SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The role of flow cytometry examination in the diagnosis and prognosis of myelodysplastic syndromes

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THE ROLE OF FLOW CYTOMETRY EXAMINATION IN THE DIAGNOSIS AND PROGNOSIS OF MYELODYSPLASTIC SYNDROMES

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The Examination takes place at at the library of the Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, at 12:00, 22nd November, 2018

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 14:00, 22nd November, 2018

1. Introduction and review of the literature

1.1.The definition and significance of myelodysplastic syndromes

Myelodysplastic syndromes are heterogeneous clonal hematopoietic stem cell disorders. The incidence rate of MDS is 5 cases per 100,000 people per year in the overall population. Since the average age at diagnosis is 70, the incidence of MDS in the age group over 60 years could be as much as 10 times higher (20-50 cases per 100,000 people per year). Considering that the incidence of MDS is underestimated in central registers due to underreporting and underdiagnosis, the true incidence of MDS could be even higher than 100 cases per 100,000 people per year in the age group over 60 years. The ageing of Europe will cause the number of MDS cases to increase year by year. Due to the heterogeneity of MDS, the cost of therapy varies widely. Regular follow-ups are recommended even in the case of patients with low grade MDS treated as outpatients. In cases whereby MDS is transformed into AML, or the patient has severe cytopenia, regular inpatient therapy is inevitable. Nowadays we have specific and efficient, although costly, therapies available, which, when applied regularly, can significantly improve the life expectancy of patients with MDS, but discontinuing the therapy causes rapid progress of the disease. For these reasons, MDS afflicts considerable and increasing burden on society, which can be moderated by finding the optimal therapeutic strategy for each patient. This is possible only by establishing the exact diagnostic and prognostic classification.

1.2. Classification systems

To facilitate the appropriate diagnosis and prognostic stratification, classification systems were developed to help establish homogenous subgroups among the heterogeneous cases of this disease. Based on the results of studies carried out in the past few decades and the dynamic development of diagnostic methods, the World Health Organization (WHO) issued a novel recommendation. Balanced and unbalanced translocations, which indicate MDS even in the absence of dysplasia, gained greater significance in the diagnosis of MDS. Although several somatic mutation associated with MDS have been described recently, only two mutation were included in the recent WHO recommendation. The *SF3B1* mutation influences the cut-off value in MDS with ring sideroblast category: If the mutation is

identified, then 5% of ring siderblasts is sufficient for the diagnosis, whereas 15% is required in cases lacking *SF3B1* mutation. The second mutation, *TP53*, can help to identify an adverse prognostic subgroup and patients with poor therapy response in the generally favorable prognostic group of "MDS with isolated del(5q)."

1.3. Diagnostic algorithm of MDS

The diagnosis of MDS is based on the recommendations of WHO in most cases. However, there are cases where the WHO-recommended criteria of MDS are not fulfilled completely, yet the diagnosis of MDS cannot be ruled out, either. To asses such cases, minimal diagnostic criteria have been proposed by the International Working Group (IWG) on MDS, which assists the recognition of conditions leading to MDS. The first step in the algorithm to diagnose MDS is the fulfillment of two prerequisite criteria: persistent peripheral blood cytopenia and the exclusion of all other hematopietic and non-hematopoetic disorders that can cause cytopenia. If both of these prerequisite criteria are met, the next step is to check three MDS-related diagnostic criteria. Even if only one of these is confirmed, the diagnosis of MDS is established. The three diagnostic criteria of MDS are the following: (i) dysplasia in one or more cell lineages in the bone marrow in at least 10% of all cells, or the presence of ring sideroblasts exceeding 15% (over 5% is sufficient if *SF3B* mutation is observed); (ii) 5-19% myeloblasts (MB%) and (iii) typical cytogenetic alterations. Regardi ng myeloblasts, a new recommendation accepts 2-19% of myeloblasts in peripheral blood as sufficient to consider this criterion met. If the MDS-related criteria of the second step in the diagnostic algorithm are not fulfilled, other diagnostic methods are necessary to establish the diagnosis such as bone marrow biopsy, histologic or immunochemical examination, detection of somatic mutation, or flow cytometry analysis.

1.4. The prognosis of MDS

In the case of MDS, there are patient-related and disease-related prognostic factors. The age, physical and mental status, and co-morbidity of patients influence the outcome.

Considering the heterogeneity of MDS, several disease-related factors have been described which impact the prognosis. Consequently, when using the WHO classification, which is predominantly based on morphological and cytogenetic examinations and considers other prognostic factors only tangentially, the prognosis of patients within each of the various

subgroups often proves to be very heterogeneous. For this reason, the International Prognostic Scoring System (IPSS) and its revised version (R-IPSS) are widely used in the daily routine and in establishing guidelines for therapy. Both scoring systems are based on the percentage of myeloblasts, karyotype examination, and the severity of cytopenia. The advantage of R-IPSS is that the underlying study analyzed a large population of patients, therefore even rare cytogenetic alterations are included in the scoring system as prognostic factors. Besides featuring their R-IPSS category, patients are also usually classified according to the recommendation of the WHO classification-based prognostic scoring system (WPSS). Just like in the case of diagnosis, new methods such as somatic mutation detection, or flow cytometry contribute to a more precise prognostic classification of patients.

1.5. Other methods supporting the diagnosis and prognostic classification of MDS

The essentiality of classical methods such as morphological and cytogenetic examination is unequivocal in the diagnosis and prognostic classification of MDS. However, other methods, such as somatic mutation, or flow cytometry examination, have been receiving a lot of attention in the past few years, and they are steadily gaining in significance in the differential diagnosis and prognosis of MDS.

1.5.1. Detection of somatic mutations

In terms of investigating the somatic mutations associated with MDS, 2014 was considered a milestone, when two working groups published their results independently. Their goals were similar, as they both wanted to explore the spectrum of somatic mutations associated with MDS by next generation sequencing of a large population of patients (Papaemmanuil et al.: n=738; Haferlach et al.: n = 944). Of the 15 most frequently detected mutations presented by the two studies, 14 were the same, and the order of frequency was also very similar.

Today there are more than 40 different mutations known which can help not only the assessment of the clonality and the identification of MDS or pre-MDS conditions but also anticipate the prognosis and the therapy responses.

1.5.2. Flow cytometry analysis

The diagnostic guidelines of MDS differentiate between mandatory, recommended, and suggested methods, with FC examination assigned to the recommended category. The changes of surface and cytoplasmic antigen expression patterns during normal haematopoiesis are well known. In the last decades several studies demonstrated that there are characteristic alterations compared to normal expression patterns which can support the diagnosis and prognostic classification of MDS. Although there is no single specific immunophenotype alteration which is sufficient in and of itself to establish the diagnosis, relying on the most common dysplastic signs, several flow cytometry scoring systems (FCSS) have been established, and they proved to be even more sensitive than morphology in detecting dysplasia. FCM can support not only the diagnosis of advanced cases – where there are excess blasts – but also helps in the identification of low-grade cases. For screening purposes, the Ogata scoring system is recommended as a "mini-panel", which is based on no more than four parameters: 1. ratio of MBs in all nucleated cells (MB%), 2. granulocytes SSC/ lymphocytes SSC (Gran/Ly) ratio, 3. ratio of CD34+ lymphoblast (LB%) in CD34 positive cells, 4. lymphocyte/myeloblast CD45 mean fluorescence intensity (MFI) (Ly/MB CD45 MFI) ratio. The high sensitivity and specificity of the screening panel was validated in a multicenter study on a large population of patients with excellent reproducibility. As the feasibility of using FC for the diagnosis and prognostic classification of MDS got verified, the International/European LeukemiaNet (ELN) Working Group for Flow Cytometry in MDS (IMDSFlow) set the aim of integrating FC into the recommendations of the WHO classification to an even greater extent. To reach this goal, it is essential to standardize and harmonize the diagnostic and prognostic use of FC, therefore ELN issued recommendations. According to the recent ELN recommendation, the immunophenotypic alterations in MDS should be analysed within 24 hours, preferably using bone marrow (BM) samples collected in heparin, or ethylenediaminetetraacetic acid (K_3-EDTA) as an alternative anticoagulant. . It is evident that delayed sample handling leads to apoptosis, then necrosis, and it is also known that K_3-EDTA and Na-heparin affect cell viability differently: several studies established that EDTA accelerates the apoptosis and necrosis of cells. Apoptotic cells exhibit characteristic changes in the nuclear, the cytoskeletal, and the membrane structure. These changes often result in altered expression patterns which resemble dysplastic signs and can cause false interpretation. Apoptosis can be avoided or minimized of course by adhering to the ELN recommendation, however, the transportation of samples and the resulting delay in sample

handling is inescapable. As the resources of flow cytometry examination – such as flow cytometer and skilled experts who can analyze and interpret the results – are available only in the national centers, the transportation of samples collected far from these centers is inescapable.

1.6. The biology of mast cells

Mast cells are a heterogeneous cell population, playing a role in various physiological processes, such as innate and adaptive immune response, tissue repair after damage (wound healing, fibrosis, and angiogenesis). Chronic mast cell stimulation, however, can cause increased impact of normal functions, which can contribute to the development of several diseases. Previous studies have detected elevated MC% by morphological examinations in the case of MDS. Tryptase expressing MCs were found to be the most elevated in the bone marrow of MDS patients. In accordance with this finding, serum tryptase level has been recommended recently as a new non-invasive biomarker in the diagnosis of myeloproliferative disorders. The role of mast cells in MDS is still unclear today.

2. Objectives

In our study, we were trying to address practical problems that arose in the application of flow cytometry in everyday routine.

1. Our aim was to examine the time-dependent immunophenotypic changes of a range of markers.
2. On the other hand – while FC works fine with samples collected in either K_3-EDTA or Na-heparin – we also wanted to learn how the application of one or the other type of anticoagulant influenced the expression patterns.
3. We especially scrutinized how different anticoagulant types and clones of antibody used for labelling affected the expression of CD11b, since previous studies published contradictory results in this respect.
4. The “mini-panel” for the screening of MDS was primarily based on the alterations of the myeloid population. We hypothesized that currently existing FCSS can be made more sensitive by adding two parameters to the “mini-panel” which give information about mast cells and erythroid precursors, which are usually affected in MDS.
5. Our further aim was to validate the power of the “mini-panel” complemented by the robust coefficient of variation of CD71 expression on erythroid precursors (CD71 rCV%) and the percentage of mast cells in a second group examined by eight-color staining method.
6. Finally, we also wanted to explore the most efficient conditions for the measurement of MCs by flow cytometric examination.

3. Materials and methods

We conducted our studies in compliance with the international and national protocols. The Hungarian Medical Research Council granted permission for our studies (reference number: 20582-2/2017/EKU).

3.1. The design of the study investigating the impact of pre-analytical factors on the interpretation of dysplastic signs

Two groups of participants were selected. In the first group twenty-three patients with suspected MDS or MPN were included in a prospective study. They were referred to the Department of Laboratory Medicine, University of Debrecen, Hungary, between December 2014 and February 2015 for detailed examination. Three tubes of bone marrow samples were collected from each patient for diagnostic purposes. One sample was collected in K_3-EDTA for morphological, flow cytometric and molecular examinations, while two tubes in Na-heparin for cytogenetic analysis. We performed our experiments on residual samples that remained after the diagnostic tests. To examine the effect of the different anticoagulants, we evaluated the de novo immunophenotype and its alterations on day 1 and day 2 in samples collected into K_3-EDTA (n=23) or Na-heparin (n=16). Samples were kept on room temperature prior to analysis.

In the second group residual peripheral blood (PB) samples of eight patients with no haematological malignancy collected in one tube K_3-EDTA and one tube Na-heparin for flow cytometry measurements were examined with different clones of CD11b monoclonal antibodies.

3.2. The design of the study for the screening of MDS by flow cytometry scoring system

In this study, two cohorts of patients were examined. By examining the first – test – cohort, we established our novel FCSS, while in the second – validation – cohort we tested it. As recently several laboratories switched to eight-color staining method from four-color staining, the parameters of FCSS were detected by a four-color setting in the test cohort and by an eight-color setting in the validation cohort. Thus we were able to explore the impact of various settings on the applicability of our FCSS.

We assigned the patients to control and MDS groups on the basis of morphological examination, cytogenetic alterations, clinical history, cytopenia and flow cytometric examination of their bone marrow samples. In the first cohort, BM samples of the patients were referred to our laboratory for FC examination between December 2009 and January 2014, while BM samples of the validation cohort were assigned between May 2014 and May 2015. Data were analysed retrospectively. The control group (n=25) included patients with autoimmune disorders (n=3), immune thrombocytopenia (n=7), therapy related disorders (n=9), iron deficiency (n=2), chronic kidney disease (n=2) or infection (n=2). Members of the control group were aged between 23 and 80 years. BM samples were collected in K_3-EDTA and were processed within 1-2 hours following aspiration.

3.3. Immunophenotype analysis

Four or eight markers were examined at the same time in a tube to evaluate the impact of pre-analytical factors and to detect the relevant parameters for the FCSS. Antibody combinations were added to 50 µL bone marrow aspirate ($1x10^6$ cells) and incubated for 15 minutes in the dark at room temperature. Then 1 mL lysing solution was added to each tube, and samples were incubated for an additional 8 minutes. Finally samples were washed once in phosphate-buffered saline (PBS) and resuspended in 500 µL 1% paraformaldehyde (PFA). 100 000 events were acquired with the help of FACS Calibur (four-color staining) or FACS Canto II (eight-color staining) flow cytometers. To make the results comparable, flow cytometers were calibrated daily, using Calibrite fluorescent microbeads and Autocomp software as recommended by the manufacturer. Data were analyzed by FACS Diva 6.1.3 and Kaluza 1.2. softwares.

3.4. Identifying the right parameters for exploring the impact of pre-analytical factors on immunophentoype

In case of MDS examinations, the gating strategy was the following: the first step was elimination of debris with the help of forward scatter (FSC) and side scatter (SSC) bivariate dot plot. Myeloblasts (MBs, CD117+/CD34+/SSCint) and lymphoblasts (LBs, CD117-/CD34+/SSClow) were identified on the basis of SSC character, CD117 and CD34 markers; then CD45 was applied in the last step, back gating. Depending on the SSC character and the

intensity of CD45, CD33, CD64, or HLA-DR expression, cells were identified as lymphocytes, monocytes, and granulocytes. CD71+/CD45- cells were classified as erythroid precursors. As for rare events, plasma cells (PCs, CD38 bright) were included in our study.

Thirty-seven different immunophenotypic variables were recorded for the samples collected in K_3-EDTA and those ones in Na-heparin for three days (de novo=day 0, day 1 and day 2), of which mean fluorescence intensity (MFI) values, robust coefficient of variation (rCV*)* and percentages of different cell types were calculated daily compared to day 0 values.
In the second part of our study, we investigated not only the impact of using different anticoagulants on the time-dependent changes of CD11b expression on granulocytes and monocytes but also the consequence of using different antibody clones. The clone of FITC labeled CD11b was ICRF44, while the clone of PE labeled CD11b was D12. The gating strategy was the following: the first step was elimination of debris with the help of forward scatter (FSC) and side scatter (SSC) bivariate dot plot. Granulocytes and monocytes were differentiated on the basis of their SSC character and CD33, CD64, CD45, and HLA-DR intensity.

3.5. The selection of FCSS parameters for screening MDS

The first step of the gating strategy was the elimination of debris with the help of forward scatter (FSC) and side scatter (SSC) bivariate dot plot. CD117 and CD34 markers were used to identify MBs and CD45 was applied in the last step, back gating. Other cell types were identified as follows: granulocyte (CD45+/SSChigh), lymphocyte (CD45+/SSC low), erythroid precursors CD45-/CD71+; LB CD34+/SSClow/CD45dim; MC CD117bright.
We elaborated the novel FCSS the following way: cut-off values published by Ogata et al. were applied for MB%, Gran/Ly SSC ratio, LB% in CD34+ cells, Ly/MB CD45 MFI ratio while we defined cut-off values for MC% (>0.039%) and CD71 rCV% (>85.8%). Samples with values above or below the relevant cut-off limits were scored by 1 point. As a next step, we investigated the diagnostic and prognostic power of this novel six-parameter FCSS and finally tested it on the validation cohort.

3.6. The detection of mast cells by flow cytometry

As the percentage of mast cells were not included in existing FCSS, we examined this population in more detail. MC can be gated easily on the basis of their bright CD117 expression. To explore the impact of pre-analytical factors on the percentage and CD117 expression of MCs, seven bone marrow samples collected in K_3-EDTA and the same number of samples collected in Na-heparin were labelled and analyzed by flow cytometer right after aspiration and on two consecutive days. Furthermore, we examined in our FCSS study the association between reactive signs (WBC, percentage of PC in BM) and increased MC%, which can be elevated in reactive processes. Finally, to exclude the relative increase in the MC% caused by elevated nucleated cell count, we compared the nucleated cell count of BM by MC%.

3.7. Statistical analysis

Considering the low number of samples non-parametric tests were used by the first study. Two related groups were compared by Wilcoxon signed-rank test, where there were more than two related groups, data were analyzed by Friedman test. Dunn's multiple comparison test was applied as post hoc test. Data distribution was evaluated by the Shapiro-Wilk test in the second study. To compare the two groups, we used Student T test for parametric and Mann Whitney U test for non-parametric data. P-value <0.05 was considered significant. We tested the specificity and sensitivity of our novel FCSS with the help of receiver-operator characteristic (ROC) curves, also calculating the areas under the ROC curves. We used Spearman´s rank test to calculate the correlation between our novel FCSS and IPSS. Pearson's Chi square test was applied to assess association between two categorical variables. Event-free survivals as determined from the date of the diagnosis to transformation to AML or death were analyzed by the Kaplan-Meier method and the degrees of difference were compared by log-rank test. Statistical analysis and the creation of figures were carried out using SPSS 20.0 and GraphPad Prism 6.0 statistical programs.

4. Results

4.1. The impact of pre-analytical factors on immunophenotype

4.1.1. Comparison of FC parameters of samples collected in K_3-EDTA and Na-heparin at day 0

When we compared the initial immunophenotype of fresh samples collected in K_3-EDTA and Na-heparin, we detected significant differences in fourteen parameters.

Six parameters were significantly higher in samples collected in K_3-EDTA as compared to samples collected in Na-heparin: MFI of CD4 on monocytes, MFI of CD117 on MBs, MFI of CD45 on lymphocytes, CD71 rCV on erythroid precursors and percentage and MFI of CD38 on PCs.

Eight parameters were significantly lower: SSC and intensity of CD45, CD11b and CD33 expression on granulocytes, MFI of CD11b, CD13 on monocytes, MFI of CD34 on MBs and MFI of CD71 on erythroblasts. Despite significant alterations in the CD34, CD117 and CD71 expression, the percentage of MBs, LBs or erythroid precursors did not show significant differences between the samples collected in K_3-EDTA and Na-heparin, so these alterations did not influence the appropriate gating of the different cell types.

4.1.2. Immunophenotypic alterations caused by delayed sample handling

Due to delayed sample processing ten parameters were significantly altered by day 1 in samples collected in K_3-EDTA, while in samples collected in Na-heparin only four parameters changed. Two of these (the MFI of CD117 on MBs and the MFI of CD38 on PCs) proved to be the most sensitive for delayed sample processing. Regardless of the type of anticoagulant, the intensity of these markers fell continuously during the time of observation.

On top of the day 1 changes similar number of additional alterations were detected by day 2 in the cases of samples collected in Na-heparin and K_3-EDTA. Fifteen parameters in scope decreased significantly in samples collected in K_3-EDTA, while three parameters increased and twelve parameters decreased in samples collected in Na-heparin.

Nine parameters changed by day 2 regardless of the type of anticoagulant: the SSC on granulocytes, the MFI of CD4, CD64, CD33 on monocytes and the ratio of CD14 positive

monocytes, the percentage of preB cells and lymphocytes, the MFI of CD45 on lymphocytes and the MFI of CD71 on erythroid precursors.

The number of stable markers throughout the 2-day period was 12 in samples collected in K_3-EDTA as compared to 18 in samples collected in Na-heparin. The following eleven parameters were the most stable regardless of the type of anticoagulant: MFI of CD16, CD10, CD33 on granulocytes, the percentage of CD10 positive granulocytes, HLA-DR on monocytes, MFI of CD45 and CD34 on MBs, the percentage of LBs, erythroid precursors and PCs and the CD71 rCV on erythroid precursors.

4.1.3. Immunophenotypic changes of CD11b caused by delayed sample handling

The intensity of PE-labeled CD11b (clone D12) expression was significantly reduced both on granulocytes and monocytes in samples collected in K_3-EDTA by day 2. This decrease was significant in the case of FITC-labeled CD11b (clone ICRF44) only on monocytes by day 2. In samples collected in Na-heparin, delayed sample handling caused the opposite phenomenon. Regardless of the type of CD11b clone, the intensity of CD11b expression of granulocytes increased significantly by day 2 in Na-heparin. This elevation was significant not only on granulocytes but also on monocytes in the case of FITC-labeled CD11b (clone ICRF44) by day 2.

4.2. Testing the diagnostic and prognostic power of our FCSS for screening MDS

4.2.1. The results of the study for the evalutation of our novel FCSS

Regarding laboratory parameters - hemoglobin level (HB), white blood cell count (WBC) and absolute neutrophil cell count (ANC) - there were significant differences between non-MDS and MDS patients groups. Platelet count did not differ significantly in the control and MDS groups, which resulted from the fact that seven ITP patients were assigned to the control group.

4.2.1.1. Testing the diagnostic power of the six-parameter FCSS in the four-color test cohort

On the one hand, when we compared MDS cases to non-MDS bone marrow samples, we detected significant differences in the MB% (p<0.001), in the Gran/Ly SSC ratio (p=0.003) and in the LB% among CD34+ cells (p<0.001), in CD71 rCV% of erythroid precursors (p=0.004) and in MC percentage (p<0.001). Ly/MB CD45 MFI ratio did not differ significantly in the two groups, which can be explained by the fact that the intensity of CD45 on MBs can either decrease or increase in MDS cases.

On the other hand, when we compared the ROC curves of the modified six-parameter FCSS and the original four-parameter FCSS described by Ogata et al, we found that considering not only the four cardinal parameters but also CD71 rCV% and MC% provided higher sensitivity (84%) while the specificity remained high (80%). The area under the receiver operating characteristic curve (ROC-AUC) for the Ogata FCSS was 0.789 (95% CI: 0.689-0.889) to discriminate MDS patients from healthy controls, which increased to 0.870 (95% CI: 0.785-0.948) for the six-parameter FCSS in the four-color labeling system.

4.2.1.2. Testing the prognostic power of the six-parameter FCSS on the test cohort

To test the prognostic power of the six-parameter FCSS, we assigned MDS patients - diagnosed on the basis of morphological examination, cytogenetic alterations, clinical history, and cytopenia – to two FCSS risk groups. Specifically, 19 patients (37% of patients) were assigned to the flow low risk (FLR) group with 0, 1 or 2 scores, while patients with more than 2 scores were assigned to the flow high risk (FHR) group (n=32, 63% of patients). When comparing laboratory parameters in the two groups, we detected significantly decreased HB (p=0.002) in the FHR group. Accordingly, some of our patients received red blood cell and/or platelet transfusions. Patients in the high-risk group were more likely to need transfusion (OR=3.51; 95% CI: 1.07-11.59; p=0.036). The percentage of MBs, which is a major parameter in most scoring systems, differed significantly between the two FCSS risk groups (p=0.009). Although cytogenetic alterations did not show significant difference between the two risks groups, those patients having the most severe cytogenetic alterations had more than 2 scores. Forty-four patients had data on clinical follow-up, two patients were excluded

because they underwent BM transplantation. We found significant difference in event-free and overall survival between the two risk groups based on the modified six-parameter FCSS (p=0.001, p=0.008, respectively)

4.2.2. Testing the six-parameter FCSS in the validation cohort

Comparison of the validation cohort to the test one regarding the six parameters of the novel FCSS revealed significant differences in two parameters: the MB% and the Ly/MB CD45 MFI ratio. The MB% was lower in the MDS group analyzed by the eight-color staining method which can be explained by the fact that in the validation cohort there were more patients in the low risk group than in the first cohort (FLR: 61% of patients in the validation while 37% of patients in the test cohort). The increased Ly/MB CD45 ratio was also more likely caused by the different composition of the patient samples rather than the application of different fluorochromes and slightly different settings in the two cohorts. We could detect significantly decreased Gran/Ly SSC ratio and LB% in all CD34+ cells, increased MC% and CD71 rCV% compared to normal, while results of the test and validation cohort did not differ significantly. To test the diagnostic power of MFC, we compared the sensitivity and specificity in the two MDS cohorts (four-color staining and eight-color staining). The sensitivity decreased on the part of the Ogata score in the MDS group analyzed by the eight-color staining method. On the other hand the sensitivity of the six-parameter scoring system was similar in the test and validation MDS cohorts, while specificity greatly increased. It means that the application of the eight-color-tube with the six-parameter FCSS can be used as an excellent screening method to detect normal samples and this way reduce the time and costs of further investigations of these samples. Furthermore, the ROC-AUC for the Ogata FCSS was 0.917 (95% CI: 0.837-0.996), which increased to 0.957 (95% CI: 0.902-1.000) when six-parameter FCSS was applied in the eight-color labeling system, suggesting that the discriminative power between MDS and normal samples of this eight-color six-parameter FCSS is excellent.

Testing the prognostic power of FCSS, we compared laboratory parameters and the MB% in the two FCSS risk groups. Similarly to the test cohort, HB level decreased, while the MB% increased significantly in the high-risk group (p=0.021 and p=0.002, respectively). The patterns of transfusion dependency in the two risk groups were also similar to those of the test cohort. In the FLR group 33% of patients required transfusion, while in the FHR group 58%

of patients did. There were no significant differences between the two groups regarding cytogenetic alterations.

Finally, in our cohorts the correlation between R-IPSS and our six-parameter FCSS ($r=0.32$, $p=0.02$ in the test cohort and $r=0.507$, $p=0.002$ in the validation cohort) was similar to the previous results.

4.3. The detection of mast cells by flow cytometry

We were able to detect mast cells in seven bone marrow samples. The MC% depended largely on the type of anticoagulant. The percentage of MC were significantly increased in samples collected in K_3-EDTA not only at day-0 but also at day-1 or day-2 ($p=0.03$, $p=0.016$, $p=0.047$, respectively). The intensity of CD117 expression on MC proved to be stable by day-1 regardless of the type of anticoagulant. Although, the intensity of CD117 expression decreased significantly by day-2 regardless of the anticoagulant, it did not influenced the MC%, as the latter is not influenced by delayed sample handling. The difference of MC percentage depending on the type of anticoagulant was not caused by a technical factor, because the gating marker (CD117) did not differ significantly in samples collected in K_3-EDTA and Na-heparin. To examine the increased MC% detected in our FCSS study, we divided the MDS samples examined in the study into two groups according to their MC ratio among all nucleated cells (cut-off 0.039%). Then we compared the following parameters: nucleated cell count of aspirated BM samples, plasma cell percentage (PC%) and WBC of peripheral blood in the two groups. There was no significant difference regarding these parameters between the patients with high ($n=21$) and low MC% ($n=30$).

5. Discussion

5.1. The impact of delayed sample handling and type of anticoagulant on the interpretation of dysplastic signs detected by flow cytometry

The most important finding of our study was that the type of anticoagulant significantly influenced not only the rate and number of alterations caused by delayed sample handling but also initial expression patterns, where apoptotic cells were not or only in a minimal amount were present. The expression patterns of various cell types proved to be more stable in

samples collected in Na-heparin, which supports the recommendation of European LeukemiaNet regarding the preferred type of anticoagulant. However, there were some parameters which were altered significantly even by day 1 in samples collected in Na-heparin. In our study, several parameters were changed due to delayed sample processing, and unless the examiner is familiar with the extent and direction of such alterations, they could be misinterpreted as dysplastic signs or the MDS cases could be categorized to advanced stage.

To best of our knowledge, we were the first to examine a wide range of markers on different cell types by eight colour labeling FC method. Previous studies usually focused on myeloid population and examined a certain marker or only a little amount of markers. Some parameters, similarly to our findings, consistently altered regardless of the type of anticoagulant. One such parameter was the decrease in the side scatter (SSC) of granulocytes during apoptosis, which can be reinforced by morphological examination. Reduced CD16, CD43 and increased CD45 expression was detected on apoptotic myeloid cells even in isolated white blood cells from citrated, heparinized or EDTA-anticoagulated whole blood. In contrast to the agreement about behaviour of these markers in the literature, contradictory findings have been published about the effects of apoptosis caused by delayed sample handling on the intensity of CD11b expression: some studies detected a rise of this antigen expression while others found loss of CD11b. These results typically depend on the design of each study. Because of CD11b is stored in cytoplasmic granules, the intensity of this marker can markedly increase during activation of neutrophils. Therefore, the first cause which can influence the results is the activation of neutrophils. This can be indicated during the sample processing (purified polymorphonuclear cell, triggered apoptosis). However, the results of the study published by Saxton et al. suggest that the root cause of activation is not always obvious. They examined six peripheral whole blood samples collected in EDTA whit or without cell stabilization solution (Cyto-Chexe). They found that intensity of CD11b increased in the case of samples without cell stabilization solution at room temperature during the examined period (4 hour). Although, Hodge et al. found that Annexin V staining, which can bind to the cell membrane to externalized phosphatidylserine (PS) in apoptotic cells, was detected only after 6 hours. This result suggests that the increased CD11b detected by Saxton et al. on non-stabilized and non-triggered cells, was not caused by apoptosis, rather spontaneous externalization of intracellular CD11b to the cell membrane. So before or during apoptosis the impact of activation should be considered.

We detected that CD11b expression changed in different directions over time, depending on the type of anticoagulant: it was over-expressed in samples collected in Na-heparin but decreased in samples collected in K_3-EDTA. Our results confirmed the second cause which can influence the change of CD11b expression during sample processing that is the type of the anticoagulant. Not only the cell viability is affected by the type of anticoagulant but also the binding of antibody to the antigen. Repo et al. found that the reason why the type of anticoagulant was able to influence the time-dependent alterations was that CD11b antibodies with D12 clones require divalent cations to bind. Our results support this explanation, as the MFI values were considerably lower in the presence of the cation chelator EDTA compared to samples drawn into heparin already on day 0 and decreased abruptly by day 1. Other CD11b clones (ICRF44) showed less of a reduction, and the type of clone did not influence the results in the case of samples collected in Na-heparin.
Furthermore, according to our results, the time-dependent changes of CD11b expression depend on the type of the sample. In bone marrow samples, CD11b expression on granulocytes did not change in samples collected in Na-heparin, and it decreased significantly on monocytes. In contrast, it increased significantly on granulocytes and monocytes in the peripheral blood samples anticoagulated with Na-heparin. This difference can be explained by the fact that the two examined cell population (neutrophils from peripheral blood and bone marrow) consist of different types of cells which have different amount of granules and intracellular CD11b pool. On the one hand, normal peripheral blood contains only mature myeloid cells, while bone marrow also contains immature variants. On the other hand, all peripheral blood samples were normal, while some of the bone marrow samples were pathological.
The majority of markers examined by us were not previously included in the studies focused on the impact of pre-analytical variables. Among these markers, probably the most important was the MFI of CD117, which was altered with both types of anticoagulants. Besides CD34 and CD45, CD117 is routinely used as a gating marker for determining MB percentage, which is currently the only FC-based parameter included in the recommendations of the WHO classification. Despite the obviously diminished intensity of CD117 in all samples on day 1 and day 2, the percentage of MBs was stable by day 1 and altered significantly only by day 2 in samples collected in K_3-EDTA.

It must be acknowledged that there are several limitations to the present study. We wanted to examine a homogenous population, but due to the heterogeneous nature of MDS, the patients enrolled with suspected MDS/MPN often ended up with a different final

diagnosis. Although, the detection of dysplastic signs play a key role not only in the diagnosis of MDS but also in MPN or AML; false interpretation of dysplastic signs on normal cells due to delayed sample processing can cause misdiagnosis of these cases. Furthermore, AML with myelodysplasia-related changes, which is an independent entity in the WHO classification, is associated with poor prognosis. Therefore the detection of dysplastic signs on normal cells or blast population is also important because it influences the treatment. Finally, we could only examine a limited number of cases, therefore further studies with large number of samples will be needed to validate our results.

In conclusion, we examined thirty-seven parameters on myeloid, erythroid and lymphoid populations, including mature and immature cell populations. We have already detected alterations in the initial immunophenotype depending on the type of anticoagulant. As dysplastic signs are identified as alterations compared to the normal pattern, the type of the anticoagulant should be always considered when comparing the samples to the patterns of normal samples. Thus for ease of reference, we recommended that only a single type of anticoagulant is used in any given laboratory.

The pre-analytical error of delayed sample processing can cause considerable immunohenotypic alterations, which can lead to the post-analytical error of false interpretation of the results. Therefore we recommend well-defined, standardized sample handling to avoid delayed sample processing. If the sample needs to be transported to a regional laboratory and delayed sample processing is inescapable, then heparin should be the preferred anticoagulant for flow cytometry, and more stable markers should be weighted more heavily in diagnosis.

5.2. Application areas of the six paramter FCSS

The most important findings of our study was that our FCSS based on only six, deliberately selected parameters can differentiate between normal, reactive and MDS cases, and it can even identify low-grade MDS affecting only one cell lineage. Our FCSS can provide information not only about myeloid but also about erythroid and lymphoid lineages. The results of the examination using four-color staining method proved to be reproducible by using eight-color staining method, so the type of the fluorescent dye and the settings of the flow cytometer did not influence the results. Our FCSS can be useful not only in the diagnosis but also in the prognostic classification of MDS. The stage of the disease correlated with the score. Previous studies have already established that the risk stratifications of their respective

FCSS are comparable to those of IPSS or R-IPSS. In our cohorts the correlation between R-IPSS and our six-parameter FCSS was similar to the results of previous studies. When we compare various FCSS and R-IPSS, two things must be taken into consideration: On the one hand, unlike the morphological examinations that R-IPSS relies on, FC proved to be a consistent and objective method for determining MB% in dysplastic bone marrow. On the other hand, the degree of cytopenia is a cardinal parameter of R-IPSS. In our study and in general practice, we did not restrict the MDS group to unsupported patients, some of them received blood cell and/or platelet transfusion within a few weeks. This influences the R-IPSS points of patients but not the scores in the six-parameter FCSS, which suggests that it can be applied in any phase of MDS, and the results of event-free survival supported this finding. In the past few decades several studies demonstrated that there are characteristic alterations compared to normal expression patterns, which can support the diagnosis of MDS. Yet no single specific marker exists for diagnosing all MDS cases due to the heterogeneity of the disease. Therefore several FCSS have been established, relying on the most common dysplastic signs. These FCSSs differed from each other in many respects. On the one hand, they focused on different cell types – most on myeloid population and some on erythroid precursors. There has been only limited attempts to investigate the megakaryocyte lineage. There is a considerable difference among the FCSS in the number of parameters, some using only a few (3-4), while others being more complex, exploiting the opportunities afforded by flow cytometry and going as high as 83 parameters. The FCSSs were established by examining patient groups of varying size and types. The specificity of these FCSSs is usually high (90-100%), while their sensitivity varies across a wide range (50-100%).

The guideline of ELN IMDSFlow highlights the "mini-panel" established by Ogata et al., which can reduce the costs of the diagnostic process through its screening potential. If we analyse the parameters of the "mini-panel" in more detail, we can understand the power of the "mini-panel" and its uniqueness compared to other scoring systems. After looking at several parameters, Ogata et al. selected four to serve as the basis for the "mini-panel". When the same ten bone marrow samples were examined in 3 different laboratories, by different settings and circumstances, the four parameters of the "mini-panel" proved to give the most reproducible results. If we look at the four parameters closely, we find the following: WHO also recommends the examination of one parameter (MB%) by flow cytometry. Two other parameters (granulocytes SSC/ lymphocytes SSC and lymphocyte/MB CD45 MFI ratio) were adjusted to lymphocytes, which made up the internal control population and were able to reduce the impact of differing circumstances of examination. The fourth parameter (LB ratio

within CD34 positive cells) determines the percentage of a progenitor cell population, just like the detection of MB%. For these reasons, the cut-off values of the “mini-panel” can be uniformly applied in all laboratories, as the values are not influenced by the different circumstances. In our study, the well-reproducible “mini-panel” was complemented by two parameters, which resulted in considerably higher sensitivity. Both parameters provide information about the altered functioning of the bone marrow, thereby enabling the diagnosis of this heterogeneous disorder by a larger number of patients. Considering that refractory anaemia is a classic and numerous subgroup within MDS ever since the definition of this syndrome, it is evident that the erythroid lineage is usually affected in MDS. This makes CD71 rCV% a suitable marker for screening patients for, even with the caveat that the cut-off level of CD71 rCV% is not stable, thus laboratories have to determine their own cut-off levels. Actually this also constitutes a limitation of our study, as our cut-off level is not readily applicable in other laboratories without previous adjustment. This was demonstrated by the fact that we also had to modify this cut-off level depending on the number of fluorochromes (85.8% for four-color labeling method vs. 115.5% for eight-color labeling method). Our other new parameter, MC%, have not been considered in the design of previous FCSS, despite the fact that methods other than MFC have already validated increased MC% in MDS as a useful diagnostic parameter. The detection of MC by flow cytometry does not require any extra markers compared to Ogata et al.’s “mini panel,” and thus it does not increase costs. Yet another advantage is that the cut-off level is not influenced by different settings.

In conclusion, we have designed a modified six-parameter FCSS derived from a single-tube MFC assay that can be a useful diagnostic and prognostic tool for the appropriate classification of patients with MDS. It enables the screening of MDS cases by providing direct information about each cell line, including myeloid, lymphoid, erythroid and mast cells, which translates to indirect information about the functional state of the bone marrow.

5.3. The impact of pre-analytical factors on the six-parameters FCSS

The type of the anticoagulant had a significant impact on the SSC of granulocytes, CD71 rCV% of erythroid precursors and MC%. When assessing these parameters, MC% was influenced the most by the type of anticoagulant: we have detected significantly higher values of this parameter in samples anticoagulated in K_3-EDTA. Percentage of CD34+ LB in CD34 positive cells, MC% and CD71 rCV% of erythroid precursors were stable in both anticoagulants.

In conclusion, it was established that samples collected in EDTA are the most suitable for the assessment of the six parameters of our FCSS, and a one-day delay in sample handling had no impact on the results gained from these samples.

5.4. Optimizing the application of MFC in the diagnosis and prognostic classification of MDS

Nowadays we have specific therapies available – such as hypomethylating agents – which can significantly improve not only the event-free but also the overall survival of patients with MDS. Two things must be considered in the application of the efficient but costly hypomethylating therapy: on the one hand, response to the therapy can be safely determined only after six months, therefore poor response will cause loss of valuable time for the patient. On the other hand, hypomethylating agents cannot eliminate the malignant clone, therefore discontinuing the therapy likely causes rapid progress of the disease. For these reasons, the precise diagnostic and prognostic classification – which serve as basis of therapeutic strategy – and the prediction of the potential therapy responses are the most important from the perspectives of both the patient and society. Beside classical methods, two types of diagnostic examinations, namely, flow cytometry and the detection of somatic mutation can supply useful information to serve this triple purpose. The detection of somatic mutations has undergone significant development lately, which is well illustrated by the fact that it was originally a recommended method in the ELN guidelines, but in 2016 the WHO selected 2 somatic mutations that should be tested in all cases. Furthermore, the 2017 guidelines of the international working group of MDS included several somatic mutations as organic components in the identification of pre-MDS states and MDS cases. This method was able to develop dynamically because it is not only an objective but also an easy-to-standardize

method. In comparison, we haven't seen a similarly rapid increase in the perceived significance of flow cytometry: it was already a recommended examination in the 2006 guidelines of IWG for MDS, and it retained the same status in the latest 2017 recommendations. Looking at the possible causes of this consistently limited role of even the most specific and sensitive FC scoring systems in the international guidelines, the following facts could be explored. Although there have been serious efforts to standardize and harmonize flow cytometry examinations, probably because of economic reasons, no widespread consensus could be reached regarding the type of sample, sample handling, the type of fluorochromes, or in terms of measurement and analysis standards. Furthermore, although this is an objective method, the correct interpretation of results involves a long learning curve, which is amply demonstrated by the breadth and depth of the most recent flow cytometry guidelines. To confirm the diagnosis of MDS, 58 immunophentotypic alterations are to be examined, where result positivity is not constituted by the presence or absence of a certain marker but rather by the decreased or increased intensity of a certain marker's expression, or an atypical expression pattern. To address these complexities, analysing software that can archive the results of several normal samples have been developed, and these unified archived patterns serve as the basis for comparison with patient samples. Such software can even directly indicate alterations with a specific numerical value, indicating how many times the standard deviation a marker's expression in a specific patient sample is from the average of the normal samples. This was analysing software makes the analysis simpler and unequivocal, and if laboratories create and use their own reference database from normal samples, then a standard interpretation protocol could be developed and applied even amongst the varying sample processing circumstances. With our studies we wanted to facilitate the widespread application of flow cytometry, in support of the goals of the International Working Group on MDS. On the one hand, we wanted to highlight the importance of standardization by examining immunophenotypic alterations caused by pre-analytical factors; on the other hand, we also attempted to contribute to the establishment of even more effective guidelines. Furthermore, we entertained the hypothesis that by archiving the results of normal samples by novel analyzing software, in time the user may be able to select such variables for their reference as the type of the anticoagulant and the time elapsed between sample collection and processing, thus making the interpretation of results more reliable and meaningful.

6. Summary

MDS is a heterogeneous disease with understandably variable clinical outcomes. Flow cytometric examination can provide useful contributions to the diagnosis, prognostic assessment, and the anticipation of the therapy's effectiveness. Our objectives were assessing the impact of pre-analytical factors on immunophenotype and improving the sensitivity of the "mini-panel" applied for the screening of MDS.

Of pre-analytical factors, we examined the immunophenotypic alterations dependent on the type of anticoagulant by immediate and delayed sample handling. Applying a wide spectrum of markers, we analyzed not only the myeloid but also the lymphoid and erythroid cell lineages and even rare events. In accordance with the recommendations of ELN, samples anticoagulated with Na-heparin showed fewer immunophenotypic alterations as a consequence of delayed sample handling. We also demonstrated that the clone of the antibody can influence the immunophenotype and its alterations.

Complementing the „mini-panel" with two additional parameters (CD71 rCV%, mastocytes %) resulted in a more sensitive flow cytometric scoring system that can identify even low-risk MDS patients efficiently. Examining the system's parameters with either 4- or 8-color staining method does not influence the applicability of the system. Furthermore, detecting the parameters remains reliable even with a 1-day delay in sample processing, regardless of the type of anticoagulant. Nevertheless, the bone marrow samples collected in K_3-EDTA proved to be the most applicable to detection of mast cells, therefore it is recommended to collect samples in K_3-EDTA for the determination of the "mini-panel" based on six parameters. Our amended system can contribute not only to the diagnosis but also the prognostic assessment of MDS cases.

With our study, we intended to support the International Flow Cytometric Working Group in their efforts to standardize and promote flow cytometric examination in the diagnosis of MDS.

7. Main new scientific findings and their clinical significance

1. To the best of our knowledge, we were the first to examine a wide range of markers (37 parameters) on different cell types by eight colour labeling FC method. We found that the type of anticoagulant significantly influenced the rate and number of alterations caused by delayed sample handling. We identified the markers that remain stable and sensitive over time.
2. The type of anticoagulant influenced the initial antigen expression patterns, where apoptotic cells were absent, or present only in very small quantities.
3. We clarified the reason behind contradictory findings in literature about the change of the intensity of CD11b expression. Our results confirmed that the type of the anticoagulant, the type of the sample (peripheral blood or bone marrow), and the clone of the antibody used in the examination influence the intensity of CD11b expression in case of delayed sample handling.
4. Confirming our hypothesis we found that by complementing the “mini-panel” with two parameters – CD71 rCV% and MC% – the sensitivity of the scoring system increased, while the specificity remained high.
5. The results of the “mini-panel” established by four-color staining method proved to be reproducible by eight-color staining method, so the results were not influenced by the setting of the flow cytometer and the type of fluorescence dyes.
6. We validated that our FCSS has not only diagnostic but also prognostic significance in relation to MDS.
7. The results of the four-parameter “mini-panel” were not influenced by the type of anticoagulant and a one-day delay in sample handling.
8. Our results have demonstrated that the most suitable sample type for MC detection by flow cytometer is bone marrow sample collected in K_3-EDTA. Therefore this type of anticoagulant is preferred in the determination of the six parameters of the extended “mini-panel”, which proved to be stable for a day.

8. List of publications

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Registry number: DEENK/278/2018.PL
Subject: PhD Publikációs Lista

Candidate: Bettina Kárai
Neptun ID: Y6ALN3
Doctoral School: Kálmán Laki Doctoral School

List of publications related to the dissertation

1. **Kárai, B.**, Miltényi, Z., Gergely, L., Széles, M., Kappelmayer, J., Hevessy, Z.: The impact of delayed sample handling and type of anticoagulant on the interpretation of dysplastic signs detected by flow cytometry.
   Biochem Med (Zagreb). 28 (2), 1-13, 2018.
   IF: 3.653 (2017)
2. **Kárai, B.**, Bedekovics, J., Miltényi, Z., Gergely, L., Szerafin, L., Ujfalusi, A., Kappelmayer, J., Hevessy, Z.: A single-tube flow cytometric procedure for enhancing the diagnosis and prognostic classification of patients with myelodysplastic syndromes.
   Int. J. Lab. Hematol. 39 (6), 577-584, 2017.
   DOI: http://dx.doi.org/10.1111/ijlh.12700
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3. **Kárai, B.**, Szánthó, E., Kappelmayer, J., Hevessy, Z.: Flow cytometry in the diagnosis of myelodysplastic syndromes.
   E-JIFCC. 23 (4), [1-8], 2012.

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List of other publications

4. Szánthó, E., **Kárai, B.**, Ivády, G., Bedekovics, J., Szegedi, I., Petrás, M., Ujj, G., Ujfalusi, A., Kiss, C., Kappelmayer, J., Hevessy, Z.: Comparative Analysis of Multicolor Flow Cytometry and Immunohistochemistry for the Detection of Disseminated Tumor Cells.
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5. **Kárai, B.**, Hevessy, Z., Szánthó, E., Csáthy, L., Ujfalusi, A., Gyurina, K., Szegedi, I., Kappelmayer, J., Kiss, C.: Expression of Coagulation Factor XIII Subunit A Correlates with Outcome in Childhood Acute Lymphoblastic Leukemia.
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DOI: http://dx.doi.org/10.1007/s12253-017-0236-0
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6. **Kárai, B.**, Jakó, J., Szánthó, E., Kappelmayer, J., Hevessy, Z.: A myelodysplasiás szindróma diagnosztikája, a prognózis megítélése.
Hematol. Transzfuziol. 46 (2), 93-102, 2013.

Total IF of journals (all publications): 9,549
Total IF of journals (publications related to the dissertation): 5,572

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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