukemia showed an increase in the absolute count of CCD34+ cells prior to AML evolution, while nonevolving cases showed stable values during the follow-up period. Recently, the presence of peripheral blasts in MDS patients with refractory anemia (RA) or refractory anemia with multi-lineage dysplasia (RCMD) was shown to be associated with lower survival rates and high risk of progression to AML. Interestingly, OS of MDS patients with 1% or more peripheral blasts was comparable with OS of patients with RA with excess blasts-1 (RAEB-1) [16]. In several studies either the percentage or the absolute count of CCD34+ cells, as measured by flow cytometry was used to define prognostic categories of MDS patients [17–19]. Cesana et al. suggested that the absolute count of CCD34+ cells is more sensitive in defining patients in different risk categories than the percentage of CCD34+ cells. They show that 6 out of 21 patients progressed to AML while the blast percentage was less than 1% CCD34+ cells but with absolute blast counts above 10/l. Follow-up studies are needed to determine whether the absolute count or the percentage of circulating peripheral blasts is more sensitive in predicting prognosis in MDS patients. There are limitations in counting blasts in bone marrow of MDS patients by flow cytometry. BM blast counts by cytomorphology and flow cytometry do not necessarily correlate in MDS. Especially in patients with moderate (secondary) myelofibrosis, a considerable amount of peripheral blood dilutes the bone marrow specimen. In addition, inappropriate puncture by the physician may influence blast cell count. Therefore, the quantification of PB blast by flow cytometry may add in the diagnosis and prognostication of MDS patients. The authors used two different methods to measure CCD34+ cells by flow cytometry. CCD34+ cells were either determined according to the Milan Protocol or the

EWGCCA-modified ISHAGE single-platform method. In the Milan Protocol blasts are measured by a single fluorescence parameter analysis and defined as CD34+. The ISHAGE protocol uses a two-colour method to define blasts by adding CD45 next to CD34. In this paper both methods proved to have comparable results. Still the definition of a blast remains an important topic. Blasts can have aberrant expression of CD34 and in some instances may even lack CD34 or CD45 expression. Immunophenotyping of blasts by the addition of monoclonal antibodies for detection of antigens such as HLA-DR and CD117 might be instrumental to quantify myeloid blasts. Important to realize is that patients with MDS can have increased numbers of circulating CD34+ cells due to the presence of (secondary) myelofi- brosis. It would have been interesting to see which of the MDS patients in the study of Cesana et al. had signs of moderate or even increased myelofibrosis by immunohistochemistry in a bone marrow trephine. It should be noted that myelofibrosis by itself is a risk factor for worse outcome in MDS. Prospective studies are still warranted to prove whether or not PB blast count is of value in MDS. As mentioned previously aberrancies found on myeloid blasts in the bone marrow could add substantially in classifying patients into prognostic subgroups. It is expected that aberrant blasts in PB adjacent to the amount of blasts in the BM may add significantly in risk assessments. In addition, a direct comparison between these blast populations may be of importance. Circulating blasts might be different or even more aberrant as compared to the bone marrow blasts with respect to antigens involved in adhesion and migration. The role of flow cytometry in the diagnosis, prognostication and monitoring of MDS is now well established and has to be validated in prospective clinical trials. The presence of circulating blasts as identified by flow cytometry with or without additional aberrancies might be of crucial importance not only at diagnosis but also in disease monitoring as an early marker of response or progression in the treatment of patients with MDS.

Conflict of interest

None.

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11

Role of flow cytometry in prognosis of myelodysplastic syndromes

6

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

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British Journal of Haematology; 2014: 167: 100-109

Abstract

Estimation of survival of myelodysplastic syndromes (MDS) and risk of progression into acute myeloid leukemia is challenging due to heterogeneous clinical course. The most widely used prognostic scoring system (IPSS) was recently revised, resulting in the revised IPSS (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 analyzed 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 WHO 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.

Introduction

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 being either the development of progressive cytopenia or by transformation into an acute myeloid leukemia (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. In the WHO classification, it was 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 selectingpatients for clinical trials. Evidence emerged that the cytogenetic subgroups, the weight that was assigned to each variable in the score and the cutoff 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 cutoff points for the percentage of blasts in the BM are adjusted and the depth of the individual cytopenias are taken into account.

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.

Particularly FC has shown advancement for prognostication of MDS (Wells et al, 2003; Scott et al, 2008; van de Loosdrecht et al, 2008). The application of FC for MDS is based on the concept that subtle disturbances in hematopoiesis that are notrecognized by morphology, can be determined by FC (Malcovati et al, 2005; 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. showing that patients with severe dyspoiesis by FC have worse prognosis compared with MDS patients with no-to-mild abnormalities (Wells et al, 2003; Scott et al, 2008; van de Loosdrecht et al, 2008). The severity of flow cytometric abnormalities in hematopoiesis 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 behavior of MDS (Wells et al, 2003; Scott et al, 2008; van de Loosdrecht et al, 2008; Kern et al, 2010; Matarraz et al, 2010; Chu et al, 2011). Currently, the feasibility of the implementation of FC for the diagnosis and prognosis of MDS is investigated by an international working party (van de Loosdrecht et al, 2009; Westers et al, 2012; van de Loosdrecht et al, 2013). 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 a refinement of prognostication in MDS.

Materials and methods

Patients

Patients meeting the minimal diagnostic criteria for MDS were included in the study (Valent et al, 2007). Between 2004 and 2012, 159 patients (103 male vs. 56 female) with MDS were included. The characteristics of the patients with MDS are described in table 1. From this cohort, data from n=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 hematologists (A.A.L. and G.J.O) very well experienced in the diagnosis of MDS by morphology. The WHO 2001, 2008 and French-American-British (FAB) classification were applied for the diagnosis (Bennett et al, 1982; Jaffe et al, 2001; Swerdlow et al, 2008). Patients diagnosed with FAB RAEB- t, which is classified as AML with 20-30% blasts in the WHO 2001 and WHO 2008, were also included in the study to make 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 et al, 1995).In cases where no metaphases could be analyzed, 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 Center, Amsterdam, Netherlands.

Patients were assigned to risk groups by applying the IPSS and IPSS-R in four and five subgroups, respectively (Greenberg et al, 1997; Greenberg et al, 2012).The adjustments that were made to the IPSS to create the IPSS-R include definition of new cutoff 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 is given. In total, 19 out of 159 patients missed one parameter of the IPSS or IPSS-R. To avoid pitfalls or bias due to missing data, a multiple imputation analysis was performed. The resultsof 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.

Table 1. Patient characteristics

	N (%)
WHO2001 classification	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	
RAEB-T	9 (6)
IPSS	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	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 anemia with or without ring sideroblasts; RCMD(RS) refractory cytopenia with multilineage dysplasia with or without ring sideroblasts; RAEB-1 refractory anemia with excess of blasts (5-10%); RAEB-2 refractory anemia with excess of blasts (10-20%); FAB French-American-British; RAEB-T refractory anemia with excess of blasts in transformation; IPSS International Prognostic Scoring System; IPSS-R International Prognostic Scoring System revised.*IPSS from 1 patient missing, because of lack of cytogenetic data and peripheral blood values. **IPSS-R from 3 patients missing, because of lack of cytogenetic data and/or peripheral blood values.

Pathologic controls and healthy volunteers

As a control group, BM samples of 61 patients with cytopenia and a confirmed diagnosis of a non-myeloid hematological disorder were collected. For the composition of the pathologic control group, the guidelines as defined by the European LeukemiaNet (ELN) working party were followed (van de Loosdrecht et al, 2009; Westers et al, 2012). The diagnoses of patients in the pathologic control group are shown in supplementary table 1. As a reference population for normal hematopoiesis, aspirates drawn from healthy controls and individuals undergoing cardiac surgery were collected and analyzed concomitantly (n=36). The median age of the pathologic 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 four months at an Hb level of ≤ 9 g/dL. For disease progression or evolution, the definition as stated by Cheson et al. was used (Cheson et al, 2006).

Flow cytometric analysis of bone marrow samples

Immunophenotyping of BM cells was performed by using four-color FC as recommended by the ELN working party (van de Loosdrecht et al, 2009; Westers et al, 2012). All samples were processed and analyzed within 24 hours. 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 DakoCytomationGlostrup, Denmark; CD15 (MMA), CD34 (8G12) from BD Biosciences (San Jose, CA); 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 FACS

Calibur 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/orCD13. 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 CD25based on cutoff values in routine immunophenotyping diagnostics of leukemia (Terwijn et al, 2009). Aberrant expression of CD7 was assessed in the context of CD13 expression. In normal hematopoiesis, 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 2 (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. was applied (Cutler et al, 2011). If clearly abnormal myeloid progenitors but less than 5% were present, in the absence of abnormalities in the mature myelomonocytic compartment, two points were scored.

Table 2. The components of the flow cytometric scoring system (FCSS)(adapted from Wells et al, 2003 and Cutler et al, 2011)

Points	Myeloidprogenitorcells
1	<5% myeloid progenitors with aberrancies defined as:
	Abnormal granularity
	Abnormal expressionof 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	Maturingmyeloidcells
1	One of the following is present:
	Abnormal granularity
	Abnormal expressionof 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	Monocyticcells
1	One of the following is present:
	Abnormal granularity
	Abnormal expressionof 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

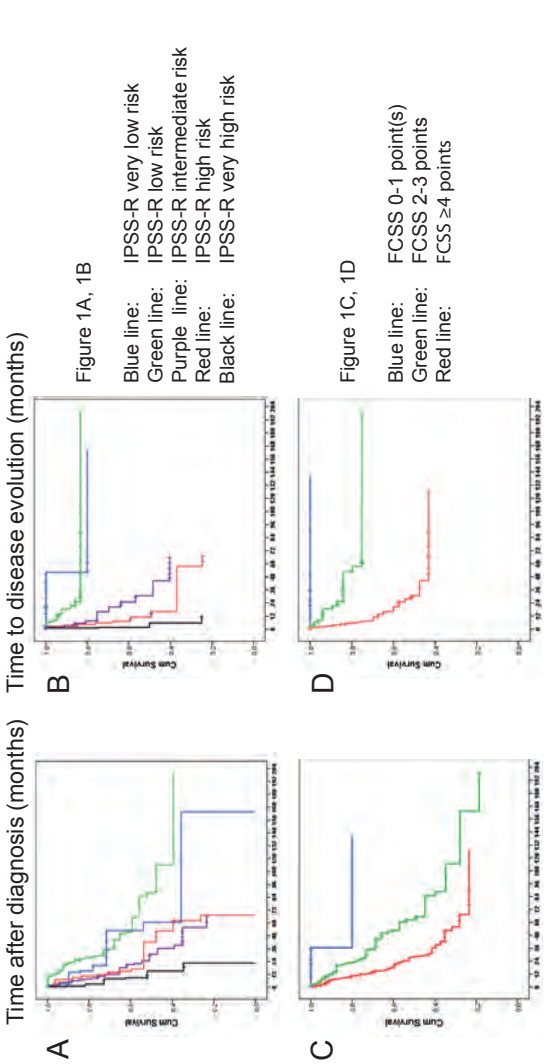
Statistical analysis

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 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 modelto express the degree of hazard of deathor disease progression for a subgroup. The OS time was defined as the period from date of diagnosisuntil 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-1 or AML. To determine whether the FCSS is of significant added value besideIPSS(-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). A p-value ≤ 0.05 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 1). 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 was35 (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. Overall survival 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 have worse OS compared with the IPSS low and intermediate-1 risk groups (Supplementary Figure 1A). Time to disease evolution was shorter for the IPSS intermediate-2 and high risk groups compared with IPSS low and intermediate-1 risk groups (Supplementary Figure 1B). Similar to the IPSS, the IPSS-R distinguished subgroups of patients with different OS (Figure 1-A-B).

Although the IPSS-R does not separate prognostic subgroups as good 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.



Survival	Median (months, (years))	Range (months)
IPSS-R study cohort (n=155)		
very low (n=22)	60.6 (5.1)	0.7-163.7
low (n=80)	88.6 (7.4)	0.5-198.6
intermediate (n=35)	30.8 (2.6)	0.7-65.9
high (n=27)	42.2 (3.5)	0.9-67.3
very high (n=11)	15.1 (1.3)	0.5-22.5
FCSS (n=139)		
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=64)	28 (2.3)	0.5-126.3

	Median (in months, (years))
IPSS-R reference cohort	
Very Low	105.6 (8.8)
Low	63.6 (5.3)
Intermediate	36 (3)
High	19.2 (1.6)
Very high	8.6 (0.8)

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

Figure 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<0.001.(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<0.001.(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<0.001.(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<0.001. IPSS-R International Prognostic Scoring System revised;The survival data of the IPSS-R reference cohort is derived from Greenberg et al. Blood 2012.

The FCSS correlates with the WHO classification

Flow cytometric scores were calculated for all patients and (pathologic) controls. The median FCSS of the total MDS cohort was 4 (range 0-8), which was significantly higher than the median FCSS of the pathologic controls (median FCSS=1, range 0-4, p<0.001) and healthy controls (median FCSS=1, range 0-2, p<0.001).

Patients with more advanced stages of MDS (RAEB-1 and RAEB-2) had relatively higher FCSS values by FC compared with patients with RA(RS) and RCMD(RS) (n=144, Spearman's correlation= 0.55, p<0. 001)(Supplementary Figure 2A). 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).

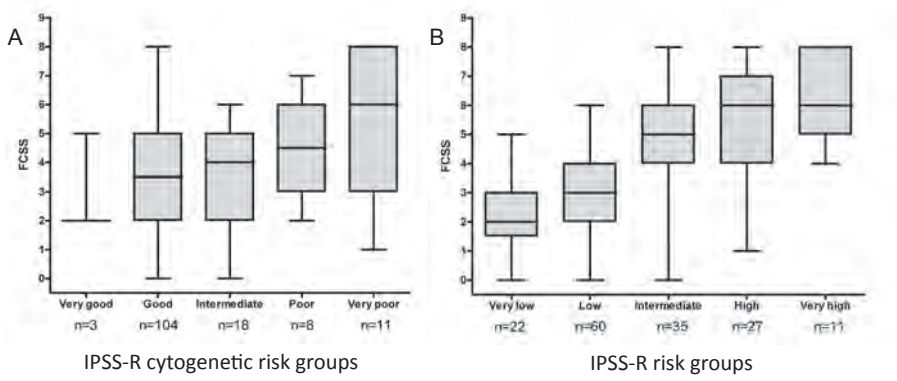


Figure 2. Distribution of FCSS scores over IPSS-R cytogenetic and IPSS-Rcategories.(A)IPSS-R cytogenetic subgroups are depicted on the x-axis, from very good to very poor and the FCSS on the y-axis. The FCSS between the IPSS-R cytogenetic subgroups was not significantly different. (B) IPSS-R risk groups 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<0.001 and borderline significant for IPSS-R intermediate vs. high risk patients, p=0.07.

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<0.001 and n=155, Pearson=0.59, p<0.001, respectively) (Figure 2 and Supplementary Figure 2B). Moreover, there was a trend for correlation between the FCSS and IPSS cytogenetic subgroups, Spearman's correlation 0.14, p=0.08. The FCSS and IPSS-R cytogenetic subgroups showed a stronger, significant positive correlation (Spearman's correlation 0.19, p=0.02) (Figure 2 and Supplementary Figure 2C). 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 of 111 patients; of these patients, 37.8% (n=42) were transfusion dependent by WPSS criteria. Follow-up with respect to the course of the disease was known of 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 favorable prognosis in patients with MDS

Three different prognostic subgroups could be distinguished within our cohort by using the defined cutoffs 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, $p=0.01$)(Figure 1C). The difference in OS between patients with no abnormalities (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 with patients with mild and severe flow cytometric abnormalities, $p<0.001$ (Figure 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 are 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 analyzing the degree of dysmyelopoiesis by FC, patients could be identified with good, intermediate and worse prognosis (Figure 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 hematopoiesis (Figure 4A). Low risk patients according to the IPSS-R and no to minimal flow cytometric aberrancies (0-1 point) have 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, $p=0.06$. In contrast to OS, the time to disease progression for IPSS-R low risk patients with either mild flow cytometric abnormalities compared or severe abnormalities was not significantly different, $p=0.24$ (Figure 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.
In a likelihood ratio-test, the combination of FCSS and IPSS-R was statistically better in predicting OS in patients with MDS than only IPSS-R as a single model, $p=0.02$. This was also the case for the combination of the IPSS and FCSS, $p=0.01$.

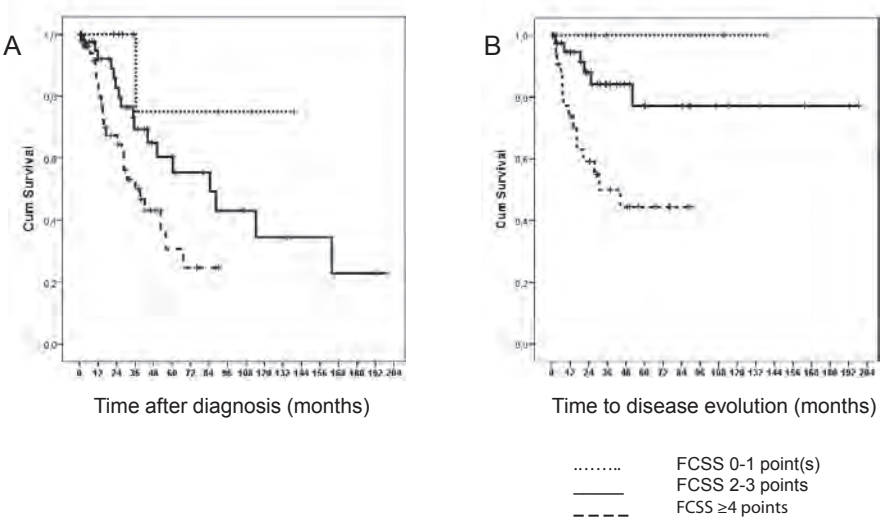


Figure 3. Overall survival and time to disease evolution by FCSS within the IPSS-R cytogenetic good risk subgroup.(A)Overall survival was significantly better in patients with a FCSS of 2-3 points (solid line, n=42/104) compared with patients with ≥4 points (striped line, n=52/104), p=0.01. Median overall 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=0.002. 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.

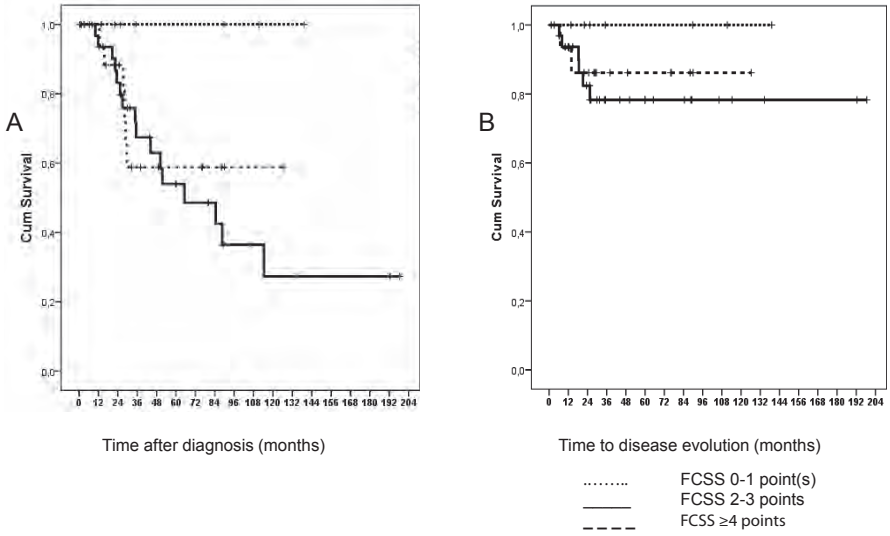


Figure 4. Overall survival and time to disease evolution by FCSS within the IPSS-R low risk group.(A)Overall survival was significantly better in patients with a FCSS of 0-1 point (dotted line, n=7/60) compared with patients with 2-3 points (solid line, n=35/60), p=0.05. Median overall survival for patients 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=0.24. Median time to disease evolution was not reached for all patient groups.

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 analysis of BM 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 classification, 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; Greenberg et al, 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 since the majority of our data is obtained after 2004, compared with the IPSS reference cohort that data collected before 1997. Furthermore, our patient group included patients receiving (intensive) chemotherapy and/or hematopoietic 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 is in the IPSS-R cytogenetic good risk group. Since one of the most important improvements in the IPSS classification is the reclassification of cytogenetic subgroups, we analyzed whether the FCSS could differentiate subgroups within this cytogenetic category. The FCSS was able to identify three groups of patients with different overall survival 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 which is reflected by lower survival of patients with RCMD(RS) compared with patients with RA(RS)(Malcovati et al, 2005). 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). Since 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, 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 (Supplementary figure 2A).

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 hematopoiesis 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 patients with MDS and give a better estimation of survival. From the likelihood ratio-test it was concluded that FCSS and IPSS-R combined appeared to givethe 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 that is 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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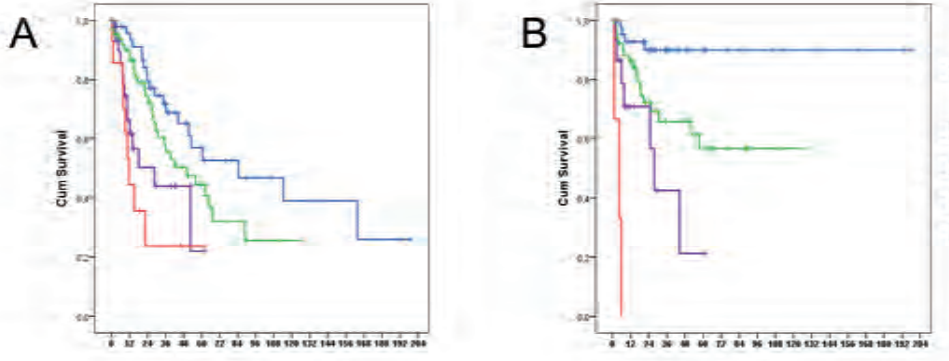
Supplementary table 1. Pathologic controls

Diagnoses	N (%)
	61
Iron deficiency	18 (29.5)
Lymphoproliferative disorder (lymphoma BM staging with no BM localization of the lymphoma)	13 (21.3)
Anemia associated with chronic disease	8 (13.1)
Idiopathic thrombocytopenic purpura	7 (11.5)
Mastocytosis	5 (8.2)
Vitamin B deficiency	2 (3.3)
Anemia associated with auto-immune disease (non-hematologic)	1 (1.6)
Anemia associated with kidney failure	1 (1.6)
Alcohol abuse	1 (1.6)
Amegakaryocyticthrombocytopenia	1 (1.6)
Idiopathicchronicneutropenia	1 (1.6)
Infectiousdisease (Leishmaniasis)	1 (1.6)
Normal BM with eosinophilia	1 (1.6)
Post chemotherapy for a non-clonal myeloid disorder without bone marrow localization	1 (1.6)

BM bone marrow

Three pathologic controls had a FCSS of four. One patient had a vitamin B12 deficiency with increased CD34+ cells which could be identified as an increased percentage of monocytic precursors. The other two patients had mastocytosis. The pathologic control with vitamin B12 deficiency and one with mastocytosis both scored extra points for more than 5% progenitor cells. The other pathologic control with mastocytosis had qualitative abnormalities in the maturation patterns of neutrophils and monocytes.

Supplementary figure 1

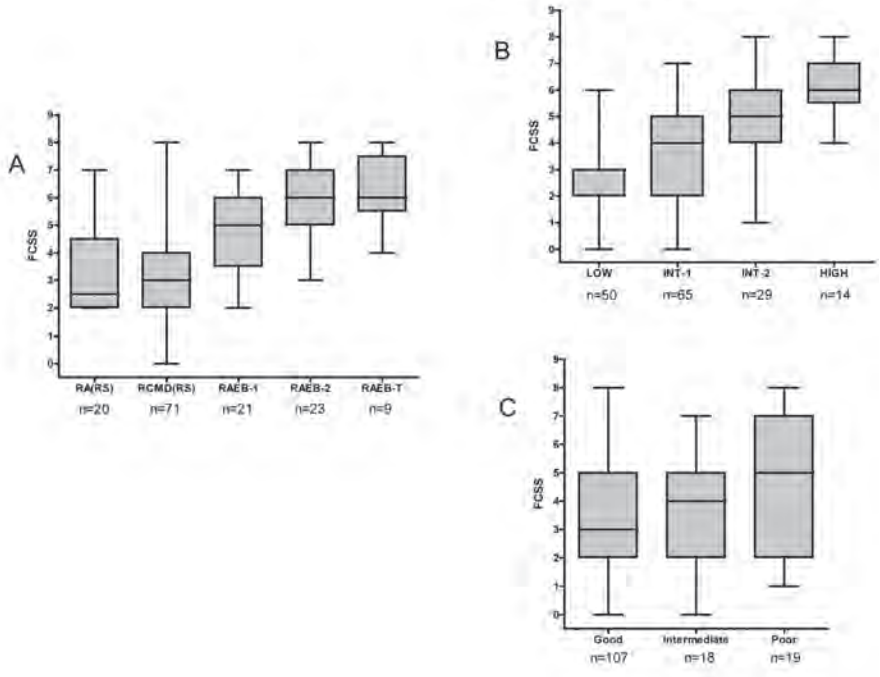


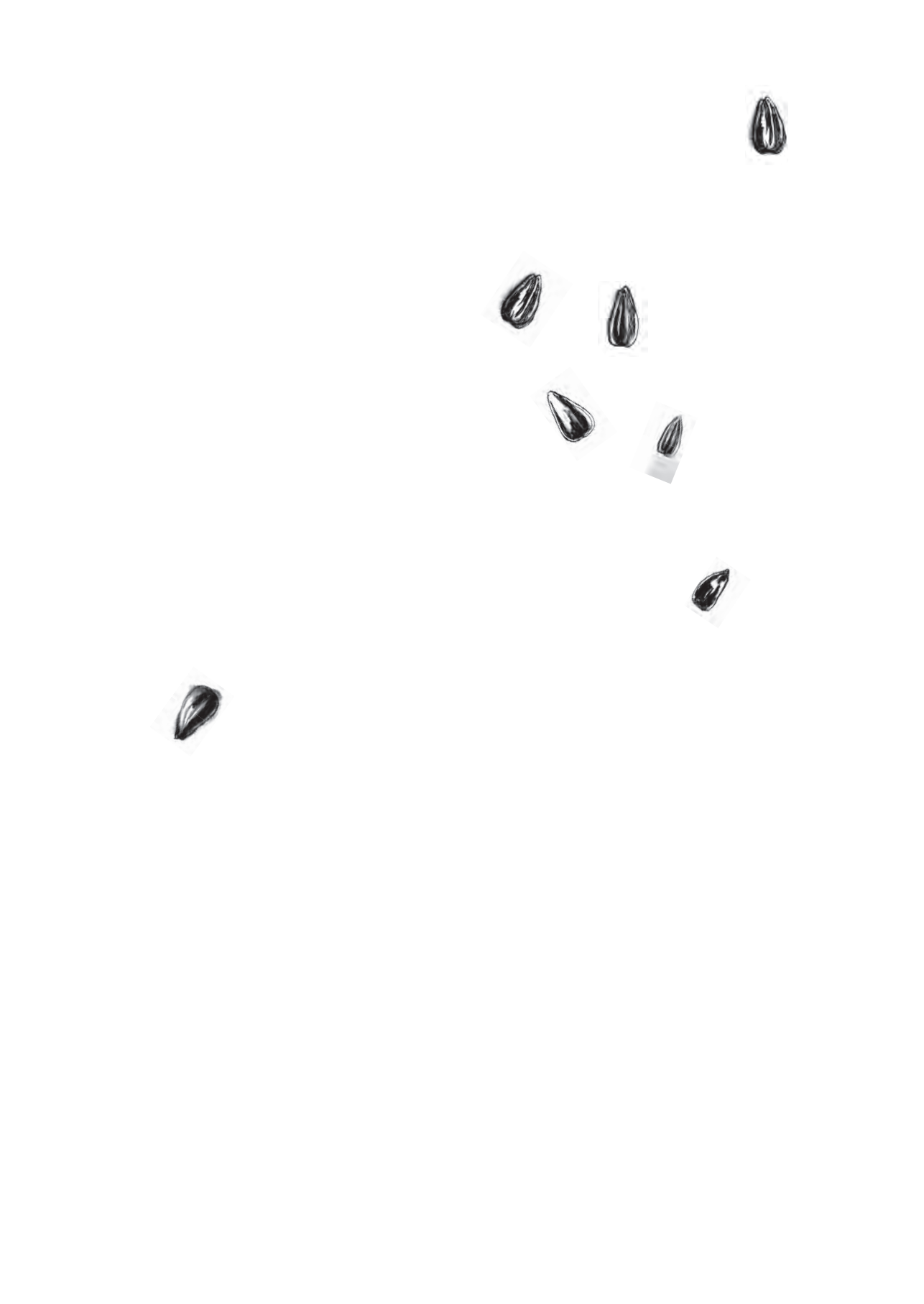
Supplementary Figure 1A, 1B

Blue line: IPSS low risk
Green line: IPSS intermediate-1 risk
Purple line: IPSS intermediate-2 risk
Red line: IPSS high risk

Survival	Median (months, (years))	Range (months)		Median (in months, (years))
IPSS study cohort (n=158)			**IPSS reference cohort**	
low (n=50)	84.6 (7.1)	0.5-198.6	Low	68.4 (5.7)
intermediate-1 (n=65)	50.3 (4.2)	0.7-126.3	Intermediate-1	42 (3.5)
intermediate-2 (n=29)	28.6 (2.4)	2.2-61.1	Intermediate-2	14.4 (1.2)
high (n=14)	11.7 (1)	0.5-61.5	High	4.8 (0.4)
Time to disease progression	**Median (months, (years))**	**Range (months)**		
IPSS study cohort (n=119)				
low (n=45)	not reached	0.7-198.6		
intermediate-1 (n=56)	not reached	0.7-126.3		
intermediate-2 (n=15)	27.8 (2.3)	1.6-61.1		
high (n=3)	4.4 (0.4)	1.1-5.7		

Supplementary figure 2





7

The myelodysplastic syndromes flow cytometric score: a 3-parameter prognostic flow cytometric scoring system

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Leukemia 2015; in press

Abstract

The prognosis of myelodysplastic syndromes (MDS) is currently estimated by using the revised international prognostic scoring system (IPSS-R). Several studies have shown that further refinement of prognostication for MDS can be achieved by adding flow cytometric parameters. However, widespread implementation of flow cytometry for the prognosis of MDS is hampered by complexity of the analysis. Therefore, the aim of this study was to construct a robust and practical flow cytometric score that could be implemented as a routine procedure. To achieve this, bone marrow aspirates of 109 MDS patients were analysed by flow cytometry. A second cohort consisting of 103 MDS patients was used to validate the MDS flow cytometric score (MFS). The parameters forming the MFS were sideward light scatter and CD117 expression of myeloid progenitor cells and CD13 expression on monocytes. Three MFS risk categories were formed. Patients with MDS and intermediate MFS scores had significantly better overall survival (OS) compared with patients with high MFS scores. The MFS further refined prognostication within the IPSS-R low risk category, by identifying patients with worse OS in case of high MFS. In conclusion, a practical three parameter flow cytometric prognostic score was constructed enabling further refinement of prognostication of MDS.

Introduction

The myelodysplastic syndromes (MDS) are a group of clonal hematopoietic stem cell disorders with a heterogeneous presentation and clinical course. The majority of patients will succumb to the consequences of ineffective hematopoiesis and a subgroup will evolve to acute myeloid leukemia (AML). The International Prognostic Scoring System (IPSS) and revised IPSS (IPSS-R) are frequently used tools to estimate clinical outcome at diagnosis and are a rationale for treatment decision making, both in clinical practice as well as in the design of clinical trials.[1,2]

The IPSS-R distinguishes five prognostic subgroups of patients with MDS based on cytogenetic abnormalities, the depth of cytopenia and bone marrow blast count.

It is anticipated that techniques such as flow cytometry and molecular analysis may have additional prognostic impact.

The flow cytometric scoring system (FCSS) allows for a numerical display of immunophenotypic aberrancies in the (im)mature myelo-monocytic lineage. Scores are generated by enumerating abnormalities and myeloid progenitor cell counts; a high score reflects a high number of aberrancies and/or a high amount of progenitors. An accumulation of flow cytometric abnormalities, resulting in high scores is associated with adverse outcome.[3-6] Previously, it was shown that patients with MDS and a high FCSS were likely to relapse after allogeneic stem cell transplantation.[3,7] Moreover, patients with a high number of flow cytometric abnormalities are likely to experience an adverse clinical outcome, even though they are in a lower risk IPSS or IPSS-R category.[4,6,8]

Flow cytometry in MDS is a technique which requires specific analytical expertise. Sophisticated flow cytometric scoring systems for MDS are available, but the laboriousness hampers general applicability.[5] Little is known which flow cytometric parameters are the most relevant for prognosis of MDS. Even though there are some indications of prognostic relevance of single flow cytometric variables, an accumulation of flow cytometric abnormalities was regarded as probably the best approach to estimate prognosis for MDS. The aim of our study was to construct a widely applicable risk-adapted flow cytometry based prognostic score for MDS. A MDS flow cytometric score (MFS) was developed in a learning cohort of patients with MDS and subsequently validated in another cohort of patients with MDS. The three parameter MFS distinguished prognostic subgroups of patients with MDS. Furthermore, refinement of the IPSS-R was achieved by applying the MFS within IPSS-R subgroups.

Materials and Methods

Patients

Bone marrow samples were collected consecutively from 109 patients with a diagnosis of MDS between 2005 and 2008 (learning cohort). Patient characteristics are summarized in table 1. Morphology was assessed by two experienced hematologists (AAvdL and GJO) and diagnoses were made according to the WHO2008 classification.[9] Patients with MDS and 20-30% blasts by bone marrow morphology, formerly classified as RAEB-t by FAB classification, were also included.[10] Cytogenetic evaluation on bone marrow samples was performed according to ISCN guidelines.[11] In bone marrow samples where no growth was found, fluorescence in situ hybridization (FISH) was applied to detect chromosomal abnormalities, as recommended for the diagnostic work up of MDS.[12,13] The FISH probes that were applied were LSI EGR1 SO/D5S23, D5S721 DC (5q31 and 5p15.2), LSI D7S486/CEP7 (D7Z1) (7q31 and centromere 7), CEP8 (D8Z2) (centromere 8), LSI p53 (17p13.1), subtelomere probes 3qter and 12pter (TotelVysion), in male patients: (CEPY) (DYZ23) centromere Y (Vysis, Abbott, United States of America) and P53 deletion probe (chromosome locus 17p13.1), Del(20q) deletion probe (chromosome loci 20q12: PTPRT and 20q13.2: MYBL2 (Cytocell, United Kingdom). Bone marrow samples of 16 healthy volunteers were analyzed by flow cytometry for reference values.The mean age of the healthy volunteer cohort was comparable with the mean age of the MDS cohort (n=16, mean 61.2, range 43-86 years and n=109, mean 64.4, range 4-90 years, respectively, p= 0.41). For the validation cohort, bone marrow samples were collected from 103 patients with a diagnosis of MDS from 2009 to 2015. Patient characteristics are summarized in table 1. Similar diagnostic procedures as described above were applied for the validation cohort.The samples that were used for flow cytometric analysis were from patients who did not receive therapy for MDS prior to flow cytometric analysis, apart from supportive care such as red blood cell transfusions.

Overall, 27/212 (13%) patients received hematopoietic stem cell transplantation (18/109 (17%) in the learning cohort and 9% in the validation cohort). All bone marrow aspirates for flow cytometric analysis were drawn prior to therapy.

Data from 24 patients in the learning cohort and 26 patients from the validation cohort were not previously reported. Data from the remaining patients was previously incorporated in studies investigating the application of flow cytometry in MDS.[4,6,14]

All samples were drawn after informed consent and in accordance with the Declaration of Helsinki. The study was approved by the Medical Ethics Committee of VU University Medical Center, Amsterdam, The Netherlands.

Table 1. Patient characteristics

	Learning cohort	Validation cohort
	N (%)	N (%)
WHO 2008 classification	109	103
RCUD	10 (9)	6 (6)
RARS	8 (7)	6 (6)
RCMD	56 (51)*	41 (40)*
RAEB-1	12 (11)	21 (20)
RAEB-2	10 (9)	17 (16)
MDS unclassifiable	6 (6)	5 (5)
deletion 5q associated		2 (2)
AML (FAB RAEB-T)	7 (7)	5 (5)
Therapy related cases	8 (7)	18 (17)
IPSS	108**	102**
low	42 (39)	23 (23)
intermediate-1	41 (38)	44 (43)
intermediate-2	15 (14)	27 (26)
high	10 (9)	8 (8)
IPSS cytogenetic risk group	93#	90#
good	71 (76)	56 (63)
intermediate	12 (13)	14 (15)
poor	10 (11)	20 (22)
IPSS-R	103***	101***
very low	15 (15)	14 (13)
low	41 (40)	32 (32)
intermediate	26 (25)	20 (20)
high	17 (16)	25 (25)
very high	4 (4)	10 (10)
IPSS-R cytogenetic risk group	93#	90#
very good	3 (3)	3 (4)
good	69 (74)	55 (61)
intermediate	13 (14)	12 (13)
poor	5 (6)	7 (8)
very poor	3 (3)	13 (14)

WHO World Health Organization; RCUD refractory cytopenia with unilineage dysplasia; RARS refractory anemia with or without ring sideroblasts; RCMD refractory cytopenia with multilineage dysplasia (with or without ring sideroblasts); RAEB-1 refractory anemia with excess of blasts (5-10%); RAEB-2 refractory

anemia with excess of blasts (10-20%); RN refractory neutropenia; RT refractory thrombocytopenia; FAB French-American-British; RAEB-T refractory anemia with excess of blasts in transformation; IPSS International Prognostic Scoring System; IPSS-R International Prognostic Scoring System revised. *In the learning cohort 5 patients had RCMD-hypoplastic and 3 patient had RCMD-hypoplastic in the validation cohort. **IPSS from 1 patient missing in the learning and validation cohort, because of lack of cytogenetic data and peripheral blood values. ***IPSS-R from 6 patients missing in the learning cohort and from 2 patients missing in the learning cohort, because of lack of cytogenetic data and/or peripheral blood values. # In the learning cohort 16 patients and 12 patients in the validation cohort could not be assigned to an IPSS(-R) cytogenetic risk category due to lack of metaphases.

Risk assessment

For risk assessment the IPSS and IPSS-R were applied.[1,2] For transfusion dependency, the definition of the WHO-based prognostic scoring system (WPSS) was used.[15] Transfusion dependency was defined as a minimum of 1 unit of packed cells every 8 weeks for at least 4 months.

Flow cytometric analysis of bone marrow samples

All samples were processed according to European LeukemiaNet guidelines.[16,17] Erythrocyte lysis was performed with ammonium chloride prior to analysis of total nucleated cells in the bone marrow. All samples were processed and analyzed within 24 hours by four-color flow cytometry.

Monoclonal antibodies that were used in this study were fluorescein isothiocyanate (FITC) conjugated: CD5 (clone DK23), CD13 (WM-47), CD16 (DJ130c), CD71 (Ber-T9) from DakoCytomation Glostrup, Denmark; CD15 (MMA), CD34 (8G12) from BD Biosciences (San Jose, CA); 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), CD25 (ACT-1), CD64 (10.1), CD235a (JC159) 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. Measurements were performed on a FACS Calibur. Data were analyzed by Cell Quest Pro Software (BD Biosciences). The combination of markers that was used is described in supplementary table 2.

Table 2. Results from univariate analysis and association with survival of each variable

Myeloid progenitors	Deviation from normal*	P values
Increased CD13 expression	> 9.8 SD	0.06
Increased CD34 expression	> 1.2 SD	0.08
Decreased CD45 expression	≤ -2.5 SD	0.01
Increased percentage of $CD56^{pos}$ cells	> 0.82 SD	<0.001
Increased CD117 expression	> 3.6 SD	<0.001
Increased percentage of $HLA\text{-}DR^{pos}$ cells	> 0.47 SD	0.06
Increased percentage of myeloid progenitors	> 5.3 SD	0.001
Decreased SSC	≤ -0.8 SD	<0.001
Maturing myeloid cells		
Increased CD13 expression	> -1.8 SD	0.098
Increased percentage of $CD34^{pos}$ cells	> 1.5 SD	0.03
Increased percentage of $HLA\text{-}DR^{pos}$ cells	> 1.4 SD	0.003
Increased number of $CD34^{neg}CD117^{pos}$ cells	> 4.6 SD	0.03
Decreased ratio between the number of neutrophils and lymphocytes	≤ -0.48 SD	0.052
Increased percentage of neutrophils	> 0.3 SD	0.06
Decreased SSC	≤ -2 SD	0.045
Monocytic cells		
Decreased CD13 expression**	≤ -1.5 SD	0.016
Increased percentage of $CD56^{pos}$ cells	> 1.3 SD	0.001
Increased SSC	> -0.74 SD	0.066
Increased HLA-DR expresssion	> -0.64 SD	0.099
Erythroid cells		
Increased percentage of $CD71^{pos}CD235a^{pos}$ cells	> -1.7 SD	0.049
Increased total number of erythroid cells	> -0.75 SD	0.07
Miscellaneous		
Decreased percentage of B cell progenitors	≤ -0.88 SD	0.001
Increased percentage of plasmacytoid dendritic cells	> -1.2 SD	0.009
Increased percentage of basophils	> 2.8 SD	0.015

Note: the variables are described in such a manner that it is associated with adverse outcome. For example, a decreased percentage of B cell progenitors ≤-0.88 SD from mean values of healthy volunteers is associated with worse OS. SD standard deviation

Definition of myeloid progenitors: CD45dim Myeloid

* These cut-off values were acquired by applying recursive partitioning (STREE)

** Decreased expression of CD13 on monocytes is associated with favourable outcome.

Cell populations were selected by sideward light scatter (SSC) and CD45 properties. Maturing myeloid cells were delineated as $CD45^{dim/bright}SSC^{int/high}$; and the monocytic compartment as $CD45^{bright}SSC^{int}CD14^{pos}$ and nucleated erythroid cells as $CD45^{neg}SSC^{low/int}$. Sideward light scatter of mature myeloid cells was expressed as a ratio to lymphoid cells. Myeloid progenitor cells were defined as $CD45^{dim}$, SSC^{low} in combination with CD34. Back gating was achieved after selection of CD34 positive myeloid progenitor cells and visualization of these cells in a CD45 vs. SSC plot. The myeloid origin of progenitor cells was checked by using myeloid markers CD13 and/or CD117 and/or HLA-DR. Notably, aberrant myeloid progenitors in MDS can aberrantly lack CD34 on myeloid progenitors. In these cases CD117 and expression of HLA-DR is combined with SSC and CD45 properties to serve as an alternative. Debris, non-viable cells and doublets were also excluded by back gating. Furthermore, B cell progenitors were identified by diminished CD45 and CD34, low SSC properties as compared to myeloid precursors, and back gating with CD19.[18] One hundred thousand white blood cell events were acquired including a minimum number of 250 events within the CD34 positive myeloid progenitor cell compartment. Aberrancies with regard to count, expression level of markers and lineage infidelity marker (LIM) expression on myeloid progenitors, B cell progenitors, differentiating erythroid, monocytic and myeloid cells were analyzed. Mean fluorescence intensities were calculated for the markers that were used in cell populations of interest.

Asynchronous or LIM expression was considered present if ≥20% of the myeloid progenitor cells formed a cluster of events and expressed CD11b, CD5, CD7, CD19, CD56 and/or CD25.[19] A cluster was distinguished from background expression by a dispersed character of the cell events upon analysis. Which is in contrast to a subpopulation of myeloid progenitor cells with abnormal marker expression that forms a group of events that are clustered together in a flow cytometric analysis plot. When a minimum number of 250 myeloid progenitors are analyzed, at least 50 cells (≥20%) are needed to have aberrant marker expression in order to meet the definition of aberrant marker expression. This is based on cutoff values in routine immunophenotyping diagnostics of leukemia. A different definition for abnormal CD7 expression on myeloid progenitors was used since in normal hematopoiesis CD7 can be expressed on $CD34^{pos}$ and $CD13^{dim}$ cells.[17] Hence, abnormal expression of CD7 on myeloid progenitors was considered present if a cluster of ≥10% (at least 25 events in case a minimum number of 250 myeloid progenitor cells was acquired) on $CD13^{pos\text{-}bright}$ myeloid progenitors was expressing CD7.[17] As a reference for CD13 expression on myeloid progenitor cells, both $CD13^{pos\text{-}bright}$ as well as $CD13^{dim}$,

myeloid progenitor cells from age-matched healthy volunteers were used. In normal hematopoiesis, CD13 expression in the myeloid progenitor compartment is heterogeneous. Early myeloid progenitor cells are CD13dim. Upon maturation, CD13 expression on myeloid progenitors increases. The normal differentiation of maturing myeloid progenitor cells forms a classic L-shaped pattern.
The FCSS was calculated according to Wells et al. with the modification proposed by Cutler et al.[3,20] Three groups of patients can be distinguished by the FCSS; i.e. those with normal to mild dysplasia (0-1 point), moderate dysplasia (2-3 points) and severe abnormalities (≥4 points).

Statistical analysis

The Student's t-test was used to assess significance of differences in age between the MDS cohort and the healthy volunteers and to assess significance in MFS flow cytometric scores between transfusion dependent and independent patients with MDS. For flow cytometric variables, outliers were checked and verified and if regarded as an outlier by Grubb's test for outliers, the value was removed. Only in the discovery phase, the outliers were excluded and there were no exclusions in the learning and validation cohort. For each flow cytometric variable in the healthy volunteer cohort, mean and standard deviation were calculated. In order to express the deviation from normal values, for each individual patient with MDS, the flow cytometric variable was transformed into a ratio by the following formula:

$$\frac{\text{Flow cytometric value of variable in patient} - \text{mean value of variable in normal}}{\text{Standard deviation of variable in normal}}$$

To determine the optimal cutoff level with the highest impact on overall survival (OS) for each variable, the statistical program STREE was used. (http://c2s2.yale.edu/software/stree/) [21] STREE uses a recursive partitioning algorithm to determine the cutoff yielding the highest separation between two groups with regard to OS. The impact on OS of the variable at a specific cutoff level was confirmed in Kaplan-Meier analyses and assessed for significance by the log-rank test. Furthermore, the Bonferroni correction was applied to the p-value of the log-rank test when multiple comparisons were made for overall survival. Overall survival was defined as time from diagnosis until date of last visit or death. A total of 29/212 subjects (14%) had more than 6 months between the diagnosis of MDS and the aspiration that was drawn for flow cytometric analysis (17/109) patients of the learning cohort and

12/103 patients of the validation cohort). Variables for the MFS were selected in a multivariate regression analysis (Cox proportional hazard model) via a forward selection procedure with a p-value for entry of ≤0.05. Only variables with a univariate p-value ≤0.1 and prevalence of the abnormality in at least 20% of patients with MDS were included in this multivariate analysis.

Of note, myeloid progenitor cells were analyzed in three different manners. By gating on $CD45^{dim}$ cells, on $CD34^{pos}$ cells and $CD45^{dim}$ and back gating by using FSC and SSC properties in combination with markers such as CD13, CD117 and/or HLA-DR. Univariate analyses for all three definitions was performed and the method of $CD45^{dim}$ with back gating on myeloid progenitor as described above was the most optimal method. This method of analysis is also the definition recommended by the ELN working party on flow cytometry in MDS.[16,17]

In one out of 109 patients, not all variables for the MFS could be analyzed due to low cell count and/or less than 250 events at flow cytometric analysis. This patient was not included in OS analyses of the MFS. Three patients were excluded from OS analyses due to missing data on OS.

The association between the MFS and transfusion dependency was statistically tested by using the Pearson Chi-square test.

Results

Risk stratification by IPSS and IPSS-R

The distribution of patients over the IPSS and IPSS-R risk categories was similar to the data of the original cohorts of the IPSS and IPSS-R.[1,2] The majority of patients was within the IPSS low/intermediate-1 risk and IPSS-R low risk category, as shown in supplementary figure 1 and supplementary table 1. Overall survival data in our study were in line with previously published IPSS and IPSS-R data. Patients within the IPSS low risk group have significantly better OS compared with patients within the IPSS high risk group, p=0.02. (Supplementary figure 1A and supplementary table 1). Similarly, there was a significant difference in OS between patients in the defined IPSS-R risk groups, p<0.001. (Supplementary figure 1B and supplementary table 1) In 9/109 patients only FISH was available due to failed karyotyping. Of these 9 patients, 3 had an abnormal karyotype as defined by the applied FISH probes. In the validation cohort, 3/103 patients had only FISH available due to lack of metaphases for conventional karyotyping. All three patients had no abnormalities as determined by FISH.

Patients with a moderate FCSS have better overall survival than patients with a high FCSS

The FCSS, originally designed as a prognostic score, distinguishes three different prognostic subgroups based on flow cytometric findings as described above.[3] Patients with MDS in our study were allocated to either normal to mild FCSS (0-1 point), moderate FCSS (2-3 points) or severe FCSS group (≥ 4 points). The FCSS identified prognostically distinct subgroups of patients with MDS, p=0.02. Figure 1 shows that patients with MDS and a moderate FCSS score have significantly better OS than patients with a severe FCSS score, p=0.009, after Bonferroni correction, p=0.03. Comparison of survival between FCSS subgroups and p-values are described in figure 1.

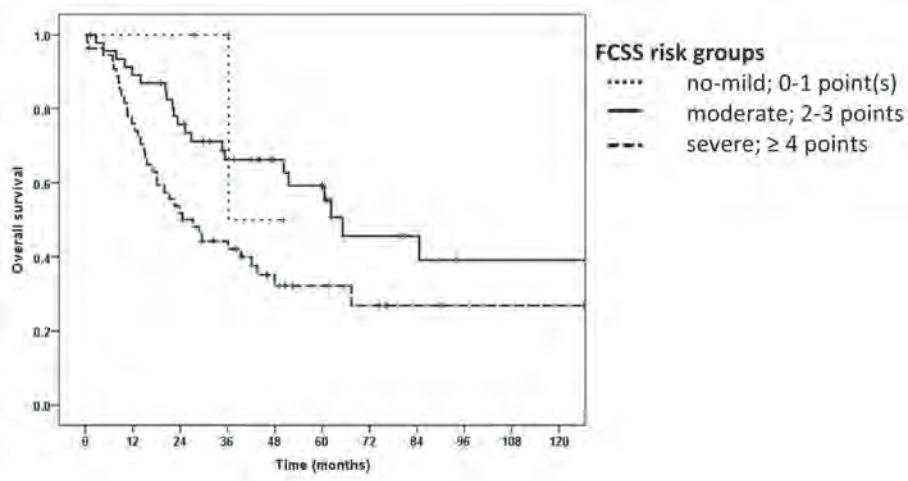

Figure 1. Survival by FCSS risk groups (A)

Overall survival was significantly better in patients with FCSS 0-1 points and/or 2-3 points, compared with patients with ≥4 points, p=0.02. Median OS time 36.3 months (n= 5), 65.2 months (n= 46) and 27.4 months (n=55), respectively. Overall survival between FCSS 0-1 points vs. 2-3 points was non significant (p=0.7), FCSS 0-1 points vs. ≥4 points was non significant (p=0.2) and FCSS 2-3 points vs. ≥4 points was significantly better, p=0.009.

Identification of flow cytometric variables with prognostic relevance and composition of a prognostic flow based model

To calculate the FCSS, it is necessary to analyze 52 flow cytometric variables. The goal of our study was to make a simplified flow cytometric scoring system that would be applicable in every laboratory without losing prognostic information. As mentioned above, a recursive partitioning algorithm (STREE) was applied to determine the

cutoff value of each flow cytometric variable that is expressed in standard deviations from the healthy control group.
The flow cytometric variables that had a p-value of ≤0.1 in univariate analysis and hence proceeded to multivariate analysis are depicted in table 2, as well as their respective cutoff values for the standardized ratios.
In contrast to the studies using the FCSS, which includes only analysis of myelomonocytic cells, we included analysis of nucleated erythroid cells in the bone marrow. Two flow cytometric variables in the erythrocyte compartment were significantly predictive for OS in univariate analysis: increased percentage of $CD71^{pos}CD235a^{pos}$ cells and increased total number of erythroid cells. Of the 52 variables, 24 were eligible for selection in the multivariate analysis. It was decided not to include the quantification of myeloid progenitors in the multivariate analysis since the quantification of myeloid progenitors or blasts is already provided by bone marrow morphology and scored for in the IPSS and IPSS-R. Three flow cytometric variables formed the final MFS model and were most significant in predicting OS by flow cytometry in MDS. Decreased SSC of myeloid progenitors of ≤-0.8 standard deviations from normal, which is a flow cytometric reflection of granularity and increased CD117 expression on myeloid progenitors of >3.6 standard deviations from normal were associated with worse OS. Furthermore, decreased CD13 expression on monocytes of ≤-1.5 was associated with better OS. Figures 2A-C display the OS curves of these three individual factors.

Since the hazard ratio of each variable was similar, 2.4 for SSC of myeloid progenitors, 2.7 for CD117 expression on myeloid progenitors and 2.4 for CD13 expression on monocytes, each variable is weighted equally in a scoring system. Table 3 provides an overview of the three parameters, with their respective standardized ratios and hazard ratios. Three groups were formed, patients with a low MFS (0 points, n=17), with an intermediate MFS (1 point, n=46) and a high MFS (2-3 points, n=42). The MFS identified subgroups of patients with MDS and significantly different outcome, $p<0.001$. Patients with low MFS had significantly better OS than patients with a high MFS score, $p<0.001$, after Bonferroni correction $p<0.001$. (Figure 2D) Similarly, OS in patients with intermediate MFS was better than patients with high MFS, $p=0.001$, after Bonferroni correction, $p<0.001$. Overall survival was also significantly better in patients with low MFS vs. intermediate MFS, $p=0.02$, after Bonferroni correction $p=0.06$.

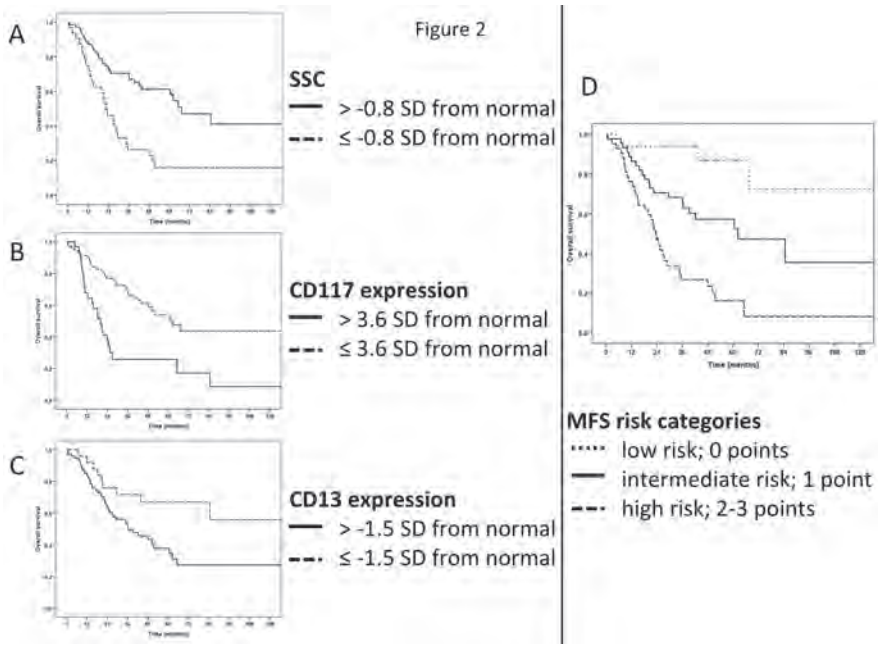

Figure 2. Survival by SSC properties and CD117 expression of myeloid progenitors, CD13 expression on monocytes and the combined MFS score (A) A decreased SSC of myeloid progenitors is associated with adverse outcome, n= 105, p<0.001. (B) An increased expression of CD117 on myeloid progenitors is associated with adverse outcome, n= 102, p<0.001. (C) A decreased expression of CD13 on monocytes is associated with favourable outcome, n=101, p= 0.02. (D) A low or intermediate risk MFS score was associated with significantly better OS compared with MDS patients with high MFS scores, p<0.001. Median OS time not reached (n=17), 62.3 months (n= 46) and 23.4 months (n= 42), respectively.

Table 3. Components of the MDS flow cytometric score (MFS)

	Deviation from normal*	Hazard ratio	Number of points
Myeloid progenitors Decreased SSC	≤ -0.8 SD	2.4	1
Myeloid progenitors Increased CD117 expression	> 3.6 SD	2.7	1
Monocytic cells Decreased CD13expression**	≤ -1.5 SD	2.4	1

Note: the variables are described in such a manner that it is associated with adverse outcome. SD standard deviation * These cut-off values were acquired by applying recursive partitioning (STREE) ** Decreased expression of CD13 on monocytes is associated with favourable outcome. Low MFS group: 0 points, intermediate MFS group: 1 point, high MFS group: 2-3 points.

The MFS identifies patients with different overall survival in IPSS-R cytogenetic good risk and IPSS-R low risk groups

Transfusion history was known for 77 patients. Patients with low and intermediate MFS scores were less likely to be transfusion dependent than patients with a high MFS, p=0.03 (Pearson Chi-square test, distribution of transfusion independent patients over MFS categories: low 13% vs. intermediate 26% vs. high 15% and transfusion dependent patients: low 3% vs. intermediate 18% vs. high 25%).

Since the majority of patients is allocated to the IPSS-R cytogenetic good risk group (n=69, 74%), the added value of the MFS within this risk group was evaluated. The MFS was able to identify patients within the cytogenetic good risk category with different outcome with regard to OS, p<0.001. (Figure 3A) Within the IPSS-R cytogenetic good risk group, patients with low MFS had significantly better OS compared with patients with intermediate MFS, p=0.048, after Bonferroni correction, p=0.144. Accordingly, in the IPSS-R cytogenetic good risk group, patients with intermediate MFS had better OS compared with patients with high MFS, p=0.003, after Bonferroni correction, p=0.009.

Similarly, the largest proportion of patients was within the IPSS-R low risk group (n=41, 40%). The MFS identified prognostically distinct subgroups of patients within the IPSS-R low risk group, p=0.003. (Figure 3B) When intermediate MFS vs. high MFS was compared within the IPSS-R low risk group, survival advantage in the intermediate group was borderline significant, p=0.051, and low MFS vs. intermediate MFS was non-significant, p=0.2 (after Bonferroni correction, p=0.153 and p=0.6, respectively.

The MFS identifies prognostic subgroups in an independent validation cohort

To validate the MFS, flow cytometric data of a second independent cohort of patients (n=103) was collected. Each of the selected three variables was transformed into a standardized ratio using the mean and standard deviation of the healthy control population. Each abnormal value scored 1 point as defined by the cutoff values in the learning cohort. In the validation cohort, patients with MDS and low scores had significantly better OS than patients with intermediate scores, n=56 and n=38, respectively, p=0.03, after Bonferroni correction p=0.06. (Figure 4) Survival rates between patients with low MFS vs. high MFS, n=9, were non-significant, p=0.6, after Bonferroni correction p=1.2. Overall survival analyses of the MFS within the IPSS-R were performed but the subgroups became too small to draw statistical conclusions. These results confirm the predictive value for OS of the MFS in an independent validation cohort of patients with MDS.

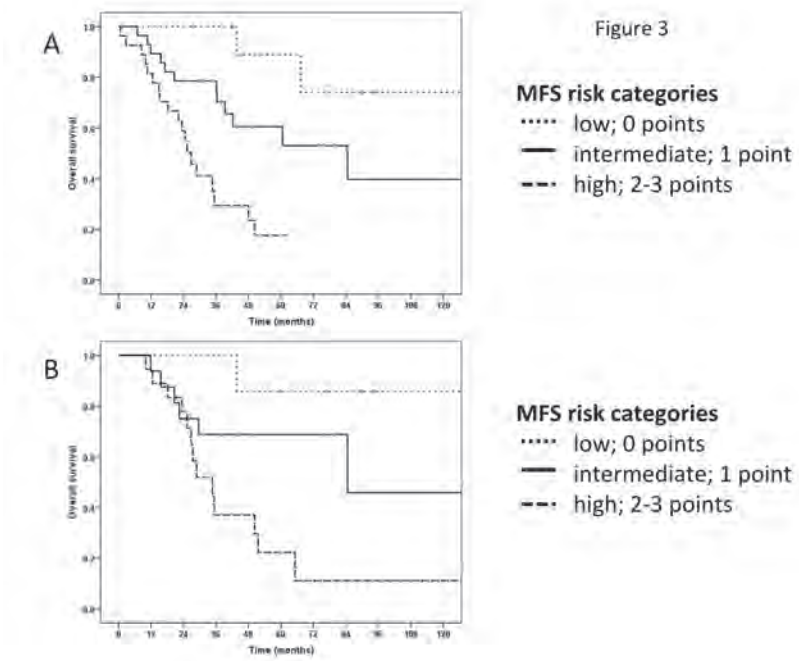

Figure 3

Figure 3. Overall survival by MFS within the IPSS-R cytogenetic good risk subgroup and IPSS-R low risk group (A) Overall survival was significantly better in patients with IPSS-R good risk cytogenetics and low/intermediate MFS scores compared with patients with high MFS scores, p<0.001. (B) Patients with IPSS-R good risk and low/intermediate MFS scores had significantly better OS compared with patients with high MFS scores, p<0.001.

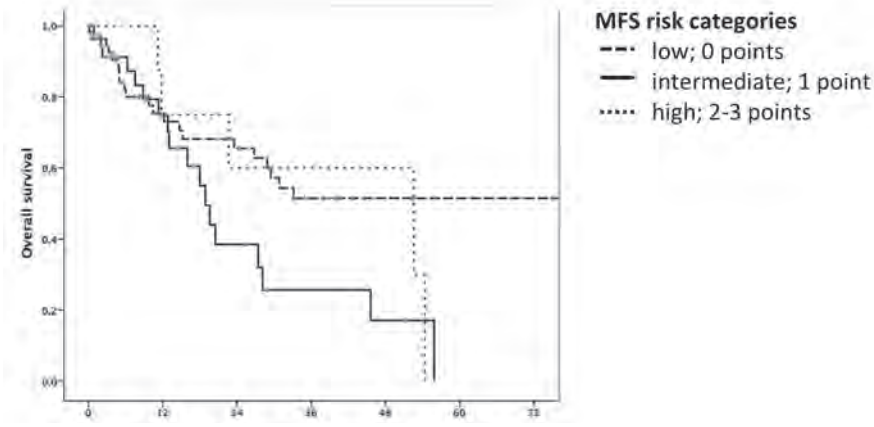

Figure 4. Overall survival by MFS score in the validation cohort

The MFS was validated in a second cohort of patients with MDS. Patients with low MFS scores had significantly better OS compared with patients with intermediate MFS scores, n=56 and n=38, respectively, p=0.03, after Bonferroni correction, p=0.06. Difference in OS between MFS low or intermediate and MFS high risk patients (n=9) was non-signifcant. Overall survival between all MFS subgroups was non-significant, p=0.07.

Discussion

Flow cytometry is recognized as a tool with prognostic significance for patients with MDS. However, the flow cytometric methods for MDS as described so far are regarded as laborious and require a high level of expertise and experience. Developing a more concise, one tube analysis for prognostication of MDS by flow cytometry would reduce costs, workload and the amount of patient material needed. Therefore, we aimed to identify flow cytometric variables with the highest prognostic impact and to design a practical prognostic flow cytometry based scoring model for patients with MDS.

In this study, a large number of flow cytometric variables were analysed in the myeloid progenitor cell population, the maturing myelomonocytic cells and erythroid lineage. To make a flow cytometric prognostic model that would be easy to handle and widely applicable, all variables were expressed as deviations from values that were obtained from healthy individuals. For future implementation it is recommended to collect bone marrow samples from age-matched healthy individuals and define reference values as described in the materials and methods section above. The number of healthy individuals should be chosen in a manner that the distribution of the values is not large and checked for outliers. In our study, bone marrow samples of 16 healthy age-matched volunteers were sufficient to generate reference values. It was decided not to include the quantification of myeloid progenitors in the statistical analysis. It is known that there is a good correlation between the blast percentage as determined by morphology and the myeloid progenitor cell count as assessed by flow cytometry. However, the values are not exchangeable and bone marrow fibrosis and peripheral blood contamination are known pitfalls for the quantification of myeloid progenitors by flow cytometry. Therefore, the power of flow cytometry is rather in the qualitative assessment of hematopoiesis than in the quantitative approach. This would make the MFS more robust and less influenced by confounders such as peripheral blood contamination (hemodilution) and/or fibrosis. In addition, in the FCSS, the percentage of myeloid progenitors is scored for in a weighted manner and can account for the score considerably.[3]

The majority of the variables that were used to construct the MFS were the same as used in studies investigating the FCSS. To benefit the robustness of the score, we decided to use strict criteria for flow cytometric variables to enter the multivariate analysis such as a p-value of ≤0.1 in univariate analysis and presence of the abnormality in at least 20% of patients with MDS. The decrease in SSC

and increase in CD117 expression of myeloid progenitors and decrease in CD13 expression of monocytes were best in predicting OS in our cohort of patients in the multivariate analysis. A low SSC of progenitors was associated with a worse OS. The myeloid progenitor compartment in normal hematopoiesis contains a heterogeneous group of progenitor cells at variable stages of differentiation with (subtle) differences in SSC and antigen expression levels. The decreased SSC might reflect the homogeneity and clonality of the myeloid progenitor cell compartment of patients with MDS. Furthermore, a low SSC might be associated with a more immature stage of the progenitors and a more stem cell phenotype.

An increased CD117 expression on myeloid progenitors was associated with worse OS. The CD117 antibody recognizes the stem cell growth factor receptor or also known as the proto-oncogene c-Kit on the surface of myeloid progenitors. CD117 is frequently (over)expressed in cancer, including leukemia.[22] Although c-Kit is essential for normal hematopoiesis, it might give malignant cells a survival benefit.[23,24]

Decreased expression of CD13 on mature monocytic cells was associated with better OS in our study. Interestingly, an adverse clinical outcome with increased expression of CD13 on mature myeloid cells was previously reported.[25] It is hypothesized that CD13 is a protein with multiple functions and that its activity on monocytes is involved in cellular adhesion as part of an inflammatory response, differentiation, proliferation and apoptosis in hematopoiesis.[26] After ligation, CD13 can function as an enzyme by cleaving the N-terminus of peptides. In this manner, cellular processes as differentiation, motility and proliferation are indirectly regulated. It is hypothesized that (over)expression of CD13 might give neoplastic cells a survival benefit and might reflect a differentiation block in hematopoiesis. Immune dysregulation is part of the pathogenesis of MDS. CD13 expression is enriched specifically on the pro-inflammatory subset of monocytes, suggesting that CD13 may regulate specific subsets of immune cells.[27] It could be hypothesized that a pro-inflammatory subset of monocytes with increased CD13 expression might contribute to immune dysregulation in bone marrow of patients with MDS.[28]

The decreased SSC and increased CD117 expression of myeloid progenitors and decreased CD13 expression on monocytes were most predictive for OS when compared with other flow cytometric variables that were tested. These immunophenotypic variables might identify (clonal) myeloid progenitor cells with proliferative capacity, resulting in an adverse clinical outcome. Furthermore, increased expression of CD13 on monocytes might have a role in maintaining/supporting the neoplastic myeloid progenitor cells.[29]

Diagnostic application of flow cytometry in MDS uses, among others aberrant expression of lymphoid antigens on myeloid progenitors to discriminate from normal, since expression is absent on cells of healthy individuals.[5] Interestingly, aberrant expression of lymphoid antigens such as CD5, CD7, CD19 or CD25 is associated with worse OS if expressed on myeloid progenitors. We have previously reported on the association between presence of aberrant myeloid progenitors and transfusion dependency or unresponsiveness to treatment with azacitidine.[4,14,30] However, these variables did not meet the criteria to enter multivariate analysis in this study.

The IPSS-R offers a refinement of prognostication, especially by the identification of patients with MDS with very good and very poor prognosis. We demonstrated that the MFS might be of additional value to the IPSS-R. The largest proportion of patients with MDS remains within the IPSS-R cytogenetic good risk group. The MFS was able to identify patients within the IPSS-R cytogenetic good risk group with different OS. Similarly, within the well-defined IPSS-R low risk group, to which the majority of patients with MDS belong, a low MFS distinguished patients with better OS compared with patients with a high MFS.

Finally, the MFS was validated in an independent validation cohort, confirming the prognostic impact of the MFS. However, there are differences between the learning and validation cohort with respect to differences in OS as distinguished by the MFS. These differences may be caused by the differences in treatment options between the two cohorts. For example, azacitidine was not yet available in 2005 when the first patients were included in the learning cohort. Furthermore, the follow up time of the learning cohort is longer than the follow up time of the validation cohort. During data collection of the validation cohort, more IPSS-R high and very high patients and patients with very poor risk cytogenetics were included as compared to the learning cohort. We ascribe this to an increased referral from non-academic medical centers of higher risk patients for treatment with azacitidine. Furthermore, the composition of hematopoietic cells in the bone marrow of low risk patients with MDS is different from that of high risk patients with MDS. Therefore, this might also have influenced the distribution of patients in MFS subgroups. As shown here, the MFS can help differentiate within that group. Despite these differences in characteristics of the learning and validation cohort, the MFS was still of prognostic significance in the validation cohort.

It was previously published that the FCSS was able to differentiate prognostic subgroups within the IPSS-R.[6] However, the MFS contains only three variables and would increase the feasibility of implementing flow cytometry for a refined

prognostication of MDS. Recently, a study was published on the prognostic relevance of a four-parameter diagnostic MDS flow cytometric score.[31-34] Interestingly, within the current study, in univariate analysis, the increased percentage of myeloid progenitor cells, decreased B cell progenitors, decreased expression of CD45 on myeloid progenitors and decreased SSC of neutrophils were significantly associated with worse OS. However, after multivariate analysis the cardinal parameters were not the most powerful prognostic parameters for OS. As described above, the percentage of myeloid progenitors were not included in our analysis.
In conclusion, we designed a flow cytometry based scoring model that consists of three variables with a high prognostic impact with respect to OS. The MFS is of added value to current existing (revised) prognostic scoring systems and should be validated in prospective clinical trials for inclusion in future prognostic models for MDS.

Acknowledgements

The authors would like to thank Kelly Schouten and Kristin Vandenberghe (Department of Hematology, Cancer Center Amsterdam (CCA), VU University Medical Center, Amsterdam, The Netherlands) for technical assistance.

Authorship contributions

CA drafted the manuscript, performed and analyzed experiments. TMW performed and analyzed experiments and revised the manuscript and validated the final version of the manuscript. CC performed and analyzed experiments. BIW guided the statistical analysis, 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

There are no relevant conflict of interest to disclose.
Supplementary information is available at Leukemia's website

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Supplementary table 1 Median overall survival by IPSS(-R) and FCSS subgroup for the learning and validation cohort.

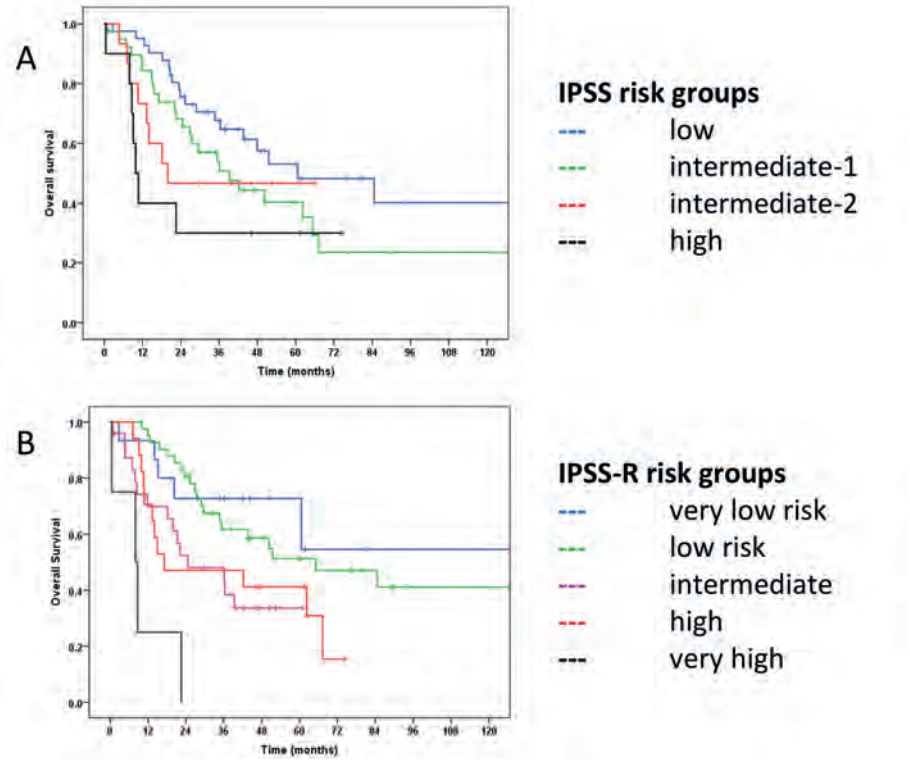
	learning cohort		validation cohort		reference cohort
	n	Median (months)	n	Median (months)	Median (months)
IPSS	106		102		
low	41	60.6	23	45.6	68.4
intermediate-1	40	39.3	44	55.8	42.0
intermediate-2	15	19.9	27	18.8	14.4
high	10	9.9	8	7.5	4.8
IPSS-R	102		101		
very low	15	60.6	11	54.3	105.6
low	41	65.2	23	not reached	63.6
intermediate	25	24.6	18	23.5	36.0
high	17	17.1	12	22.6	19.2
very high	4	8.0	9	9.7	9.6
FCSS	106				
no to minimal (0-1 point)	5	36.3			
mild (2-3 points)	46	65.2			
severe (≥4 points)	55	27.4			

Supplementary table 2 Flow cytometric panel with antibody combinations that were used

	FITC	PE	PerCP	APC
1.	PBS	PBS	CD45	PBS
2.	CD16	CD13	CD45	CD11b
3.	CD34	CD11b	CD45	HLA-DR
4.	CD36	CD33	CD45	CD14
5.	CD36	CD64	CD45	CD14
6.	CD15	CD10	CD45	CD34
7.	CD34	CD117	CD45	CD13+CD33
8.	PBS	PBS	CD45	CD34
9.	CD5	CD19	CD45	CD34
10.	CD2	CD56	CD45	CD34
11.	CD13	CD7	CD45	CD34
12.	CD13	CD25	CD45	CD34
13.	CD34	PBS	CD45	PBS
14.	CD34	CD123	CD45	HLA-DR
15.	CD71	CD235a	CD45	CD117

*For the MFS, CD13-PE and CD117-PE were used.

Supplementary figure 1. Survival by IPSS and IPSS-R risk groups



III

Role of flow cytometry in response prediction and treatment monitoring in myelodysplastic syndromes

8

Aberrant Immunophenotype of Blasts in Myelodysplastic Syndromes is a Clinically Relevant Biomarker in Predicting Response to Growth Factor Treatment

Westers TM, Alhan C, Chamuleau MED, van der Vorst MJDL, Eeltink C, Ossenkoppele GJ, van de Loosdrecht AA

Blood 2010; 115: 1779-1784

Abstract

Myelodysplastic syndromes (MDS) are a group of clonal disorders of the bone marrow characterized by peripheral cytopenias. Standard treatment in low and intermediate risk-I MDS is supportive therapy consisting of regular transfusions and growth factors, i.e. erythropoietin (Epo) and G-CSF. Since flow cytometric analysis of MDS bone marrow samples can identify clinically relevant subgroups regarding transfusion dependency and disease progression, we addressed the question whether flow cytometry (FCM) was instrumental in predicting response to a standardized Epo/G-CSF regimen. In forty-six patients with low and intermediate-I risk MDS that were treated with Epo/G-CSF, low Epo level and low transfusion need were associated with response to Epo/G-CSF. Interestingly, aberrant phenotype of myeloblasts identified non-responders among patients with highest response probability according to the predictive model of Hellström-Lindberg et al. Moreover, aberrant FCM of myeloblasts acted as a significant biomarker for treatment failure in multivariate analysis. A new predictive model based on FCM combined with previously validated Epo levels is proposed defining three subgroups with 94%, 17% and 11% response probability. In conclusion, FCM may add significantly to well-known predictive parameters in selecting MDS patients eligible for Epo/G-CSF treatment. This is of relevance regarding prevention of treatment failure and favoring the choice for alternative therapies.

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders of the bone marrow characterized by peripheral cytopenia in one or more cell lineages. Standard treatment for patients with low and intermediate risk-I MDS is supportive therapy consisting of regular transfusions and growth factors, i.e. erythropoietin (Epo) and human recombinant granulocyte-CSF (G-CSF). Administration of Epo has been shown to ameliorate hemoglobin (Hb) levels and reduce red blood cell (RBC) transfusion requirements in patients suffering from anemia with improvement of quality of life and overall survival.[1;2] Addition of G-CSF has been shown to induce responses in patients resistant to Epo alone.[3-6] Approximately 40-50% of anemic MDS patients show erythroid response to combined administration of Epo and G-CSF.[2;7-11] Response to growth factor treatment can be predicted by a model based on pre-treatment serum Epo levels (<100, 100-500 and >500 U/L) and transfusion need (< or ≥ 2 units per month).[8] This validated scoring system distinguishes three patient groups: one with a high probability of erythroid responses (74%), one intermediate (23%) and one poor (7%).[9]

Recently, it was demonstrated that flow cytometric (FCM) analysis of MDS bone marrow samples adds significantly in the distinction of clinically relevant subgroups in MDS with respect to transfusion dependency and progression of disease.[12-14] Aberrant expression of the lymphoid antigen CD7 on myeloid blasts, for instance, correlates with poor clinical outcome.[13;15;16] Therefore, we addressed the question whether FCM analysis was instrumental in predicting response to a standardized Epo/G-CSF regimen.

Material and Methods

Patient characteristics and routine diagnostics

Forty-six patients with low and intermediate-I risk MDS (median age: 69 years, range: 40-90 years) were enrolled in this study from 2004 until 2007 and followed-up until December 2008. Diagnosis of MDS was made by two experienced hematologists (AAvdL and GJO) according to WHO 2001 classification.[17] Bone marrow samples were evaluated for chromosomal anomalies according to ISCN guidelines.[18] In those cases where no metaphases could be analyzed additional fluorescence in situ hybridization was performed according to recently published recommendations.[19]

Patients were monitored with respect to peripheral blood cell counts, blast counts in bone marrow and occurrence of any event related to MDS, e.g. transfusion requirements. Epo levels were assessed by radioimmunoassay (EPO-Trac™, Antwerp, Belgium). All samples were drawn after written informed consent; the study was approved by the local Medical Ethics Committee. Risk assessment was based on the international prognostic scoring system (IPSS) and WHO classification-based prognostic score system (WPSS).[20-22] Patient characteristics are summarized in table 1.

Table 1. Characteristics of responders and non responders to Epo/G-CSF treatment

	responders	non-responders	P-value‡
number of patients	18	28	
age*	69 (47-87)	68 (40-90)	0.964
gender (m/f)	10 / 8	20 / 8	
WHO(2001)†			0.248
RA(RS)	8 (44%)	10 (36%)	
RCMD(RS)	10 (56%)	16 (57%)	
RAEB-1		1 (3.5%)	
MDS-U		1 (3.5%)	
IPSS†			0.183
low	12 (67%)	13 (46%)	
int-1	6 (33%)	15 (54%)	
WPSS†			<0.001
very low	5 (28%)	0 (0%)	
low	11 (61%)	11 (39%)	
int	2 (11%)	11 (39%)	
high		5 (18%)	
not classifiable		1 (3.5%)	
prior transfusion support (number of patients) †	2 (12%)	18 (62%)	0.010
blast percentage*	1.9 (0.6-4.2)	2.2 (0.5-9.7)	0.257
Hb (mmol/L)*	5.9 (4.8-6.3)	5.1 (4.1-6.6)	0.001
time from diagnosis to study entry (months)*	0 (0-58)	0 (0-78)	0.426
serum Epo at study entry (U/L)*	76 (19-587)	187 (33-6000)	0.001
ferritin at study entry (mg/L)*	576 (47-1992)	786 (47-3543)	0.168
LDH at study entry (U/L)*	341 (192-569)	350 (205-2599)	0.276
aberrant flow cytometry†	2 (11%)	21 (72%)	<0.001

* median and range are depicted; † percentages between brackets depict percentage of cases within responder or non-responder group; ‡ P-values indicate comparison between responders and non-responders; abbreviations: m, male; f, female; MDS, myelodysplastic syndromes; RA, refractory anemia; RCMD, refractory anemia; RS, ring sideroblasts; RAEB-1 or RAEB-2, refractory anemia with excess blasts type 1 or 2; IPSS, international prognostic scoring system; WPSS WHO-based prognostic scoring system; Hb, hemoglobin; Epo, erythropoietin; LDH; lactate dehydrogenase. Note: data of some patients (25/46, i.e. 9 RA(RS), 14 RCMD(RS), 1 RAEB-1 and 1 MDS-U) were used in a previous study.[13]

Treatment

All patients started with Epo if symptomatic at an Hb of less than 10 g/dl (6.2 mmol/L) independent of endogenous erythropoietin level. Epo (NeoRecormon®, Epoetin-beta, Roche Netherlands BV, Woerden, The Netherlands) was started at a dose of 30.000 IU once weekly. In absence of an increase in Hb of at least 1g/dl (0.62 mmol/L) within 6 weeks, Epo dose was escalated to 60.000 IU according to Hellström-Lindberg et al.[4] If still no response was achieved within 12 weeks, G-CSF (Neupogen®, Filgrastim, Amgen BV, Breda, The Netherlands) was added (300-480 µg dependent on weight, 3 times weekly). Dose reduction of G-CSF was performed if leukocyte counts increased above $30x10^9$/L.

Response criteria

Erythroid response was evaluated according to IWG2006 response criteria.[23] In short, erythroid response is defined by an increase of Hb level by more than 1.5 g/dl (0.93 mmol/L) in patients with baseline Hb below 11g/dl (6.8 mmol/L) or a relevant reduction of red blood cell (RBC) transfusions of at least 4 per 8 weeks as compared to number of transfusions in the previous 8 weeks. Only transfusions given for Hb of ≤9.0 g/dl (5.6 mmol/L) were taken into account. Transfusion dependency was evaluated and defined as requirement of 3 units of packed cells per month for at least a period of 4 months. Disease progression was defined as an increase in WHO subgroup to at least RAEB-1 and/or AML within 18 months after diagnosis of MDS. Both time to response and response duration were documented.

Flow cytometric analysis of bone marrow samples

Bone marrow samples drawn at diagnosis were analyzed by four-color flow cytometry (FCM) as previously described.[13] Analysis was performed on total nucleated bone marrow cells; ammonium chloride lysis of erythrocytes was performed prior to the staining procedure as proposed by the European LeukemiaNet Working Party.[24] Monoclonal antibodies used in this study included fluorescein isothiocyanate (FITC) conjugated: CD5 (clone DK23), CD16 (DJ130c) from Dakocytomation Glostrup, Denmark; CD7 (M-T701), CD15 (MMA), CD34 (HPCA2), HLA-DR (L243) from Becton Dickinson (BD), San Jose, CA; 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); peridinin-chlorophyll protein (PerCP) conjugated: CD45 (2D1) from (BD); allophycocyanin (APC) conjugated: CD11b (D12), CD13

(L138), CD14 (MoP9), CD33 (P67.6), CD34 (HPCA2), HLA-DR (L243) from BD and CD117 (104D2) from DakoCytomation as described previously.[13] Samples were analyzed using a FACS Calibur (Becton Dickinson (BD), San Jose, CA); data were analyzed using Cell Quest Software (BD). Different cell compartments were identified using CD45 expression and sideward light scatter (SSC).[24] Main focus was analysis of myeloid blasts; myeloid blasts were defined as $CD45^{dim}SSC^{low/int}$ with expression of CD34 and/or a myeloid marker such as CD13 or CD117; at least 250 events within this compartment were acquired.[13; 24] Upon analysis CD34-APC positive blast cells were back gated in the CD45/SSC plot. Back gating strategies were performed to exclude debris, non viable cells and doublets. This gate was also used to check myeloid commitment using CD117 or CD13, and CD19. Marker expression in a defined subpopulation was determined as compared to isotype controls or unstained cells; based on currently used cut offs in routine immunophenotyping diagnostics of leukemia a cut off of 20% was applied in the evaluation of aberrant marker expression.

Statistical analyses

Comparison between responders and non-responders were statistically tested using Fisher exact test for categorical data and Mann Whitney U test for continuous data; univariate and multivariate regression analyses were performed to analyze value of markers predictive for response (SPSS 15.0 software).

Results

Response to growth factor therapy in IPSS and WPSS risk groups

Most patients were scheduled to Epo 60.000 IU/week (n=44) and 42 patients additionally received G-CSF. G-CSF dose was temporarily reduced in 5 patients. Of all the patients in this study, 39% (18/46) responded to the standardized Epo/G-CSF regimen according to IWG2006 criteria, with a median time to response of 3 months and a median duration of 12 months (range: 3.5-51 months). Four patients became transfusion independent. Disease progression was observed in 9 patients; 2 patients in the responder (11%) and 7 in the non-responder group (25%). Patients with a low risk IPSS showed hematological improvement (HI) in 48% of the cases (12/25, table 1.) and patients with intermediate-1 risk MDS in 29% (6/21). When WPSS was used to classify patients, all of the very low risk patients (WPSS 0, n=5) responded

to treatment, whereas 50% response was seen in low risk patients (WPSS 1, n=22). Only 18% of the intermediate risk patients (WPSS 2, n=13) and none in high risk group (WPSS 3, n=5) were responsive to treatment. In one patient (classified by WHO morphology as MDS-U), WPSS risk group could not be determined.

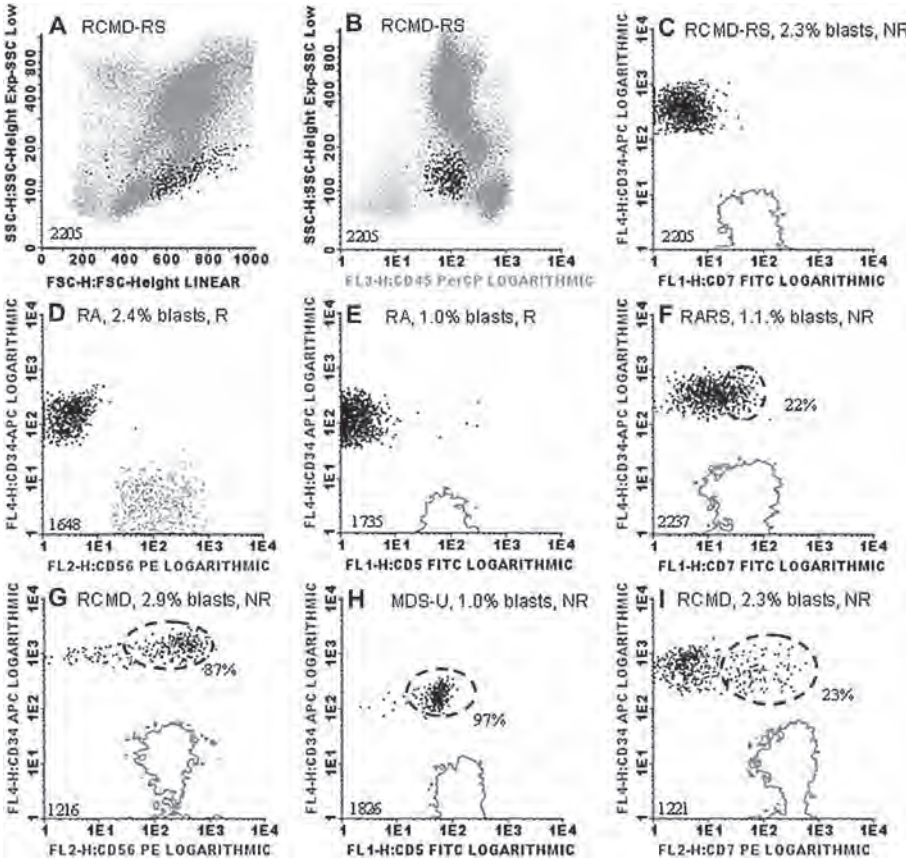


Figure 1. Examples of immunophenotypic analysis of myeloid blasts in low and int-1 risk MDS. In panel A. forward light scatter (FSC) and sideward light scatter (SSC) properties of a bone marrow sample of a MDS patient is depicted (unique patient numbers are depicted in lower left corners). FSC (X axis) and SSC (Y axis) reflect size and granularity, respectively. Panel B. shows CD45 staining (X axis) vs SSC (Y axis) in this patient; CD45 is expressed on all white blood cells (highlighted in dark grey and black). CD34 positive myeloid blasts, characterized by their diminished level of CD45 and low to intermediate SSC are highlighted in black in all panels. In panels C. to I, CD34 is depicted on the Y-axes and lymphoid markers on the X-axes. FL1 (fluorescence 1), FL2, FL3 and FL4 indicate FITC, PE, PerCP and APC conjugated antibodies, respectively. Solid grey lines indicate expression levels of lymphoid markers CD5, CD7 and CD56 in the reference lymphocyte population (X-axes in panel C. to I.). In panel D., the NK cell population was too small to generate a contour line; therefore the reference population is depicted as grey dots. Marker expression was compared to unstained cells or appropriate isotype controls. Dashed black lines are depicted in case more than 20% of myeloid blasts expressed a lymphoid marker (aberrant flow cytometry); percentages of aberrant blasts are indicated as percentage of CD34 positive cells. R: Epo responder, NR: non-responder. Graphs were generated using Infinicyt software (Cytognos, Salamanca, Spain).

Response to growth factor therapy in relation to flow cytometry characteristics

Median blast percentage in patients' bone marrow samples at diagnosis was 2.0% of total white blood cells (range: 0.5-9.7%) as assessed by FCM; no differences in blast percentages were observed between responders and non-responders (table 1, P=0.472). A dense cluster of at least 20% of the myeloid blast fraction (mainly consisting of CD34 positive cells) that showed expression of e.g. lineage infidelity markers was considered as aberrant (figure 1.). Median percentage of aberrant blasts in these cases was 43% (range: 22-97%, supplementary figure S1). Observed immunophenotypic aberrancies[12] on myeloid blasts in this patient group were the expression of a lineage infidelity marker (CD5 (n=1), CD7 (n=15) or CD56 (n=3), CD5 in combination with CD56 (n=1)), loss of CD45 in addition to CD7 expression (n=1) or loss of myeloid antigen CD33 (n=1). Strikingly, aberrancies within the myeloid blast compartment were mainly seen in non-responding patients (21/28, 75%). Only 2 out of 18 patients that responded to treatment (11%) showed an aberrant phenotype (CD7); however, response duration in these particular patients was just 3.5 and 6 months as compared to a median of 12 months in all responders. Overall, 70% of patients (16/23) with normal myeloid blasts by FCM responded to Epo/G-CSF treatment; while 91% of the patients (21/23) with aberrant FCM were non responsive. Thus, aberrant immunophenotype of myeloid blasts is highly associated with treatment failure (P<0.001).

Response to growth factor therapy in relation to endogenous Epo levels

Several studies validated the application of serum Epo levels at diagnosis to predict response to treatment.[9-11] Epo/G-CSF treatment is recommended if Epo levels are below 500 U/L and no or low transfusion need.[25] In line with this, levels of endogenous Epo differed significantly between responders and non-responders within our patient group (table 1., P=0.001). Only 44% of patients (17/39) that had Epo levels below the threshold of 500 U/L responded to treatment, while response at higher Epo levels was only 14% (1/7). In recently published studies, response rates were highest in those patients that had serum Epo level below 200 U/L.[2;11] Other reports state that Epo levels below 100 U/L are indicative of a higher probability of response to Epo/G-CSF.[7;9;26] This cut-off level of 100 U/L appeared to be most discriminatory in our patient group: at an Epo level below 100 U/L, 71% of patients (15/21) were responsive to Epo/G-CSF treatment; while 88% of the patients (22/25) with Epo levels above 100 U/L failed to respond. Thus, Epo levels over 100 U/L are highly associated with treatment failure (P<0.001).

Additional markers in response prediction

Several other parameters are known to influence response to therapy, e.g. longer disease duration prior to Epo/G-CSF treatment affects outcome.[10] Furthermore, patients requiring less than 2 units of RBC per month have a higher probability of response to Epo/G-CSF.[9] Most patients in this study were newly diagnosed as MDS, time to treatment was similar in the responder and non-responder group (P=0.426, table 1), though Hb was significantly lower in non-responders as compared to responders (P =0.001, table 1.). As a result, transfusion dependency before treatment was more frequent among non-responders as compared to responders (P =0.010). Transfusion dependency is often associated with the risk of iron overload which adversely affects survival of the patients.[21;27] Ferritin levels correlated significantly with transfusion need before growth factor treatment (table 1., P =0.004). Of note, only few patients received additional iron chelation therapy.

When according to the current validated predictive model[9] transfusion requirement was taken into account next to Epo levels, response rates were 53% in the group with a high probability to respond (17/32), 10% in the intermediate (1/10) and 0% in the poor probability-to-respond group (0/4). Interestingly, the presence of aberrant myeloid blasts might have identified 11/15 non-responders among those patients that were supposed to have a good probability to respond.

Combination of validated response markers and flow cytometry in response prediction

Since aberrant FCM was significantly associated with treatment failure, it might add to the well known validated predictive response parameters to select those patients that are likely to respond to Epo/G-CSF. As illustrated in table 2., 13/14 patients with low Epo levels and normal FCM responded to therapy. Of note, six patients were non-responders despite their high response probability based on their low Epo level (<100 U/L); five of these patients might have been identified as non-responders based on the presence of aberrant myeloid blasts. Approximately one third of patients responded that had either aberrant FCM and low Epo levels or normal FCM and Epo levels above 100 U/L; while none of the patients that had Epo levels above 100 U/L and aberrant FCM responded to treatment.

Table 2. Epo levels and immunophenotype of myeloid blasts at start of Epo/G-CSF treatment

	Epo < 100U/L		Epo > 100U/L	
	nFCM*	aFCM*	nFCM*	aFCM*
responders	13	2	3	0
non-responders	1	5	6	16
response rate	94%	29%	33%	0%

*nFCM, normal immunophenotype of myeloid blasts; aFCM, aberrant immunophenotype of myeloid blasts

Flow cytometry as biomarker in a new predictive model for response

As aberrant FCM seems to be so strongly associated with treatment failure, a multivariate logistic regression analysis was performed to analyze whether aberrant FCM is an independent predictor of response. All variables with a P value less than 0.1 in univariate analysis (data not shown) were included in the multivariate analysis. Despite significance in the univariate analysis, Hb level and WPSS were not included in the multivariate analysis since Hb level is biased by pre-treatment transfusions and WPSS evaluates transfusion dependency. Transfusion dependency was included in the analysis. Epo levels were logarithmically transformed because of non Gaussian distribution. In the final multivariate model aberrant FCM, Epo level and transfusion requirement before treatment were entered. In our cohort, only aberrant FCM and Epo levels were significant predictors of response to Epo/G-CSF treatment (table 3.). When Epo levels were grouped according to the model of Hellström-Lindberg et al.[9], probability to respond was 37 times less in case of aberrant FCM and approximately 10 times less in case of higher Epo levels (P =0.001, Odds ratio 0.027 and P =0.003, Odds ratio 0.099, respectively).

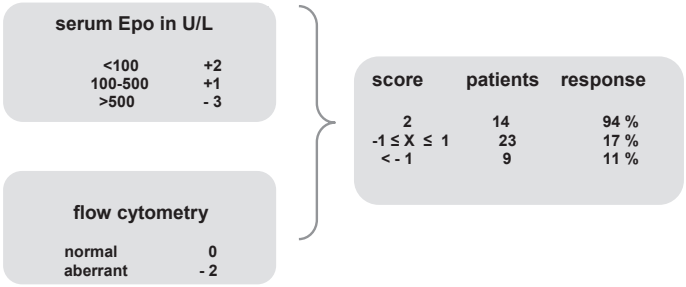


Figure 2. Endogenous Epo levels and flow cytometry of myeloid blasts as biomarkers in the prediction of response to Epo/G-CSF treatment in low/int-1 risk MDS. Points granted for Epo level in this model are exactly as in the validated model of Hellström-Lindberg et al.9 Normal and aberrant FCM score 0 and -2 points, respectively. Applying this new model defines three subgroups with 94%, 17% and 11% probability to respond to growth factor treatment.

Table 3. Multivariate logistic regression analysis of prediction of response to Epo/G-CSF

	P-value	Odds ratio	95% CI*
aberrant flow cytometry	0.001	0.035	0.005 – 0.274
serum Epo at study entry	0.019	0.245	0.076 – 0.795
pretreatment RBC transfusions	0.291	0.294	0.030 – 2.850

* CI, confidence interval

Based on our data, we propose a new predictive model (figure 2.); this model is solely based on previously validated Epo thresholds[9] in combination with normal or aberrant FCM of myeloid blasts. Score for Epo levels was applied according to the model of Hellström-Lindberg et al.; i.e. <100 U/L: 2 points, 100-500 U/L: 1 point and >500 U/L: -3 points. Regression analysis of FCM against Epo at a cut off of 100 U/L revealed approximately the same regression coefficients, P-values and Odds ratios (data not shown); therefore, same weight of points was applied: in case of aberrant FCM 2 points were subtracted, no points were added in case of normal FCM. Application of the proposed model distinguishes three subgroups with 94%, 17% and 11% probability to respond to a standardized regimen of Epo/G-CSF. The first subgroup concerned patients with normal FCM and Epo levels below 100 U/L (n=14); the second subgroup had Epo levels below 100 U/L in combination with aberrant FCM or Epo levels between 100 and 500 U/L with either normal or aberrant FCM (n=20); the third subgroup had highest Epo levels, most of them with aberrant FCM (n=4). Median response duration in the first subgroup was 14.5 months as compared to 5 months in the other subgroups.

Discussion

Flow cytometric analysis of MDS bone marrow samples was shown to add significantly in the recognition of clinically relevant subgroups with respect to transfusion dependency and progressive disease.[13] In this study, we showed that immunophenotypic analysis of myeloid blasts is also instrumental in predicting response to a standardized Epo/G-CSF regimen in low and int-1 risk MDS. In fact, aberrant immunophenotype of myeloid blasts was highly associated with treatment failure. The most common aberrancy was expression of lymphoid antigen CD7; a marker known to be associated with poor clinical outcome.[13;15;16]

Most responders had low Epo level and low transfusion requirement, hallmarks of the current validated predictive model.[9] Interestingly, flow cytometry (FCM) could identify non-responders among those patients that should have a good probability to respond according to the latter model. Multivariate regression analysis identified aberrant FCM of myeloid blasts and increased Epo level as significant biomarkers for prediction of treatment failure. Therefore, a new predictive model based on FCM and previously validated Epo levels was proposed (figure 2). By applying this model, we were able to identify three subgroups with 94%, 17% and 11% probability to respond. The first subgroup concerned 14 patients with normal FCM and Epo levels below 100 U/L; the second subgroup of 23 patients had Epo levels below 100 U/L in combination with aberrant FCM or Epo levels between 100 and 500 U/L with either normal or aberrant FCM; the third subgroup of 9 patients had highest Epo levels, most of them with aberrant FCM. Of note, a cut off of 20% of aberrant marker expression, common in routine diagnostics, was used. Smaller percentages of aberrant blast cells might be of importance, since it can not be excluded that these represent a dysplastic or pre-leukemic clone; this is being evaluated by regularly monitoring bone marrow samples.

Based on recent studies, low and int-1 risk MDS patients that are responsive to treatment with Epo/G-CSF might benefit from an increased overall survival (OS).[2;10;11] Whether or not duration of response or time to response is predictive for this OS benefit is not yet elucidated. From our data we hypothesize that 94% of patients with a predictive score of 2 are highly likely to respond to Epo/G-CSF thereby selecting patients who might benefit from a prolonged OS. On the other hand, patients with a very low probability to respond to treatment should not be selected for Epo/G-CSF treatment. Patients with an intermediate score might be selected for Epo/G-CSF for a maximum of 6 months. Our data demonstrated that median response duration in these patients was only 5.5 months (range 3.5-6 months) as compared to a median of 14.5 months (range 6-51 months) in those patients with a high probability to respond. Our proposed model might have major financial impact, since it can identify patients less likely to respond to an expensive Epo/G-CSF regimen superior to the current predictive model.

In MDS bone marrow residual normal and dysplastic hematopoiesis coexist. A preferential outgrowth of normal progenitors might correlate with response to treatment.[28] So, next to the value of FCM in predicting response to growth factor treatment, FCM might contribute to the management of low/int-1 risk MDS patients. Changes in immunophenotypic aberrancies over time might provide information on

response to treatment or further progression of disease, especially when no other disease parameters such as molecular and cytogenetic parameters are available.[29] Preliminary data indicate that immunophenotypic MDS-related abnormalities in bone marrow cells are no longer detectable or decrease in number in responding patients when compared to pre-treatment analysis.[30]

To conclude, our data underscore observations that Epo/G-CSF is an effective first line treatment regimen in a subgroup of patients with low and int-1 risk MDS with a low serum Epo level and low transfusion need. Notably, aberrant immunophenotype of myeloid blasts acted as a significant and cost-effective biomarker for response to Epo/G-CSF treatment in combination with Epo levels at validated thresholds. Importantly, FCM analysis of myeloid blasts in MDS bone marrow is a relatively easy technique for any laboratory with experience in analysis of leukemia samples and monitoring minimal residual disease. Thus, FCM may add significantly to the well known validated predictive parameters in the selection of patients likely to respond to Epo/G-CSF. In patients with MDS with an intermediate or poor response probability to Epo/G-CSF, one might consider alternative treatment strategies. Prospective studies are currently being conducted to validate the role of FCM in the diagnosis, prognostication and disease monitoring of low/int-1 risk MDS during Epo/G-CSF and new emerging drugs such as lenalidomide and 5-azacitidine.

Acknowledgements

The authors thank Claudia Cali, Linda van Dreunen and Yvonne van der Veeken for technical assistance, Hans Berkhof (Department of Epidemiology and Biostatistics, VUMC, Amsterdam) for statistical assistance, and Drs van Maanen (Westfries Gasthuis, Hoorn, The Netherlands), Timmers (Ziekenhuis Amstelveen, The Netherlands) and van der Linden (Kennemer Gasthuis, Haarlem, The Netherlands) for their contribution to patient accrual. This study was supported (in part) by a Young Investigators grant (2007-2008) from the Myelodysplastic Syndrome Foundation Inc., USA to AAvdL and an unrestricted educational grant of Roche BV, Woerden, The Netherlands. Authors state that there are no conflicts of interest to disclose.

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Absence of aberrant myeloid progenitors by flow cytometry is associated with favorable response to azacitidine in higher risk myelodysplastic syndromes

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Cytometry B Clinical Cytometry 2014; 86: 207-215

Abstract

Background: In intermediate-2 and high risk patients with myelodysplastic syndromes (MDS), treatment with azacitidine is associated with hematological responses and prolonged overall survival in patients who respond to therapy. However, only half of the patients that are treated will benefit from this treatment. It is a major challenge to predict which patients are likely to respond to treatment. The aim of this study was to investigate the predictive value of immunophenotyping for response to treatment with azacitidine of Int-2 and high risk MDS patients.

Methods: Bone marrow aspirates were analyzed by flow cytometry in 42 patients with Int-2 and high risk MDS, chronic myelomonocytic leukemia or low blast count acute myeloid leukemia before treatment and after every third cycle of azacitidine. A flow score was calculated using the flow cytometric scoring system (FCSS).

Results: The presence of myeloid progenitors with an aberrant immunophenotype was significantly associated with lack of response ($p=0.02$). A low pretreatment FCSS was associated with significantly better overall survival compared with a high pretreatment FCSS ($p=0.03$). A significant decrease in FCSS was observed in patients with complete response after three cycles azacitidine compared to patients with progressive disease ($p=0.006$).

Conclusions: Absence of aberrant myeloid progenitor cells at baseline and/or a decrease in the FCSS during treatment identified Int-2 and high risk MDS patients who are likely to respond to treatment with azacitidine.

Introduction

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of bone marrow (BM) disorders characterized by ineffective hematopoiesis resulting in peripheral cytopenias and increased risk of acute myeloid leukemia (AML). The WHO classification, International Prognostic Scoring System (IPSS) and the revised IPSS (IPSS-R) provide prognostic information in MDS patients (1). By using IPSS, MDS patients can be divided into four risk groups with respect to survival and progression to AML: low, intermediate-1 (Int-1), intermediate-2 (Int-2) and high risk (2). Approximately 29% of newly diagnosed MDS cases are Int-2 and high risk MDS (3). Intermediate-2 and high risk patients may benefit from intensive treatment approaches. At present, the only curative treatment is allogeneic stem cell transplantation. However, the majority of MDS patients are not eligible for intensive treatment schedules due to increased age in combination with co-morbidities (4). Low intensity treatment regimens are currently available for these patients. Azacitidine has been approved for treatment of Int-2 and high risk MDS, CMML and AML patients with 20-30% blasts. Recently, prognostic scores were designed for MDS patients treated with azacitidine, using platelet doubling, defined as an at least two-fold increase in platelet count after the first cycle of azacitidine or performance status, cytogenetics, the presence of circulating blasts and red blood cell transfusion dependency as components of the score (5,6). Lower response rates were found in patients with abnormal karyotype and >15% BM blasts. Previously, it was shown that a flow cytometric scoring system (FCSS) was able to estimate survival and relapse after allogeneic hematopoietic stem cell transplantation in patients with MDS (7,8). The FCSS is a scoring system that allows for a numerical display of immunophenotypic aberrancies in the (im)mature myelomonocytic lineage in the BM (7). Scores are generated by weighed scoring for the number of abnormalities in the myelomonocytic compartment and for the percentage of progenitor cells; i.e. high scores reflect high number of aberrancies and/or high percentages of progenitor cells. Furthermore, flow cytometric analysis of BM cells in low and Int-1 risk MDS is instrumental to identify subgroups with distinct clinical behavior regarding transfusion dependency and progression (9). A clinical decision model was designed using presence of aberrant myeloid progenitors determined by flow cytometry (FC) in BM in combination with endogenous erythropoietin (EPO) levels to predict response to growth factor treatment (10). Low and Int-1 risk MDS patients with

normal myeloid progenitors and low endogenous EPO have the highest probability of responding to growth factor treatment.
In this study, we aimed to investigate the role of FC in prediction of response and treatment monitoring of Int-2 and high risk MDS patients treated with azacitidine.

Material and Methods

Patient selection

Patients were eligible for treatment with azacitidine if diagnosed with MDS, MDS/myeloproliferative neoplasm (MPN), CMML or AML with 20-30% blasts and not eligible for standard induction chemotherapy and/or allogeneic stem cell transplantation regimens. Since azacitidine is also approved for patients with CMML and AML with 20-30% blasts, these patients were also included in the study. However, the study describes a series of patients that is enriched for Int-2 and high risk MDS. From 2009 to 2012, 42 patients (28 male/14 female; median age 71 years (range 56-82)) were enrolled in this retrospective cohort study. Diagnosis were made according to WHO2008 classification (11). MDS patients were assigned into prognostic groups by applying the IPSS and an adjusted IPSS with extra weight to poor risk cytogenetics (2,12). Bone marrow samples were evaluated for chromosomal anomalies according to International System for Human Cytogenetic Nomenclature guidelines (13). In those cases where no metaphases could be analyzed, additional fluorescence in situ hybridization (FISH) was performed as recommended (14).
All samples were drawn after informed consent and in conformance with the Declaration of Helsinki. The study was approved by the Institutional Medical Ethics Committee of VU University Medical Center, Amsterdam, Netherlands.

Treatment

All patients were treated according to standard protocols with azacitidine (75 mg/m2/day), administered subcutaneously during the first seven days of a 28 day cycle. Dose modifications were applied according to standard guidelines.

Response criteria

Response was evaluated according to IWG2006 criteria (15). Responses were categorized according to hematologic improvement and disease status. Hematologic improvement was further categorized into erythroid (HI-E), platelet (HI-P) and

neutrophil response (HI-N), according to IWG2000 criteria to investigate whether the degree of hematologic improvement was of relevance (16). Disease status comprised patients with complete remission (CR), stable disease (SD) and progressive disease (PD).

Flow cytometric analysis of bone marrow samples

Immunophenotypic analysis was performed using four-color FC. Bone marrow samples for FC were drawn at baseline, after every third cycle or in case of suspicion of disease progression. Analysis was performed on total nucleated BM cells after ammonium chloride lysis of erythrocytes as proposed by the European LeukemiaNet Working party (17,18). All samples were processed and analyzed within 24 hours. 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); 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), CD25 (ACT-1), 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. Measurements were performed on a FACS Calibur and data were analyzed by Cell Quest Pro Software (BD Biosciences).

Cell populations of interest were selected by sideward light scatter (SSC) and CD45 properties. Mature myeloid cells were defined as CD45dim(inished) and SSChigh. Monocytes were identified by CD45bright and SSCintermediate in combination with CD14 or CD33bright expression. Myeloid progenitor cells were defined as CD45dim, SSClow in combination with CD34 and/or expression of a myeloid marker such as CD13 and/or CD117 (17,19). B cell progenitors were discriminated from myeloid progenitors by lower CD45, lower SSC properties and back gating with CD19 and were excluded from myeloid progenitor analysis (17,18). A minimum number of 250 events within the myeloid progenitor compartment was measured. The myeloid progenitors were considered as positive for asynchronous or lineage infidelity marker expression (LIM) if there was a cluster of ≥20% of cells with expression of CD11b, CD5, CD19, CD56 and/or CD25 based on cut-off values

in routine immunophenotyping diagnostics of leukemia (20). Aberrant expression of CD7 was assessed in the context of CD13 expression. In normal differentiating hematopoietic cells, CD7 can be expressed on CD34posCD13dim cells. Abnormal expression of CD7 on myeloid progenitors can be distinguished from normal expression by quantifying CD7 on CD13bright cells. If CD7 was present on 10% or more of CD13 pos-bright myeloid progenitors, the myeloid progenitors were regarded positive for this aberrant marker.

Bone marrow aspirates of 16 age-matched healthy volunteers and patients undergoing cardiothoracic surgery (median age 65, range 45-79) were used as a reference (after informed consent). Aberrant expression of a marker was defined as ≥ two standard deviations increase or decrease compared with the mean expression level in the age-matched control group.

The observed number of aberrancies in the maturing myelomonocytic compartment and the percentage of myeloid progenitors were transformed into a weighed flow score using the FCSS as described previously (7). In short, for aberrancies in the differentiation of maturing myelomonocytic cells a maximum of five points can be scored. Additionally, the percentage of myeloid progenitors is scored for in a weighted manner, to a maximum of four points. In 14 patients, fresh BM samples were not available for flow cytometric analysis at baseline and after three cycles of azacitidine. In these cases, BM mononuclear cells that were frozen after density gradient centrifugation were analyzed. The FCSS could not be calculated in these cases because mature myeloid cells are removed by the latter procedure. Therefore, solely analysis of aberrant marker expression on myeloid progenitors was performed. Myeloid progenitors after density gradient centrifugation were not used for quantification of myeloid progenitors because the proportions are not comparable with whole BM samples. Previously, it was shown that aberrant marker expression on myeloid progenitor cells from fresh samples is comparable with that of cells that have been frozen and thawed (21). In two patients the baseline FCSS could not be calculated because of unsuccessful BM aspiration. The circulating myeloid progenitors in the peripheral blood were analyzed for aberrancies in these cases.

The flow cytometric approach for prognostication and prediction of response to treatment was compared with a clinical prognostic scoring system designed for patients treated with azacitidine (5). The scoring includes four clinical factors: the Eastern Cooperative Oncology Group (ECOG) performance status, presence of circulating blasts, red blood cell transfusion dependency and cytogenetics.

Statistical analysis

The relationship between response and flow cytometric aberrancies was tested using the Mann-Whitney U test for continuous data and Fisher's exact test for categorical data. Differences in overall survival (OS) were assessed by Kaplan-Meier analysis and significance by using log-rank testing. The OS was defined as time from start of treatment with azacitidine until death or for patients who were alive at time of data analysis, until the date of last visit. Statistical calculations were performed by SPSS 15.0 (SPSS, Chicago, IL). A P-value lower than 0.05 was regarded significant.

Results

Patient characteristics

The diagnoses according to WHO 2008 classification were RCMD (n=4), RAEB (n=21), AML with ≤30% blasts (n=12), MDS/MPN (n=1) and CMML (n=4) (Table 1). The risk categories according to the IPSS were Int-1 (n=4), Int-2 (n=17) and high (n=16). In total, 45% (17/38) of patients with known cytogenetics had normal karyotype. Median follow up after initiation of the first cycle of azacitidine was 10.1 months (range 1.0-28.5). Median number of cycles received was 6 (range 1-28). At time of data analysis, 30/42 (71.4%) patients had discontinued treatment. Four patients discontinued treatment due to hematological toxicity, one patient decided to stop treatment after one cycle; in three patients, treatment was stopped at physician's discretion and one patient received allogeneic stem cell transplantation after three cycles. Overall, 19 patients stopped because of PD after median follow up of 8.9 months (range 1.0-26.8). At time of data analysis, 18 patients were deceased. One patient died of progression of a non-hematological malignancy after 9.6 months and was excluded for OS analyses.

Table 1. Patient characteristics

WHO2008 classification diagnosis	n=	%
RCMD	4	10
RAEB-1	6	14
RAEB-2	15	36
AML (≤ 30% blasts)	12	29
MDS/MPN	1	2
CMML-1	3	7
CMML-2	1	2
IPSS*		
Intermediate-1	4	11
Intermediate-2	17	46
high	16	43
Cytogenetic category°		
good	17	46
intermediate	7	19
poor	9	24
not available	4	11
Response**		
complete response	6	15
stable disease	17	44
progressive disease	16	41

WHO World Health Organization; IPSS international prognostic scoring system; FCSS flow cytometric scoring system; MPs myeloid progenitors; RCMD refractory cytopenia with multilineage dysplasia; RAEB refractory anemia with excess blasts; AML acute myeloid leukemia; MDS/MPN myelodysplastic/myeloproliferative overlap syndrome; CMML chronic myelomonocytic leukemia; *In cases where cytogenetic analysis was not available, the minimum IPSS category is given. ° Cytogenetic category according to the IPSS classification. The karyotype of the patient with MDS/MPN patient and one patient with CMML-1 was normal. There were two patients with CMML-1 and one with CMML-2 with a non-complex karyotype. ** Response could not be evaluated in these patients because of follow up time less than two months.

Response to treatment with azacitidine

According to IWG2006 response criteria for MDS, six patients showed CR, 17 patients had SD and 16 patients had PD; three patients were excluded because response could not be evaluated because these patients received less than three cycles.

Overall survival in patients with CR or SD was significantly better compared with patients with PD (median OS not reached vs. median 9.8 months [range 1.0-26.8], $p < 0.001$, respectively).

No significant difference was observed in HI between the group with known karyotype (n=31) with a normal karyotype 10/15 (67%) and patients with karyotypic abnormalities 7/16 patients (44%), including complex karyotypes (≥3 aberrations, p=0.2).
Erythroid response was achieved in 13/34 patients, of which 9 patients achieved major HI-E after median of two cycles (range 2-4) and minor HI-E was observed in four patients after median number of 3.5 cycles azacitidine (range 3-4).
Platelet response was achieved in 12/32 patients, of which 11 had major response and one patient had minor response. Median number of cycles to achieve HI-P was 2.5 (range 1-5). Major HI-N was achieved in 5/21 patients and minor HI-N was present in one patient after a median number of three cycles (range 2-4).
HI-E, HI-P and/or HI-N could not be evaluated in cases where pretreatment peripheral blood values were either above reference values or patients were transfusion independent (14).
Any HI was seen in 54% of patients (19/35) (after median of two cycles (range 1-4). Median response duration of patients who showed any HI was 8 months (range 3-28.5). The majority of patients (11/18, 61%) that showed HI, had combined HI-E, HI-N and/or HI-P. Nine patients with initial HI lost response or progressed after median time of 6.9 months (range 4-14) and median number of 9 cycles (range 5-15).
The clinical prediction model for response to treatment with azacitidine, as proposed by Itzykson et al., which could be calculated for a subgroup of patients (n=23) due to availability of the parameters, was not able to identify patients who responded to treatment in our cohort (5).

Absence of aberrant myeloid progenitors is associated with hematologic improvement in patients treated with azacitidine

Median percentage of myeloid progenitors by FC, defined as CD45dim, SSClow in combination with CD34 and/or expression of a myeloid marker such as CD13 and/or CD117, was 8.9% (range 1.1%-54%) (17,19). Of note, progenitor counts as detected by FC correlate with morphologic blast count, but do not necessarily generate the same percentage. Median percentage of myeloid progenitors as detected by FC did not differ between patients with PD, SD and CR. At baseline, 64% (27/42) of patients had LIM expression on myeloid progenitors such as CD5 or CD7; in 77% (26/34) of patients aberrant over or under expression of myeloid markers such as CD13, CD34, CD45, CD117, and/or HLA-DR was observed. Overall, 79%

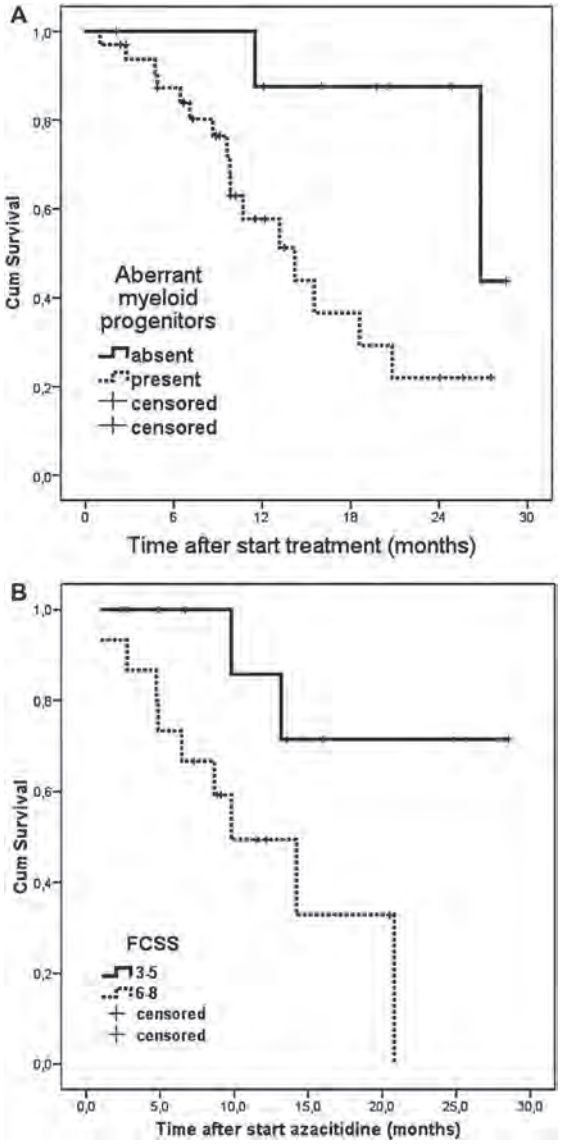

Figure 1. Absence of immunophenotypically aberrant myeloid progenitors and a pretreatment low FCSS is associated with better overall survival in patients treated with azacitidine. Kaplan-Meier plot (A) of patients treated with azacitidine with (n=33) or without (n=9) aberrant myeloid progenitors at baseline. Patients without aberrant myeloid progenitors at baseline had a significantly better overall survival (full line, median survival= 26.8 months) compared with patients with aberrant myeloid progenitors, (dotted line, median survival= 14.2 months), Log-rank test p=0.03. Kaplan-Meier plot (B) of patients treated with azacitidine with a pretreatment FCSS of 3-5 (n=11) and patients with a FCSS of 6-8 (n=15). Patients with a FCSS of 3-5 at baseline had a significantly better survival, (full line, median survival= not reached) compared with patients with a FCSS of 6-8, (dotted line, median survival= 9.8 months), Log-rank test p=0.03.

(33/42) of the patients had aberrant myeloid progenitors defined as either LIM or aberrant expression of lineage-associated markers. As noted above, at baseline the percentage of myeloid progenitors by FC did not differ between response groups. In contrast, aberrant myeloid progenitors were significantly more frequent in those patients with SD and PD. In the CR group, only 33% (2/6) had aberrant myeloid progenitors, compared with 88% (15/17) in SD group and 88% (14/16) in the PD group (Fisher's exact test, p=0.01). Furthermore, absence of aberrant myeloid progenitors was significantly associated with achievement of any major HI (Fisher's exact test, p=0.02). Interestingly, although 10 patients with aberrant myeloid progenitors had major HI, there was a trend towards shorter response duration compared with patients without aberrant myeloid progenitors (median response duration 6.5 months (range 3-12.4) vs. 13.2 months (range 4-28.5), respectively, p=0.08). These data indicate that immunophenotype of myeloid progenitors is more informative in response prediction than percentage of myeloid progenitors.
Patients with aberrant myeloid progenitors received a median of six cycles azacitidine (range 1-16) and had median survival of 14.2 months after initiation of treatment. In contrast, patients without aberrant marker expression received a median of 12 cycles (range 2-28) and had median survival of 26.8 months (Fig. 1A). Patients with aberrant myeloid progenitors received less cycles because of disease progression and/or death. In other words, patients who received less cycles because of disease progression or death were mainly found in the group with aberrant myeloid progenitors. Thus, OS of patients without aberrant myeloid progenitors was significantly better compared with patients with aberrant myeloid progenitors.

Interestingly, loss of aberrant myeloid progenitors during treatment with azacitidine was associated with response to treatment in contrast to persisting LIM expression on myeloid progenitors in patients that did not respond. Figure 2 shows an example of this finding in one patient with loss of aberrant myeloid progenitors and concomitant response to azacitidine, in contrast to a patient with persisting aberrant myeloid progenitors and lack of response.

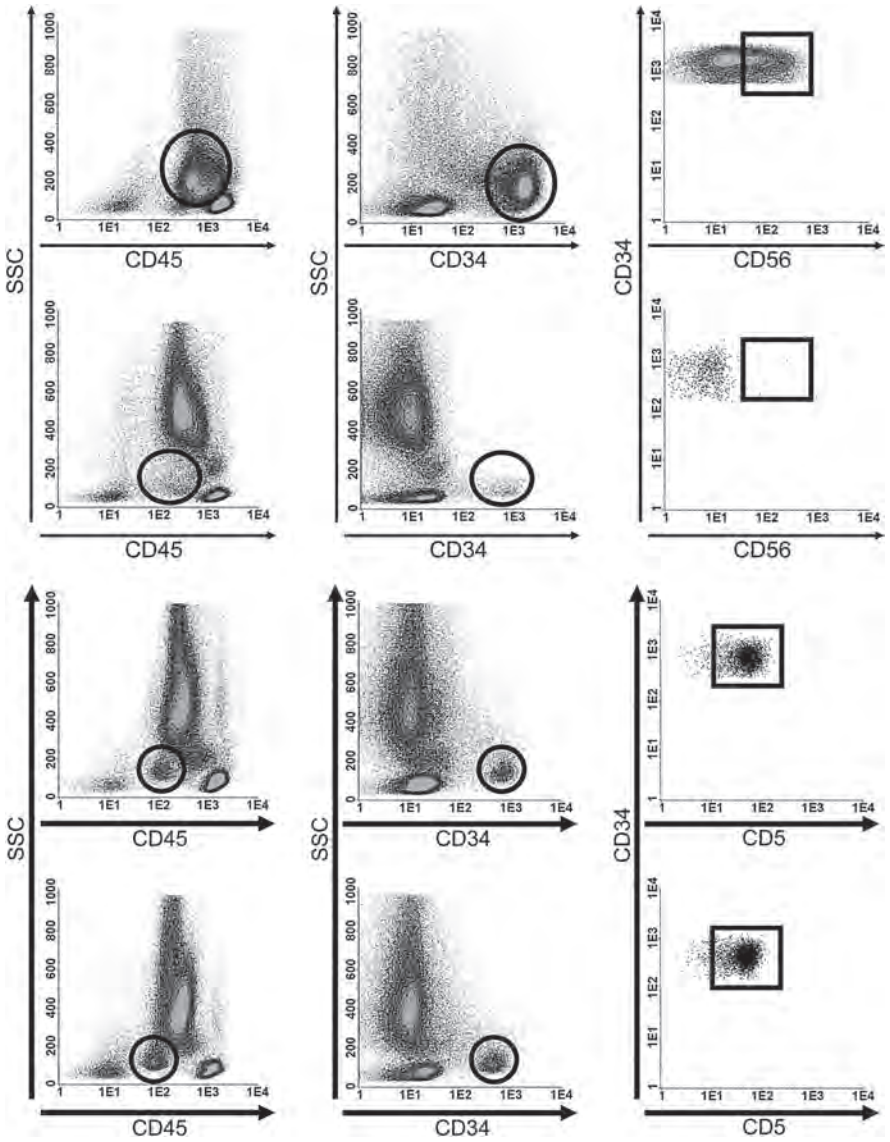

Figure 2. Disappearance or persistence of myeloid progenitors with aberrant immunophenotype corresponds with the nature of response to azacitidine. In the CD45 vs. SSC (sideward light scatter) graphs the myeloid progenitors can be identified by CD45dim, SSClow characteristics (encircled). To assess aberrancies, the myeloid progenitors in these examples are gated by using CD34 (CD34 vs. SSC graphs). In the upper row, a patient (A) with complete response during treatment had an aberrant expression of CD56 (X-axis) on the myeloid progenitors at baseline (indicated by squares, defined as CD45dim, SSClow and CD34pos). After six cycles of azacitidine, these aberrant myeloid progenitors could not be detected (second row). In the third row, at baseline, a patient (B) with stable disease during treatment had aberrant expression of CD5 (X-axis) on the myeloid progenitors at baseline (indicated by squares, defined as CD45dim, SSClow and CD34pos). After six cycles of azacitidine the aberrant myeloid progenitors were still present (fourth row).

Decrease in FCSS during treatment with azacitidine is associated with response

Cumulative aberrancies in the myelomonocytic compartment as assessed by FC are reflected in the FCSS. The baseline FCSS was not significantly different between patients achieving CR, SD and PD (median FCSS=5.5, range 3-8, median FCSS=4, range 2-8, median FCSS=7, range 5-8, respectively).

Patients who achieved CR showed a significant decrease in the FCSS after three cycles of azacitidine compared with patients with PD (median FCSS=1.5, range 1-3 and median FCSS=6.5, range 3-8, respectively p=0.004) (Fig. 3). Decrease in FCSS was sustained after six cycles azacitidine in patients with CR.

Median FCSS for patients with SD was decreased after three cycles (FCSS=4, range 1-6, p=0.03, compared with patients with PD and not significantly different from patients with CR) (Fig. 3). Decrease in FCSS could not only be assigned to a decrease in the percentage of myeloid progenitors; a decrease in the number of aberrancies in the differentiation of myeloid and monocytic cells was also observed.

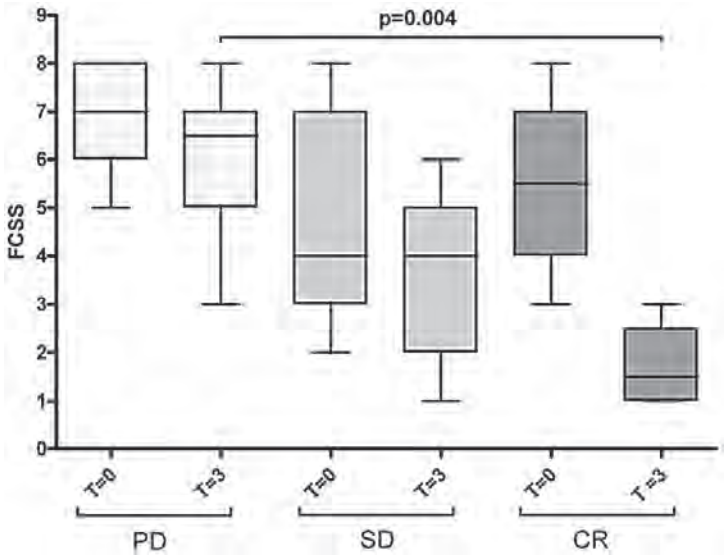

Figure 3. Decrease of the FCSS correlates with response to azacitidine treatment. The FCSS represents a weighed score for aberrancies of myelomonocytic cells and the percentage of myeloid progenitors: higher scores indicate a higher degree of disruption of hematopoiesis.6 The FCSS was measured at baseline and after every third cycle. The FCSS at baseline was comparable in the response categories progressive disease (PD, light grey boxes, n=9), stable disease (SD, grey boxes, n=10) and complete response (CR, dark grey boxes, n=4). The response criteria were based on IWG2006 criteria.12 The FCSS is significantly decreased in patients with CR compared with patients with PD after 3 cycles of azacitidine (p=0.004).

There was an association between occurrence of HI-E, HI-P and decrease of FCSS upon azacitidine treatment (Fig. 4). This is remarkable, since the FCSS only takes dyspoiesis of myeloid cells into account. The majority of patients with HI-E (7/9, p=0.01) and HI-P (6/8, p=0.02) had improvement of FCSS upon treatment. Furthermore, patients with HI-N, all showed decrease in the number of aberrancies in the mature myeloid compartment (data not shown). Overall, this indicates that HI-N is reflected by improvement of neutrophil dyspoiesis as detected by FC.

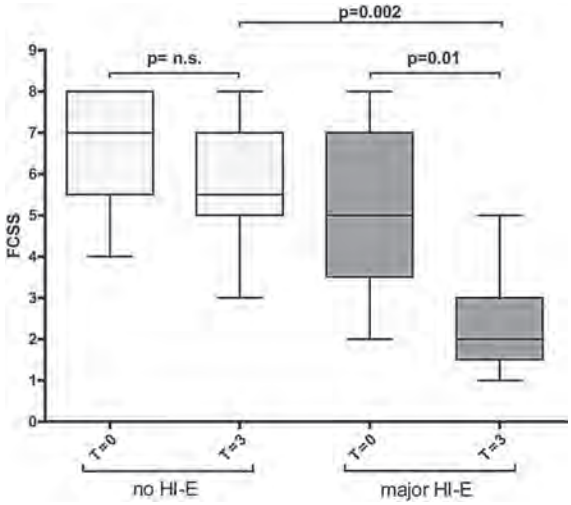

Figure 4. Changes in the FCSS correlate with erythroid response in patients with MDS treated with azacitidine. The FCSS was measured at baseline and after every third cycle.6 The FCSS at baseline was comparable for patients without (no HI-E, light grey boxes, n=10), with minor (minor HI-E, not depicted in figure, n=1) and major (major HI-E, dark grey boxes, n=8) erythroid response according to IWG2006 response criteria.12 The FCSS is significantly decreased after three cycles of azacitidine in patients with major erythroid response compared with patients without erythroid response, p=0.002. One patient with minor HI-E is not depicted in the figure. The FCSS was three at baseline and four after three cycles of azacitidine. n.s. not significant

High pretreatment FCSS is associated with worse overall survival in patients treated with azacitidine

All BM samples were analyzed by FC at baseline and after every third cycle. Median FCSS at baseline was six (range 2-8). No difference in FCSS was observed between IPSS Int-2 and high risk groups (median FCSS=5 (range 3-8) and 7 (range 4-8), respectively) and between IPSS cytogenetic risk categories good, intermediate and poor. The FCSS did not differ significantly between adjusted IPSS cytogenetic risk groups (12). Among patients with baseline FCSS of 2-5, OS was significantly better compared with patients with pre-treatment FCSS of 6-8, p=0.03 (Fig. 1B).

Discussion

Myelodysplastic syndromes are heterogeneous with respect to clinical course and response to treatment. It was shown previously that aberrancies as detected by FC can be instrumental in prognostication (9, 22) Furthermore, FC can be applied for clinical decisions in low and Int-1 risk MDS patients by identification of patients that are most likely to respond to growth factor treatment (10). Therefore, we studied the clinical relevance of FC for prediction of response to azacitidine in Int-2 and high risk MDS. Absence of aberrant myeloid progenitors was significantly associated with favorable response to treatment with azacitidine. In our patient group, 79% had myeloid progenitors with aberrant immunophenotype. Moreover, there was a significant association between absence of aberrant immunophenotype and achievement of CR. Furthermore, presence of aberrant myeloid progenitors was significantly associated with lower probability of achieving long-term major HI-E. These findings are in line with data reported on low and Int-1 risk MDS patients (10). In this study, the absence of aberrant myeloid progenitors was predictive for HI-E to growth factor treatment. Remarkably, although a proportion of patients with aberrant myeloid progenitors had major HI-E, response duration of these patients tended to be shorter than that of patients without aberrant myeloid progenitors. An association between abnormal karyotype and lower response rates and complex karyotypic abnormalities with shorter response rates was described by Itzykson et al (5). However, in our group abnormal cytogenetics show a trend but did not significantly correlate with absence of response to azacitidine.

The rate of any HI was 54% in our patient group and majority of patients had combined HI-E, HI-P and/or HI-N (61%). These response rates are in line with published data (23-25). As described earlier, the number of cycles to obtain response varies from five to nine (26,27). The FCSS was significantly decreased after three cycles azacitidine in patients with CR compared with PD, while patients with SD or PD did not show significant change in FCSS after three cycles. Decrease of FCSS in responsive patients was not only caused by decrease in percentage of myeloid progenitors but also by a qualitative improvement of dyspoiesis in maturing myelomonocytic cells. Majority of patients with HI-E and HI-P had significant decrease of dysmyelopoiesis as reflected by an improvement of the FCSS. This is of interest, since erythroid and megakaryocytic cell analysis is not included in FCSS. This might indicate that in MDS, in which a common hematopoietic progenitor cell is affected, ineffective hematopoiesis is restored upon treatment with azacitidine in responsive patients.

Significantly worse OS was found in patients with high pretreatment FCSS (6-8) and/or myeloid progenitors with aberrant immunophenotype. This might be a reflection of the degree of disruption of normal hematopoiesis. These findings are in line with previously published studies; which showed that flow cytometric scores, including the presence of aberrant myeloid progenitors is associated with disease progression, shorter transfusion free survival and/or overall survival (28-30). Notably, FCSS was similar between subgroups within IPSS and IPSS cytogenetic risk groups. Therefore, FC may provide valuable prognostic information in addition to currently validated prognostication systems.

In conclusion, the presence of myeloid progenitors with aberrant immunophenotype identified patients with higher risk MDS that are unlikely to achieve CR and long-term major HI upon treatment with azacitidine. Hence, presence of myeloid progenitors with aberrant immunophenotype was associated with worse OS compared with patients without aberrant myeloid progenitors. Decline in FCSS during treatment was correlated with clinical response to azacitidine which indicates that FC might be applied to monitor MDS during treatment and might identify patients who would benefit from prolonged treatment with azacitidine.

Acknowledgements

The authors would like to thank Claudia Cali, Kelly Schouten and Kristin Vandenberghe (Department of Hematology, VU Institute of Cancer and Immunology (V-ICI), Cancer Center Amsterdam (CCA), VU University Medical Center, Amsterdam, The Netherlands) for technical assistance. Tanja M van Maanen-Lamme, Linda Luppens-de Graaf, (Department of Internal Medicine, Westfries Gasthuis, Hoorn), Aart Beeker and Bart de Valk, (Department of Internal Medicine, Spaarne Ziekenhuis, Hoofddorp) for providing bone marrow samples.

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IV

10

General discussion and future perspectives

General discussion

This thesis aimed to contribute to the implementation of flow cytometry in myelodysplastic syndrome (MDS). Previous publications have shown that flow cytometry might have an added value for the diagnosis and prognostication of MDS. (1-3) However, the elaborateness of the available methods and complexity of the interpretation of flow cytometric data have delayed implementation. Therefore, the international and European LeukemiaNet working party for standardization of flow cytometry in MDS (IMDS-Flow) was established to develop guidelines and perform multicenter studies for validation. In this thesis, the guidelines as developed by the working party were included in chapter two, three and four. These guidelines aid in standardization of flow cytometric procedures for the analysis of bone marrow (BM) samples of patients with MDS and aid in achieving concordance between laboratories. Nevertheless, technical issues can influence accurate interpretation of flow cytometric analyses. Therefore, technical considerations for flow cytometric analysis of BM samples of patients with MDS were provided in chapter five. Furthermore, pitfalls were described by giving examples of flow cytometric BM analyses of patients with MDS. The information described in these chapters can help in facilitating laboratories that are not (yet) experienced in the implementation of flow cytometry for MDS. Chapter six addresses the application of flow cytometry for analysis of circulating myeloid progenitors in MDS. The technical issue of inadequate BM samples might be solved by analysing circulating myeloid progenitors in peripheral blood samples. The presence of circulating myeloid progenitors and/or an aberrant immunophenotype of circulating myeloid progenitors might have diagnostic and prognostic value for MDS.

Previous studies have shown that flow cytometry can identify prognostic subgroups of patients with MDS. (1, 2, 4) So far, flow cytometric analyses are not part of any clinical prognostic scoring system for patients with MDS. In chapter seven, the flow cytometric analysis for MDS was applied for the purpose of prognostication. A study by our group was described, showing the added value of flow cytometric analysis for prognostication of MDS within the revised International Prognostic Scoring System (IPSS-R) for MDS. (5) The IPSS-R is refined by changing the classification and weighing of cytogenetic abnormalities, as well as scoring for the depth of the cytopenia. Interestingly, the FCSS, representing an enumeration of flow cytometric abnormalities and the number of myeloid progenitors was able to identify prognostic

subgroups within these well-defined IPSS-R risk groups. Patients with low flow cytometric scores have better overall survival compared with patients with high scores, even in the well-defined subgroups of the IPSS-R.
The study as described in chapter seven indicated that in future clinical prognostic scoring systems for MDS, flow cytometric analyses of BM samples should be incorporated; the prognostic impact cannot be ignored. The question is, in what form it should be incorporated in future prognostic scoring systems. As mentioned above, the elaborateness of the available flow cytometric analyses withholds widespread applicability. Until now, the majority of flow cytometric studies focussed on the enumeration of phenotypic abnormalities which provides a measurement for the degree of dysregulation of hematopoiesis or distance from normal. However, some phenotypic aberrancies might be of more significance than others in detecting dysregulation of hematopoiesis. In the study, as described in chapter eight, a large number of flow cytometric variables were measured in a learning cohort. Statistical analyses identified the SSC and CD117 expression of myeloid progenitors and CD13 expression on monocytes as the most discerning for overall survival of patients with MDS. A MDS flow cytometric score (MFS) was constructed and validated in a validation cohort. The MFS is a practical method with only three variables that might provide a solution for the complexity of flow cytometric analyses for MDS and an answer to the reserve for a general implementation.
In recent years, it was shown that somatic mutations as found in patients with MDS have prognostic impact. This raises the question whether flow cytometric analyses of MDS can still have a role in prognostication of patients with MDS. An answer to this question might be the integration of techniques such as flow cytometry and molecular analyses provides the most comprehensive view with regard to prognostication, besides the established IPSS-R. Especially since in a number of patients with MDS no molecular abnormalities are found. The number of patients with molecular abnormalities varies in literature from 50% to 90%. (Recurrent) molecular abnormalities are more frequently found in patients with higher grade MDS. In the near future, a study integrating the IPSS-R, flow cytometry and molecular analyses should provide an answer.

In clinical practice, the IPSS-R serves as guidance for risk stratification and therapeutic regimens. Lower risk patients with MDS may not require treatment if the level of cytopenia is asymptomatic or remains above certain cut off points. This patient group may require transfusions or treatment with growth factors. In previous studies,

a relationship with transfusion dependency and the presence of abnormal myeloid progenitors as detected by flow cytometry was described. (2) Low risk MDS patients with less than 5% blasts can already have abnormal blasts by flow cytometry. (1, 2, 6, 7) More importantly, these low risk MDS patients had an adverse clinical outcome with a higher risk for transfusion dependency and/or progressive disease, independent of existing and validated prognostic classification systems. (2) The study in chapter nine describes that patients with normal myeloid progenitors were more likely to respond to treatment with EPO and/or G-CSF compared with patients with myeloid progenitors with lineage infidelity marker expression. The presence of aberrant myeloid progenitors might indicate the presence of a stem cell with clinical and prognostic unfavourable behaviour. Flow cytometric data on myeloid progenitors were added to the existing prediction model by Hellström-Lindberg et al. next to endogenous EPO levels, whereas transfusion need was removed. (8) The latter is a much debated variable due to questions on objectivity. A patient with MDS and co-morbidities might experience symptoms due to anemia at higher hemoglobin levels than a MDS patient without co-morbidities and thus will not (strictly) meet the definition of transfusion dependency. (9) This may affect interpretation of response. The flow cytometric model can aid in selecting patients with MDS who will benefit the most from EPO and/or G-CSF treatment.

In general, IPSS-R higher risk patients can be treated with intensive chemotherapeutic regimens preferably followed by allogeneic stem cell transplantation. However, the majority of patients with MDS is older than 70 years (median age 75 years) with significant co-morbidities which makes them not eligible for intensive treatment options. New therapeutic strategies are emerging for MDS, and a more refined diagnostic and prognostic procedure is of importance to select patients who are likely to respond to treatment. Azacitidine is a chemotherapeutic agent with cytotoxic and demethylating modes of action. In chapter ten, we showed that higher risk patients with normal myeloid progenitors as assessed by flow cytometry were likely to respond to azacitidine (for a longer time period) than higher risk patients with MDS with abnormal myeloid progenitors. It would be of interest to design subsequent studies that allocate patients with MDS to treatment modalities based on flow cytometric characteristics. Patients with MDS and aberrant myeloid progenitors, might benefit more from intensive treatment modalities. Likewise, MDS patients without aberrant myeloid progenitors might not require intensive treatment regimens. For example, patients with MDS refractory anemia and excess of blasts-1 (RAEB-1) might be allocated to either supportive care/reduced intensity

treatment regimens or intensive treatment depending on the presence aberrant myeloid progenitors as defined by FC.
The above mentioned studies confirmed that aberrancies in the (myeloid) progenitor cell compartment might be of more relevance for prognostication of MDS patients than aberrancies in the mature myelo-monocytic compartment. (2, 4) To a certain extent this is in line with the findings from chapter eight, in which the MFS was constructed and showed that abnormal myeloid progenitors as defined by decreased SSC and CD117 expression were most predictive for overall survival. Of note, aberrant myeloid progenitors as defined by lineage infidelity marker expression such as CD5 or CD7 were not included in the analysis because these markers did not meet the inclusion criteria for multivariate analysis. The presence of lineage infidelity markers has prognostic impact for patients with MDS as shown in chapter 9 and 10. In the studies in this thesis the cut off point for lineage infidelity marker expression was set at 20% for the majority of markers. In subsequent studies it would be of interest to investigate the prognostic impact of lineage infidelity markers by determining the most optimal cut off point. Moreover, further prospective studies are warranted to validate the prognostic relevance of flow cytometric findings in MDS patients in general.
In summary, the research in this thesis provided information that is a prerequisite to implement flow cytometry for MDS in laboratory and clinical practice: from standardization of techniques to implementation of results in diagnostics, prognostics and treatment decision making.

Future perspectives

This thesis encompassed studies that can serve as a guideline for the application of flow cytometry for the laboratory and clinical practice of MDS. The above mentioned studies and many others that were published resulted in the inclusion of flow cytometry (FC) as a recommended diagnostic criterion to establish a diagnosis of MDS. (10, 11) The 'Ogata'-score is a step towards implementation of flow cytometry as a diagnostic tool in the (clinical) practice of MDS. (3) The sensitivity and specificity of the 'Ogata'-score might be improved by adding lineage infidelity marker expression and/or abnormalities of the erythroid and/or megakaryocytic lineage. (12) So far, for technical reasons, erythropoiesis and megakaryopoiesis were not extensively investigated. The members of the international and European

LeukemiaNet working party for flow cytometry in MDS are exploring methods to analyse nucleated erythroid cells and thrombocytes. (13) This might contribute to the diagnosis of MDS in cases where the diagnosis based on standard tools is indecisive. Flow cytometric analyses of BM of patients with cytopenia needs to be further developed and validated, also in new disease categories such as idiopathic cytopenia of unknown significance. Furthermore, refinement of prognostication may be accomplished. Developments in flow cytometric analysis software might be helpful in objective quantification of the distance from normal and discrimination of MDS cases from normal or malignant hematopoietic disorders.

Although not included in this thesis , flow cytometry offers possibilities in providing a gateway to further research in the pathophysiology of MDS. Steps into exploring immunology, adhesion molecules and microenvironment in MDS were already made. (14-16) Since, MDS is considered a stem cell disorder from which the dysplastic BM cells are derived; interactions between the hematopoietic stem cells and the bone marrow microenvironment and immune cells might contribute to disease progression and adverse clinical outcome. In line with this, detection of stem cells with an aberrant immunophenotype as a reflection of clonal hematopoiesis might aid in the diagnosis and prognosis of MDS. With regard to prognosis, flow cytometry should be included in the next prognostic scoring systems, together with molecular and single-nucleotide polymorphism analyses to contribute in refining prognostication. The strength would be in the combination of several techniques that each provide valuable information.
This thesis describes the evolution of flow cytometry in MDS from laboratory practice to clinical implementation. The studies as described in this thesis can serve as a guideline for laboratories and clinicians that are seeking to implement flow cytometry for MDS and can form the basis for further research.

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Nederlandse samenvatting voor medisch niet-ingewijden

Het myelodysplastisch syndroom (MDS) bestaat uit een groep van beenmergziekten die wordt gekarakteriseerd door gestoorde bloedcelaanmaak resulterend in bloedarmoede (anemie) en/of een tekort aan bloedplaatjes (trombopenie) en/of witte bloedcellen (neutropenie). Patiënten overlijden vaak aan de gevolgen van tekorten in bloedcellen of acute myeloïde leukemie (AML). De geschatte incidentie is 3,3 per 100.000 toenemend op hogere leeftijd tot 50 per 100.000. De incidentie lijkt toe te nemen, deels als gevolg van een betere bekendheid van het ziektebeeld en de tendens om beenmergziekten bij oudere patiënten beter in kaart te brengen. De etiologie van primaire MDS is in de meeste gevallen onbekend. Bij patiënten die (beroepsmatig) zijn blootgesteld aan benzeen, haarverf, roken en straling is een verhoogde incidentie te zien. Daarnaast kan MDS secundair voorkomen na therapie met bestraling en/of chemotherapie. Een toegenomen incidentie met leeftijd en risico op MDS bij syndromen als Fanconi anemie en dyskeratosis congenita suggereert een stoornis in het herstel van DNA-schade als onderliggend mechanisme.

Diagnose

De goudstandaard voor het stellen van de diagnose 'MDS' is met behulp van cytomorfologie. Door middel van een beenmergpunctie worden cellen geaspireerd, uitgestreken op een glaasje en vervolgens zichtbaar gemaakt met een kleurstof voor onderzoek met een microscoop. Bijna altijd zijn er tekenen van verstoorde aanmaak (dysplasie) van rode bloedcellen (dyserytropoëse), witte bloedcellen (dysgranulopoëtische) en bloedplaatjes vormende cellen (dysmegakaryopoëtische) aanwezig. Onderzoek naar afwijkingen in het DNA (cytogenetica) van de bloedvormende cellen is obligaat bij het stellen van de diagnose, zeker bij minimale dysplastische afwijkingen. Bij ongeveer 50% van de patiënten met MDS worden genetische afwijkingen gevonden. Ontbreken van chromosomen of delen van chromosomen 5, 7, 20, Y of een drievoudig voorkomen van chromosoom 8 komen het meest frequent voor doch ook vele andere, waaronder complexe chromosoom afwijkingen, zijn beschreven.

Classificatie

MDS wordt ingedeeld volgens het WHO-classificatiesysteem op basis van de bevindingen in het perifeer bloed en beenmergonderzoek. Cytomorfologie, de goudstandaard en grondslag van de WHO-classificatie, is gebaseerd op kenmerken die

niet exclusief zijn voor MDS. Vooral bij het laagrisico-MDS zijn subtiele morfologische afwijkingen moeilijk te onderscheiden en worden mogelijk onvoldoende herkend. Het combineren van diagnostische methoden, zoals cytogenetisch onderzoek, heeft geleid tot een verbetering van de classificatie; toch blijft er een aanzienlijke groep over die niet te classificeren is. In de afgelopen jaren zijn minimale diagnostische criteria geformuleerd in een consensusrapport voor MDS waarin flowcytometrie (FCM) is opgenomen als co-criterium. Flowcytometrie is in staat om afwijkingen te detecteren die met behulp van cytomorfologie niet zichtbaar zijn. Met behulp van FCM kunnen op deze manier dysplastische kenmerken in de witte bloedcelreeks worden gedetecteerd bij een deel van de patiënten die met microscopisch onderzoek alleen afwijkende rode bloedcellen hadden. Dit is van belang aangezien de aanwezigheid van dysplasie in meerdere cel reeksen relevant lijkt voor de prognose.

Prognose

De 'International Prognostic Scoring System' (IPSS) is een systeem voor risicostratificatie van MDS-patiënten en wordt daarnaast gebruikt als leidraad voor keuze van therapie. De parameters waaruit de IPSS bestaat, zijn het percentage blasten (jonge voorlopercellen) in beenmerg, de cytogenetische afwijkingen en het aantal cellijnen dat afwijkingen vertoont. Afhankelijk van de prognostische betekenis worden punten toegekend aan iedere parameter. De totale score verdeelt de patiënten in 4 risicogroepen: laag-, intermediair-I-, intermediair-II- en hoogrisico-MDS. De kans op progressie naar AML neemt parallel aan de risicogroepen toe. De IPSS risicostratificatie blijft in ongeveer 30% van de gevallen onvolledig omdat er geen cytogenetisch onderzoek beschikbaar is als gevolg van technische beperkingen. Vervolgonderzoek met behulp van interfase fluorescentie-insitu-hybridisatie (FISH)-techniek kan uitkomst bieden om de belangrijkste chromosomale afwijkingen te detecteren. De WPSS (een op de WHO gebaseerd prognostisch scoringssysteem) weegt naast de WHO-diagnose ook de transfusiebehoefte.

Recentelijk verschenen studies wijzen op de prognostische betekenis van de aanwezigheid van blasten in perifeer bloed van MDS-patiënten. De aanwezigheid van meer dan 10 blasten per µl in perifeer bloed gemeten met FCM is geassocieerd met een kortere leukemievrije overleving. De prognose van patiënten met laagrisico-MDS waarbij blasten in het perifeer bloed aantoonbaar zijn, lijkt vergelijkbaar met die van patiënten met hoogrisico-MDS.

Flowcytometrie in MDS

Flowcytometrie is gebaseerd op de eigenschappen ofwel het fenotype van cellen zoals grootte, inhoud en eiwitten op het celoppervlak. De grootte correspondeert met de 'forward light scatter'(FSC) en de inhoud met 'sideward light scatter' (SSC). De FSC zal toenemen naarmate een cel groter is en de SSC zal toenemen naarmate een cel meer korrels bevat. Daarnaast kunnen de eiwitten op het celoppervlak gemerkt worden met stoffen waaraan een label zit dat zichtbaar gemaakt kan worden met behulp van een flowcytometer en de daarbij behorende sofware.
De toepassing van FCM voor MDS is gebaseerd op het concept dat de uitrijping van bloedcellen door verschillende stadia gaat waarbij het fenotype mee evolueert. De verandering die bloedcellen doormaken tijdens de ontwikkeling tot rijpe cellen is in gezonde individuen een gecontroleerd proces. Een afwijkende bloedcelvorming zoals bij MDS zal dan ook resulteren in een van normaal afwijkend fenotype van bloedcellen.
Dit proefschrift heeft zich toegespitst op de toepassing van FCM voor de diagnose, prognose en monitoren van therapie van patiënten met MDS. Er zijn een aantal voorwaarden waaraan FCM zal moeten voldoen voordat het algemeen toegepast kan worden binnen de diagnostiek en risicostratificatie voor MDS. Deel I van dit proefschrift beschrijft het onderzoek dat is gewijd aan standaardisatie en technische aspecten van de toepassing van FCM voor MDS. Een deel van dit onderzoek is gedaan binnen de werkgroep 'Flowcytometrie in MDS' van de Nederlandse Vereniging voor Cytometrie en de internationale en Europese LeukemiaNet MDS-werkgroep (IMDS). Vanuit deze samenwerkingsverbanden zijn aanbevelingen gedaan voor onder andere de manier waarop beenmergmonsters worden bewerkt, combinaties van merkers om eiwitten te labelen en de manier waarop analyses worden gedaan. Laboratoria die interesse hebben in de toepassing van FCM voor MDS kunnen de aanbevelingen gebruiken voor implementatie.

In deel II wordt de stap gezet naar de klinische toepassing van FCM voor MDS. Een op flowcytometrie gebaseerd prognostisch score systeem (FCSS) is in staat om prognostische subgroepen te onderscheiden binnen het gereviseerde internationale prognostische score systeem. De FCSS kent punten toe aan afwijkende bloedcel uitrijping en het aantal jonge voorlopercellen; een hogere score correspondeert met een slechtere uitkomst voor de patiënt. Hieruit vloeit voort dat in toekomstige prognostische score systemen voor patiënten met MDS, flowcytometrische analyse van beenmerg kan bijdragen aan verdere verfijning van het score systeem.

De meerderheid van de flowcytometrische score systemen bestaat uit tientallen parameters. De bewerkelijkheid van onder andere de FCSS heeft niet uitgenodigd tot snelle implementatie. In hoofdstuk 7 wordt een flow cytometrisch score systeem beschreven dat bestaat uit slechts drie parameters met behoud van prognostische betekenis. Het MDS flow score systeem is toegankelijker voor implementatie in laboratoria en in klinische prognostische score systemen.

De behandeling voor patiënten met laag- en intermediair-I-risico MDS bestaat uit ondersteunende therapie in de vorm van transfusies en/of groeifactoren die als doel hebben de bloedcelaanmaak te stimuleren zoals erytropoietine (EPO) al dan niet in combinatie met granulocyten stimulerende factor (G-CSF). In deel III, hoofdstuk 8 is een model gemaakt met onder andere flowcytometrie als variabele, dat een inschatting geeft van de kans dat een patiënt met MDS zal reageren op groeifactoren. Patiënten met laagrisico of intermediair-I-risico MDS met afwijkende jonge voorlopercellen in het beenmerg, gemeten met flowcytometrie hebben een kleinere kans op respons op groeifactoren dan patiënten zonder afwijkende voorlopercellen.
Genezing van MDS is alleen mogelijk door middel van een stamceltransplantatie van een donor. Helaas komt het merendeel van de patiënten met MDS hiervoor niet in aanmerking vanwege het tegelijk voorkomen van andere aandoeningen en de intensiteit van het traject van een stamceltransplantatie. Behandeling met azacitidine, een chemotherapeuticum dat het teveel aan blasten terug brengt en de bloedcelaanmaak stimuleert door (indirect) in te grijpen op het DNA kan voor deze groep (tijdelijk) uitkomst bieden. Patiënten met MDS en intermediar-II- of hoog-risico MDS komen hiervoor in aanmerking. Het onderzoek dat wordt beschreven in hoofdstuk 9 laat zien dat het mogelijk is om met behulp van flowcytometrie een inschatting te geven van de kans op respons op azacitidine. De aanwezigheid van jong voorlopercellen met een afwijkend fenotype is informatief voor de clinicus en geeft een inschatting van de kans op respons op therapie bij patiënten met MDS.

Conclusie

Dit proefschrift en de studies die daarin opgenomen zijn kunnen dienen als een richtlijn voor laboratoria die flowcytometrie willen implementeren voor MDS. De vertaalslag van laboratorium naar de dagelijkse klinische praktijk is gemaakt door het bieden van flowcytometrische modellen om een inschatting te maken van de prognose en kans dat een patiënt zal reageren op therapie. De opkomst van moleculair biologische technieken maakt duidelijk dat toekomstige prognostische systemen elementen hiervan zullen bevatten. De kracht zal uiteindelijk zitten in de combinatie van verschillende technieken die ieder voorzien in prognostische informatie.
Niet opgenomen in dit proefschrift is de rol van het beenmergmilieu, immuunsysteem en stamcellen waaruit MDS-cellen voortkomen. De aanwezigheid van afwijkende jonge voorlopercellen en de relatie met klinische uitkomst kan een basis bieden voor verdere verdieping naar de ziektemechanismen van MDS.

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Curriculum vitae

Canan Alhan was born on March 30th 1980 in Velsen. In 1999 she graduated from the Gymnasium Felisenum in Velsen-Zuid. Because of numerus fixus she was not admitted to study Medicine and decided to study Biomedical Sciences at the Vrije Universiteit of Amsterdam. That year inspired her to perform research and participate in the Honours Programme during her Medicine study that she started in 2000. In 2004 she paused her medical training for a year to perform research at Sanquin, under supervision of Y. Souwer and S. M. van Ham. The subject of investigation was the immune system in patients with B cell chronic lymphocytic leukemia. In 2007 she received her medical degree and started working as a PhD student. The research performed during that period has led tot his thesis. In 2013 she started training to become an internist at VU University Medical Centre in Amsterdam with Y. M. Smulders as head of the training committee.

Canan Alhan werd geboren op 30 maart 1980 te Velsen. In 1999 behaalde zij haar gymnasium diploma aan het Gymnasium Felisenum te Velsen-Zuid. Vanwege uitloting voor de studie Geneeskunde begon zij aan de studie Biomedische Wetenschappen aan de Vrije Universiteit te Amsterdam. Dit jaar is beslissend geweest in de keuze om tijdens de studie Geneeskunde, waar ze in 2000 aan begon, onderzoek te doen en deel te nemen aan het Honours Programme. Na het behalen van het doctoraal begon ze in 2004 aan een onderzoeksjaar bij Sanquin onder begeleiding van Y. Souwer en S.M. van Ham waarbij ze onderzoek deed naar het immuunsysteem bij patiënten met B-cel chronische lymfatische leukemie. In 2007 behaalde ze het artsexamen waarna ze in 2008 begon aan het onderzoek dat heeft geleid tot dit proefschrift. Vanaf 2013 is zij in opleiding tot internist (opleider Y.M. Smulders) bij VU Medisch Centrum te Amsterdam.

Dankwoord

In Tate Modern, Londen heb ik in 2010 een expositie gezien van Ai Weiwei. De vloer van een enorme hal was bezaaid met miljoenen porseleinen zonnebloempitten. Iedere zonnebloempit was met de hand beschilderd door arbeiders in een werkplaats in Jingdezhen, China. Ai Weiwei gebruikt zijn kunstwerken om onder andere te protesteren tegen het regime in zijn land. In mijn dankwoord zullen geen woorden van protest vóórkomen, alleen maar dankbetuigingen! De vele zonnebloempitten hebben een fabriekshal kunnen vullen. Zo hebben ook veel mensen bijgedragen aan de totstandkoming van dit proefschrift.

Mijn dank gaat uit naar alle patiënten die hun bloed en beenmerg hebben afgestaan voor dit onderzoek.

Beste Arjan, niet alleen ben je mijn promotor, maar je bent in de jaren van mijn promotieonderzoek ook een mentor geweest. Je staat bekend om je enthousiasme, maar bezit ook relativeringsvermogen en vindingrijkheid wanneer er minder reden is tot enthousiasme. Met bewondering kijk ik naar hoe jij het werk in de kliniek weet te combineren met onderzoek en een gezinsleven. Hartelijk dank voor de ruimte die je hebt geboden voor wetenschappelijke en persoonlijke ontwikkeling.

Beste Gert, naast Arjan, mijn promotor. Dank voor de interesse die je toonde en tijd die je nam om te horen hoe het vorderde op het lab. Je kreeg vaak als laatste manuscripten te zien en ik heb me verbaasd over hoe jij toch nog waardevolle suggesties had, ook al was een stuk tig keer heen en weer geweest tussen mij, Arjan, Marisa en andere co-auteurs.

Beste Marisa, het is fijn dat jij als mijn co-promotor plaats neemt op het podium. We hebben vijf jaar lang intensief met elkaar samen gewerkt. Jouw betrokkenheid bij diagnostiek en onderzoek maakt jou van grote waarde voor de afdeling Hematologie. Ik heb heel veel van je geleerd. Op momenten dat ik er soms niet uitkwam met een manuscript of presentatie was jij altijd bereid om er samen voor te gaan zitten. Mijn dank is groot.

Peter, ik schreef je dat ik me geen promotie kon voorstellen zonder jouw aanwezigheid op het podium. Ik heb nog in de rieten stoeltjes gezeten en voor ik

het wist had ik daar een 'life changing ' keuze gemaakt, met een beetje hulp van jou. Ik had nog veel kunsten van je willen afkijken. Dank je wel voor het duwtje, maar ook de scherpe vragen en het stimuleren tot onderzoek uit fascinatie.

Professor Kramer, beste Mark, hartelijk dank dat je plaats hebt willen nemen in mijn commissie. Als hoofd van de afdeling interne geneeskunde van VUmc heb je vooral te maken met bestuurlijke aangelegenheden, maar het vak hematologie zit in jouw bloed en dat is nog met regelmaat te merken. Ik hoop nog veel van je te kunnen leren.

De overige leden van de promotiecommissie: Professor Ellen van der Schoot, Gerrit Jan Schuurhuis, Gerwin Huls en Vincent van der Velden hartelijk dank voor de tijd en aandacht die jullie hebben besteed aan het lezen van mijn proefschrift.

Mike Loken and Denise Wells, both of you belong to the group of pioneers in the field of flow cytometry in myelodysplastic syndromes. Your experience and knowledge in this field is admirable. Thank you for the time and effort you took to read my thesis.

Alle stafleden van de Hematologie van VUmc dank ik voor de inbreng tijdens besprekingen, maar ook voor de getoonde interesse buiten de besprekingen om.

Birgit Witte, hartelijk dank voor alle statistische adviezen en bijdragen die je hebt geleverd aan dit proefschrift. Laurens Groenewegen, dank dat ik af en toe gebruik mocht maken van je kennis van Access. Beste Corien, jij zag de patiënten waar wij in het laboratorium het bloed een beenmerg van kregen. Regelmatig spraken wij elkaar over hoe het met hen ging. De stipjes op het scherm kregen een verhaal en dat verhaal viel vaak niet mee. Dank je wel voor de prettige samenwerking. Je bent een fantastische nurse practitioner voor de patiënten.

Beste Claudia, hartelijk dank dat jij op de dag van mijn promotie naast mij wilt staan als paranimf. Je bent een stille kracht en constante factor voor de groep. Kristin Vandenberghe en Kelly Schouten, hartelijk dank voor jullie bijdrage!
Ik heb me ontzettend thuis gevoeld op de afdeling hematologie en dat is (mede) te danken aan de mensen die er werken. Ik dank iedereen van de afdeling hematologie van het CCA voor de goede werksfeer. Een aantal mensen licht ik er uit. Angelika,

je hebt niet de makkelijkste taak als hoofd van het laboratorium. Ik dank je voor jouw toegankelijkheid, interesse en de gezelligheid tijdens congresbezoeken aan het buitenland. Yvonne, ik hoop nog heel lang samen naar het Amsterdamse Bostheater te kunnen voor een picknick, bijpraten en natuurlijk theater! Lieve Sander, we kenden elkaar al van voor onze tijd bij de hematologie. Het is mooi om te zien dat jij je hebt ontwikkeld tot een technische vragenbaak voor flowcytometrie. Adri, ik denk dat iedere afdeling zo iemand als jij zou moeten hebben. Je beheerst veel technieken, je bent collegiaal en multi-inzetbaar (van analist tot toneelspeler en regisseur). Je hebt de meest aanstekelijke lach die ik ooit heb gehoord. En jij hebt, net als alle anderen bijgedragen aan 'gezond aquariumwater', dank daarvoor!

Mijn tijd bij de afdeling hematologie heeft mij niet alleen een proefschrift, maar ook vrienden opgeleverd. Lieve Monique en Willemijn, we hebben veel met elkaar gedeeld en ik hoop dat we dat in de toekomst ook zullen blijven doen. Denise, Niels, Marvin, Dave, Jurjen, Han, Rolf, Anna, Bo en Costa, jullie hebben mij een hoofd vol herinneringen en een onvergetelijke tijd gegeven, ik ben jullie dankbaar! Beste Eline, jij hebt het MDS-project van mij overgenomen en het eigen gemaakt. Ik hoop dat er vele mooie publicaties zullen volgen. Ik kijk uit naar jouw boekje! Veel succes wens ik de aio's die nu bezig zijn met hun promotieonderzoek, Nathalie, Wendelien, Carolien en Rocco.

Lieve Dénise, (noem mij bij mijn diepste naam), ik ben je ontzettend dankbaar voor je vriendschap en voor het maken van de kaft van mijn proefschrift. Zoveel talent, zulke prachtige werken en zo een mooi mens. Ik heb geen moment getwijfeld.

De vrienden die ik heb verwaarloosd, het gevoel altijd en overal tekort te schieten. Dank voor jullie begrip en voor het aanhoren van mijn klaagzangen. In het bijzonder noem ik Susan, Peter-Ben en Funda, dank voor jullie vriendschap.

Mijn opa, mijn lieve opa, Süleyman Akcaova, aan wie dit proefschrift is opgedragen. Sine qua non. Je pakte je koffers eind jaren '60 en vertrok naar Nederland om te werken in de bouw en later, tot aan je pensioen bij de Hoogovens. Na jarenlang de boerderij en het kroost alleen bestierd te hebben vond mijn oma het tijd om een beslissing te nemen. In Nederland of Turkije wonen, maar wel met z'n allen. Aldus geschiedde. Wat jullie mij mee hebben gegeven, al dan niet via mijn ouders is van onschatbare waarde. Ne kadar teşekkür etsem azdır. Nur içinde yatın.

Lieve mama en papa, jullie arbeidsethos, loyaliteit, bescheidenheid, omgangsvormen, onafhankelijkheid en wijsheid hebben mij (mede) gevormd tot wie ik ben. Dankbaar ben ik jullie.

Kardeş, lief broertje, Ural, ik probeer het mezelf af te leren om jou broertje te noemen. Inmiddels ben je een man met een baan, verloofde en een huis. Wat ben ik trots op je! Dank je wel dat je, min of meer gedwongen ;-), naast me wilt staan vandaag.

Tot slot is er liefde, heel veel liefde.