Exploration of Flow-Cytometric Methods for the Study of Dyserythropoiesis. A book of Science, Fiction, and Greek Philosophy.

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Exploration of Flow-Cytometric Methods for the Study of Dyserythropoiesis

A book of Science, Fiction, and Greek Philosophy

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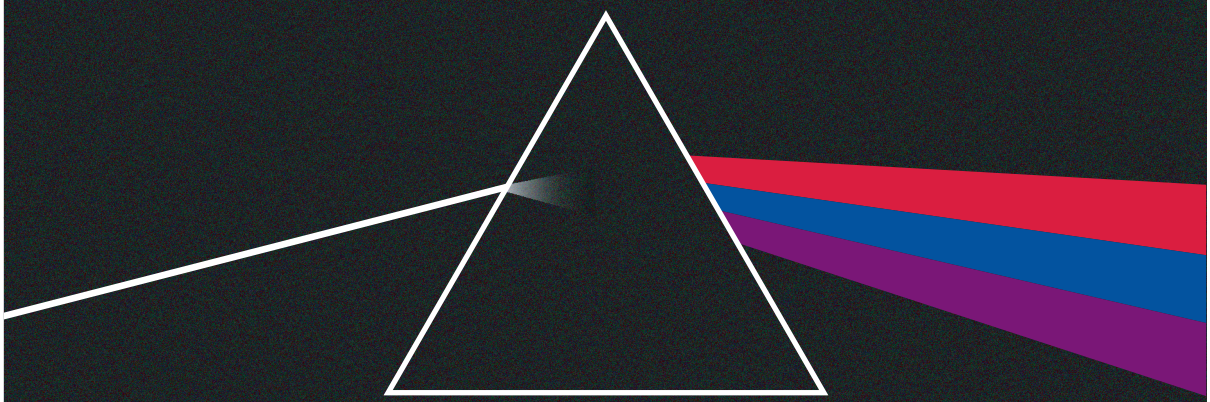

FC has been romantically involved with AML, but they have recently agreed to make their relationship open. AML has already started dating the twins qPCR and dPCR. FC has been liking AML's cousin, MDS, for quite some time now; she actually gets so excited when she meets him that she just can't hide it. But, MDS is complicated and full of syndromes that make the way to his heart hard to find. To make matters worse, a new threat has arrived in town: NGS, irresistible thanks to her perfect genes, is out to take over the field.

Join us in this book of science, fiction, and Greek philosophy to discover whether the romance between FC and MDS will reach its happy ending.

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Exploration of Flow-Cytometric Methods for the Study of Dyserythropoiesis

A book of Science, Fiction and Greek Philosophy

Despoina Violidaki

LUND UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 30th January 2026 at 09.00 a.m. in Föreläsningssalen, Department of Clinical Pathology, Lund, Sweden

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Title and subtitle: Exploration of Flow-Cytometric Methods for the Study of Dyserythropoiesis. A book of Science, Fiction, and Greek Philosophy.

Abstract:

Myelodysplastic syndromes/neoplasms (MDS) are heterogeneous clonal disorders of the hematopoietic stem cell, characterized by ineffective hematopoiesis and an increased risk of progression to acute myeloid leukemia. Diagnosis is based on the presence of cytopenias, morphologic dysplasia in bone marrow (BM) and genetic aberrancies. Flow cytometry (FC) has proven helpful in MDS diagnosis, as well as in prognostication and prediction. In this context, erythropoiesis has been less explored. Also, most FC studies applied lysis-based protocols, adversely affecting erythroblasts.
In Study I, we developed a non-lysis approach, the ERY panel, for the study of erythropoiesis. BM population sizes obtained with this panel showed better agreement with the morphological counts. In contrast, lysis-treated samples showed a prominent effect on the percentage, side scatter and marker expression of erythropoiesis. Analyzing BM from 68 MDS patients, 43 non-clonal cytopenias and 29 normal BM, we identified MDS-defining aberrancies: lower mean fluorescence intensity (MFI) and higher coefficient of variation (CV) for CD36, and higher CV for CD105.
In Study II, Imaging Flow Cytometry (IFC) was used to translate morphologic hallmarks of dyserythropoiesis into objective, measurable parameters. By comparing 26 MDS BM samples with 12 normal BM, cytoplasmic complexity, megaloblastoid changes and abnormally formed nuclei, including binucleated forms, were defined as MDS features.
In Study III, we applied an unsupervised clustering algorithm, Flow Self-Organizing Maps (FlowSOM), to BM from 11 MDS patients and five non-clonal cytopenias and compared the detected clusters with a reference normal BM established in a previous study. Eighteen aberrant clusters were identified, characterized mainly by aberrant CD36 and CD71 expression.
In Study IV, FlowSOM was applied to 34 MDS and 11 normal BM. Decrease of the immature erythroid compartment and two mature clusters with aberrant CD36 and CD71 expression were the most discriminating. Their combination defined five FC patterns; increasing complexity correlated with the number of gene mutations. During treatment with azacitidine, increasing FC improvement was observed over time.
In conclusion, these studies establish the importance of dyserythropoiesis in MDS diagnosis, demonstrate the robustness of a non-lysis approach, and provide a foundation for the application of the less explored methods IFC and FlowSOM.

Key words: myelodysplastic neoplasms, myelodysplastic syndromes, erythropoiesis, dyserythropoiesis, flow cytometry, imaging flow cytometry, unsupervised cluster analysis, self-organizing maps, FlowSOM

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MADE IN SWEDEN

To the real explorers of the family,
my children

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I. **Violidaki D**, Axler O, Jafari K, Bild F, Nilsson L, Mazur J, Ehinger M, Porwit A. Analysis of erythroid maturation in the nonlysed bone marrow with help of radar plots facilitates detection of flow cytometric aberrations in myelodysplastic syndromes. *Cytometry B Clin Cytom.* 2020;98(5):399-411.

II. **Violidaki D**, Axler O, Nilsson L, Porwit A, Ehinger M. Translation of the Morphological Hallmarks of Dyserythropoiesis to Objective Morphometric Parameters by Imaging Flow Cytometry. *Int J Lab Hematol.* 2025;47(6): 1089–1098.

III. Porwit A, **Violidaki D**, Axler O, Lacombe F, Ehinger M, Béné MC. Unsupervised cluster analysis and subset characterization of abnormal erythropoiesis using the bioinformatic Flow-Self Organizing Maps algorithm. *Cytometry B Clin Cytom.* 2022;102(2):134-142.

IV. **Violidaki D**, Béné MC, Porwit A, Nilsson L, Ehinger M. Unsupervised cluster analysis of erythropoiesis in flow cytometric follow-up of high-risk MDS patients during treatment. *Manuscript.*

The following article is not included in this thesis but was used as a background for Studies III and IV:

Béné MC, Axler O, **Violidaki D**, Lacombe F, Ehinger M, Porwit A. Definition of Erythroid Differentiation Subsets in Normal Human Bone Marrow Using FlowSOM Unsupervised Cluster Analysis of Flow Cytometry Data. *HemaSphere.* 2021;5(1):512

Abbreviations

5-aza	5-azacitidine
AML	Acute myeloid leukemia
ALL	Acute lymphoblastic leukemia
BM	bone marrow
BF	brightfield
CD	Cluster of differentiation
CMML	Chronic myelomonocytic leukemia
FC	Flow cytometry
FISH	Fluorescence in situ hybridization
FSC	forward scatter
G-CSF	Granulocyte colony - stimulating factor
HSCT	Hematopoietic stem cell transplantation
ICC	International Consensus Classification
IFC	Imaging flow cytometry
IPSS	International Prognostic Scoring System
MDS	Myelodysplastic syndromes/neoplasms
MFC	Multiparameter flow cytometry
MFI	Mean fluorescence intensity
MRD	Measurable residual disease
MyP	Myeloid progenitors
NBM	Normal bone marrow
NGS	Next generation sequencing
PB	peripheral blood
SSC	side scatter
WHO	World Health Organization

Nomenclature

There is considerable heterogeneity in the literature regarding the nomenclature of hematopoietic cell types. Looking back at my own articles, I found that I was not entirely consistent in my use of terminology either. I will therefore try harder in this thesis.

Erythropoiesis: Technically, the term refers to the *process* of production of red blood cells; however, I (and many others) use it to refer to the cells that comprise this lineage, aka the erythroid precursors or erythroblasts.

Erythroid precursors (or progenitors). The hematopoietic cells responsible for the production of red blood cells. This is the preferred term in flow cytometry. Designations like nucleated erythroid cells, nucleated red cell precursors or immature red blood cells can also be met in literature contributing to confusion.

Erythroblasts: The morphological counterpart of erythroid precursors.

Immature erythroid compartment: Collectively, the $CD117^{+}/CD105^{+}$ and the $CD117^{-}/CD105^{+}$ erythroid precursors, corresponding to the morphological proerythroblasts and basophilic erythroblasts respectively.

Mature erythropoietic compartment: The $CD117^{-}/CD105^{-}$ erythroid precursors corresponding to the morphological polychromatic and orthochromatic erythroblasts.

Erythrocytes: The enucleated red blood cells.

Myelopoiesis: In this thesis, I use this word to collectively refer to the granulocytic and monocytic lineage. (In a broader sense that I do not embrace, the word can be used for the total of hematopoietic bone marrow cells.)

Myeloid progenitor cells (MyP): The $CD34^{+}$ progenitors, corresponding to the morphological blasts, including mainly – but not exclusively – the *myeloblasts*.

Introduction

MDS

Syndrome (σύνδρομο): A set of medical signs and symptoms which are correlated with each other and often, but not always, are associated with a particular disease or disorder.

Dysplasia (δυσπλασία): In the microscopic level, the word refers to the abnormal growth of cells, implying a clonal or preneoplastic nature. The term has erroneously been used interchangeably with "atypia", the latter meaning deviation from the typical, the normal.

It was not until 1976 that the French-American-British group (FAB), in their seminal article *Proposals for the Classification of the Acute Leukaemias,*[1] described

> a range of less acute disorders usually presenting in persons above 50 years of age with symptoms of variable duration that were described collectively as the dysmyelopoietic or myelodysplastic syndromes (MDS); for these patients, urgent treatment was not indicated.

Until then these disorders were mostly referred to by the term *preleukemia.*[2–4]

The word *syndrome* was wisely coined by the FAB group to reflect the complexity of this group of disorders in terms of clinical manifestations, diagnosis and prognosis. This initial description was expanded to result in the first classification of Myelodysplastic Syndromes in 1982.[5] The five entities included in the initial FAB classification (Table 1) were based on morphologic description of dysplasia and percentage of blasts in BM; monocyte count in PB was also considered as chronic myelomonocytic leukemia (CMML) was also included in this classification.

A lot has happened since. The classification mission was taken over by the World Health Organization (WHO), which refined classification criteria, added new entities and updated existing ones; and international prognostic systems were proposed to facilitate risk stratification.

Fast forward and we are in 2022. Today there are not one but two classification systems: the 5th edition of the WHO *Classification of Haematolymphoid Tumours*[6] and the *International Consensus Classification of Myeloid and Lymphoid Neoplasms*[7] (Table 1). And both decided that it was time to replace the term *myelodysplastic syndromes*—in use for 40 years—with *myelodysplastic neoplasms*, to better reflect the clonal nature of the disease. Thankfully, they retained the historical abbreviation *MDS*.

Table 1. Classification systems of MDS, then and now

FAB 1982	WHO 5th edition 2022	ICC 2022
Refractory anemia (RA)	**MDS with defining genetic abnormalities**	**MDS with defining genetic abnormalities**
RA with ring sideroblasts (RARS)	MDS with low blasts and del(5q)	MDS with del(5q)
RA with excess blasts (RAEB)	MDS with low blasts and *SF3B1* mutation	MDS with mutated SF3B1
Chronic myelomonocytic leukemia (CMML)	MDS with biallelic *TP53* inactivation	MDS with mutated TP53
RAEB in transformation (RAEB-T)	**MDS, morphologically defined**	**MDS, no otherwise specified**
	MDS with low blasts	Without dysplasia
	MDS, hypoplastic	With single lineage dysplasia
	MDS with increased blasts	With multilineage dysplasia
	MDS with fibrosis	With excess blasts
		MDS/AML
		With mutated *TP53*
		With MDS-realted gene mutations
		With MDS-related cytogenetic abnormalities
		No otherwise specified

So, what is MDS?

Myelodysplastic syndromes/neoplasms (MDS) are a heterogeneous group of clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, resulting in one or more cytopenias, morphologic dysplasia in the bone marrow, and an increased risk of progression to acute myeloid leukemia (AML).[6,7] MDS has an annual incidence of 4 cases per 100 000. Patients with MDS have a median age of 77 years at diagnosis, while patients younger than 50 years comprise <10%.[8,9]

Today, the somatic genomic landscape of MDS is well defined: nearly all patients carry at least one driver lesion, involving pathways such as epigenetic regulation, RNA splicing, transcriptional control, DNA repair, cohesin function, and signal transduction, leading to impaired maturation and increased apoptosis of hematopoietic

precursors.[10–12] Germline predisposition syndromes are now also recognized in up to 15% of MDS cases.[10,13,14]

The prevailing view in the clonal evolution of MDS identifies clonal hematopoiesis (CH) as a steppingstone, i.e. driver mutations in the multipotent stem/progenitor cell, frequently found in healthy older individuals without the clinical phenotype of MDS. These CH clones are considered to predispose to progression to MDS and AML; their expansion and/or accumulation of additional mutations ultimately promote leukemogenesis. The most frequently reported mutations in CH occur in the key epigenetic modifiers *DNMT3A*, *TET2*, and *ASXL1*.[15–17]

Apart from the genetic aberrations, the role of inflammation in MDS pathogenesis has also been underscored. The interplay between immune dysregulation and clonal hematopoiesis is complex. Chronic inflammation plays a central role, driven by abnormal cytokine secretion – particularly TNF-α, IL-6, and IL-1β – which promotes apoptosis, ineffective hematopoiesis, and further immune activation. Somatic mutations common in MDS (e.g., *TET2, DNMT3A, ASXL1, SF3B1, TP53*) interact with the inflammatory signaling and disturb macrophage and stromal cell function, further contributing to disease progression. The bone-marrow microenvironment, including stromal cells, macrophages, and endothelial cells, sustains and augments the inflammatory reaction and supports clonal selection.[18–22]

Clinical presentation and differential diagnosis

The clinical manifestations of MDS are variable and depend directly on the type and severity of the cytopenias. Fatigue is the most common symptom of anemia; other anemia-related symptoms include dizziness, weakness, angina, and loss of appetite. Infections may develop as a consequence of neutropenia, while thrombocytopenia may lead to bleeding, easy bruising, and petechiae. General symptoms such as fever, weight loss, and night sweats are uncommon and may indicate disease progression. Nevertheless, some patients remain asymptomatic, and their cytopenias are detected incidentally during routine medical examinations.

The differential diagnosis of MDS is broad and includes conditions and disorders that can manifest with cytopenia and/or dysplasia:

Nutritional deficiencies (iron, vitamin B12, folate, copper); hypothyroidism; drugs including cytotoxic and myeloablative regimens; toxic agents (alcohol and heavy metal exposure); infections (Parvovirus B19, human immunodeficiency virus); hemolytic anemia and peripheral cell destruction.

Bone marrow failure syndromes and clonal disorders: congenital disorders (more relevant in the pediatric population; sideroblastic anemia; aplastic anemia (AA); paroxysmal nocturnal hemoglobinuria (PNH); cytopenia of undetermined significance (CCUS); and overt myeloid neoplasms, such as acute myeloid leukemia (AML) and myelodysplastic/myeloproliferative neoplasms (MDS/MPN).

Diagnosis

Despite the changes in names and classification systems over time, diagnosis of MDS has always been based on i) the presence of persistent, unexplained cytopenia(s), ii) morphologic dysplasia in BM and PB, iii) genetic abnormalities. Flow cytometry is considered helpful adjunct for diagnosing MDS, but its role as a primary diagnostic tool in this context remains unclear.

Laboratory tests

Cytopenia in at least one hematopoietic lineage is required for a diagnosis of MDS. The cytopenia-defining values are: hemoglobin (Hb) <13 g/dL in male patients and <12 g/dL in female patients; absolute neutrophil count $<1.8 \times 10^9$/L; and platelets $<150 \times 10^9$/L.[23]

Anemia in MDS is usually macrocytic, reflected by the increase in mean corpuscular volume (MCV); reticulocyte count is usually not increased.

Other tests that are essential in the diagnostic work-up of MDS to exclude other causes of cytopenia include iron studies; folate and vitamin B12 levels; liver and kidney function tests to exclude underlying disease; inflammatory markers (C-reactive protein and erythrocyte sedimentation rate); viral testing for hepatitis B and C, cytomegalovirus, human immunodeficiency virus and parvovirus B19 infections.[24]

Morphologic assessment

The morphologic hallmark of MDS is dysplasia; a threshold of 10% of cells in the respective lineage is used to define significant dysplasia. Dysplasia in at least one hematopoietic lineage is required for a diagnosis of MDS.[25–27] The dysplastic features per lineage are presented in Figure 1 and Table 2.

The gold standard for the morphologic assessment of dysplasia is the evaluation of BM smears; the blast count and presence of ring sideroblasts are also assessed in smears. Trephine biopsies add additional information regarding cellularity, overall architecture and fibrosis; megakaryocytic dysplasia is also better appreciated in biopsies or clots.

Biopsies can also reveal BM engagement by non-hematopoietic disorders, like metastatic or inflammatory infiltrations.

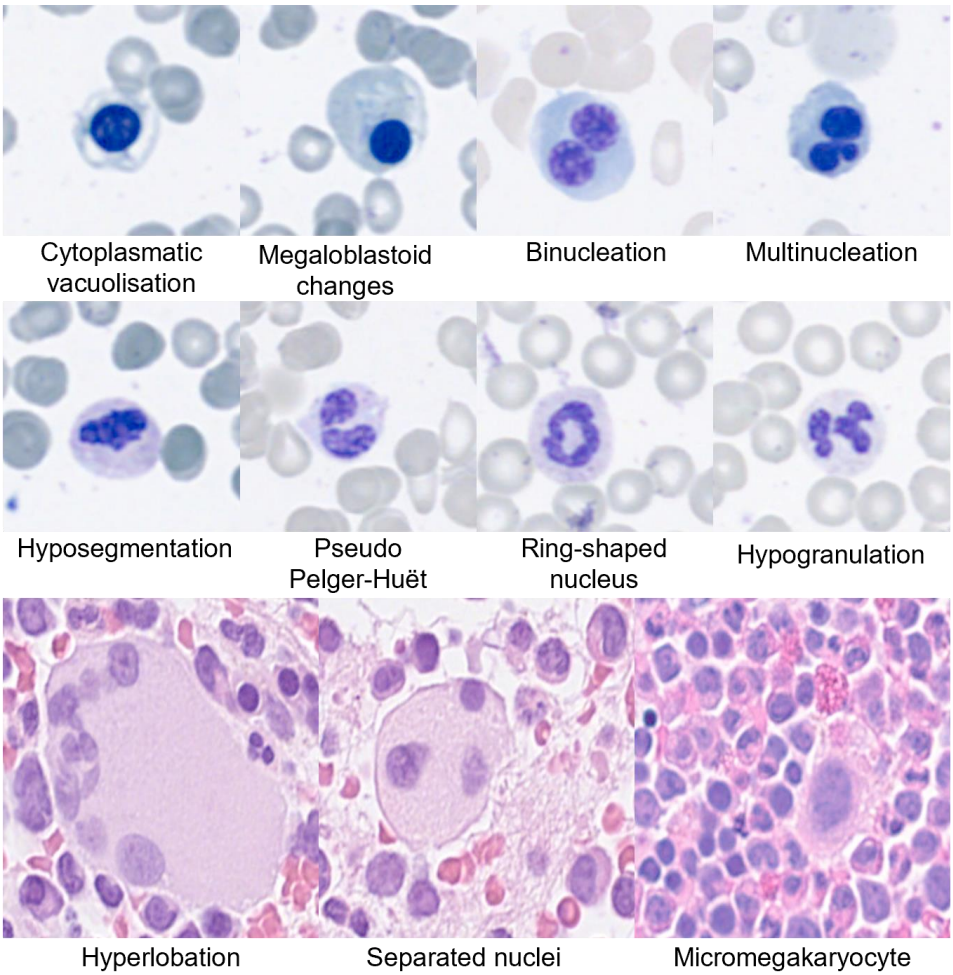

Figure 1. Moprhological features of dysplasia

Table 2. Morphologic features of dysplasia

Lineage	
Erythropoiesis	Ring sideroblasts
	Cytoplasmic vacuolization
	Megaloblastoid changes
	Bi- and multinucleation
	Nuclear budding
	Internuclear bridging
Myelopoiesis	Abnormal nuclear segmentation: hyper- or hyposegmentation (pseudo-Pelger-Huët); ringed-shaped nuclei
	Hypo- or hypergranulation
	Auer rods
Megakaryopoiesis	Micromegakaryocytes
	Hypo- or hyperlobation
	Multiple separated nuclei

Genetic abnormalities

Cytogenetic abnormalities based on karyotyping and FISH analyses are detected in about 50% of patients with MDS.[28,29] The most common of them include unbalanced abnormalities, like +8, -7/del(7q), del(5q), del(20q), -Y, i(17p), -13/del(13q), del(11q), del(12p)/t(12p), del(9q), idic(X)(q13), and balanced ones, like t(1;3)(p36;q21), t(2;11)(p21/q23), inv(3)(q21;q26) and t(6;9)(p23;q34).

Of note, some of these, namely -Y, del(20q) and +8, are not sufficient for an MDS diagnosis in the absence of dysplasia. Conversely, the translocations t(8;21), t(15;17) and inv(16)/t(16;16) exclude MDS and establish an AML diagnosis.[28,29]

The diagnostic accuracy of MDS has increased significantly after the introduction of DNA sequencing methods. Genetic variations can be detected in up to 95% of MDS patients. Over 50 recurrent gene mutations have been identified in MDS. Many are not specific to the disease but occur in other myeloid neoplasms, and a subset has limited or uncertain clinical significance. The three most recurrent genes are *TET2*, *ASXL1*, and *SF3B1,* mutated in at least 20% of MDS patients, followed by *DNMT3A*, *SRSF2*, *RUNX1*, and *TP53* which are detected in 10% to 20% of cases.[10,11] The number of mutations related to MDS is expected to raise as the capacity of the sequencing platforms increase.

The ICC has recommended a gene panel for the investigation of MDS and MDS/MPN,[7] presented in Table 3.

Table 3. Genes recommended by the ICC for the investigation of MDS and MDS/MPN

Gene Function category	Genes
Histone modification	*ASXL1, EZH2, PHF6, BCOR, BCORL1, KMT2A-PTD*
DNA methylation	*TET2, DNMT3A, IDH1, IDH2*
RNA splicing	*SF3B1, SRSF2, U2AF1, ZRZS2, PRPF8*
DNA repair control	*TP53, PPM1D, RAD21*
Signaling	*CBL, KRAS, NRAS, JAK2, PTPN11, GNB1, FLT3-ITD, FLT3-TKD, NF1, ETNK1, KIT*
Transcription factors	*RUNX1, CEBPA, GATA2, WT1, ETV6*
Cohesin complex	*STAG2*
Other	*SETBP1, DDX41, NPM1, UBA1, CSF3R*

Although molecular techniques have significantly improved diagnostic accuracy in MDS, conventional cytogenetics remain mandatory because of their prognostic implications.

Prognosis

There are two international prognostic scoring systems (IPSS) applied today in MDS, the Revised IPSS (IPSS-R), and the molecular IPPS (IPSS-M).

The IPSS-R is the revised version of the original IPSS that was established in 1997.[30,31] The prognostic factors included in this scoring system are hemoglobin, absolute neutrophil count (ANC) and platelet counts in PB; percentage of myeloid blasts in BM; and cytogenetic risk group. The latter is a 5-tiered system allocating the cytogenetic abnormalities in five risk groups, presented in Table 4.

Table 4. Cytogenetic risk groups according to IPSS-R.

Prognostic groups	Cytogenetic abnormalities
Very good	-Y, del(11q)
Good	Normal, del(5q), del(12p), del(20q), double including del(5q)
Intermediate	del(7q), +8, +19, i(17q), any other single or double independent clones
Poor	−7, inv(3)/t(3q)/del(3q), double including −7/del(7q), complex: 3 abnormalities
Very poor	Complex: > 3 abnormalities

The patients are subsequently stratified into five groups based on the risk of mortality and transformation to acute myeloid leukemia (AML): very low, low, intermediate,

high, and very high risk. Importantly this scoring is applied only at diagnosis, in untreated patients, since treatment can alter the measured factors.

The IPSS-M was developed in response to the growing need to integrate accumulating molecular prognostic information into MDS risk stratification.[32] This system practically uses IPSS-R as a background, applying almost the same factors – i.e. cytogenetic risk, myeloid blasts, hemoglobin and platelets but not the neutrophil count – and incorporates the molecular information, based on 31 genes. These are divided in two categories with different weights: the driver, "main effect" genes with higher weight, including *ASXL1, CBL, DNMT3A, ETV6, EZH2, IDH2, KRAS, NPM1, NRAS, RUNX1, SF3B1, SRSF2,* and *U2AF1*; and the "residuals" genes (*BCOR, BCORL1, CEBPA, ETNK1, GATA2, GNB1, IDH1, NF1, PHF6, PPM1D, PRPF8, PTPN11, SETBP1, STAG2*, *WT1*), of lower prognostic impact. *TP53* mutation number and Loss of heterozygosity, MLL PTD and FLT3-ITD or TKD are treated as separate categories. This system applies six prognostic categories: very low, low, moderate low, moderate high, high, and very high risk.

In the original study introducing IPSS-M, 2678 patients, originally stratified with IPPS-R, were revaluated with IPSS-M; 46% of them were restratified, 74% of whom to a higher risk group and 26% to a lower one.[32]

Both systems are included in the national recommendations and guidelines worldwide. Increasing evidence however highlights the superiority of IPSS-M in terms of outcome prediction.[33]

Practical and user-friendly online calculators are nowadays available for both systems.

Treatment

There are two therapeutic directions in MDS, depending on the risk evaluated by the scoring systems: lower and higher risk. The lower-risk category includes patients with very low and low risk according to IPSS-R, while the higher risk category includes patients with high and very high risk. For patients with IPSS-R intermediate risk, treatment decisions are based on the severity of cytopenias, symptoms, transfusion needs, and presence of specific genetic markers.[34]

Treatment in lower-risk MDS

Treatment in this group aims primarily to improve blood counts, reduce symptoms and transfusion needs, avoid complications, and maintain quality of life, rather than curing the disease. Supportive care including transfusions, iron chelation and infection management are essential. Erythropoiesis-stimulating agents are the first-line treatment

for anemia, particularly in patients with low transfusion requirements and low endogenous erythropoietin (EPO) levels.[35] Combination with G-CSF in selected cases can have a synergistic effect.[36] Regarding thrombocytopenia, platelet transfusion has limited utility and is associated with transfusion-related adverse effects; thrombopoietin receptor agonists do not yet have a clear role in MDS treatment.[37] Lenalidomide, an immunomodulatory agent, is the preferred option for patients with isolated del(5q). Promising evidence supports the role of luspatercept – a protein inhibiting SMAD signaling and thereby accelerating erythroblast maturation – in lower-risk MDS.[38] In carefully selected settings, hypomethylating agents (HMA) can be considered for patients with lower risk MDS; this therapeutic option, however, is approved in the USA but not in Europe.[39]

Treatment in higher-risk MDS

The primary therapeutic goal in this group is to extend survival and delay or prevent progression to AML. Allogeneic stem cell transplantation remains the only curative option and should be considered early for eligible patients, based on age, comorbidity, performance status, and genetic risk.[40] For those who are not transplant candidates, HMA, namely azacitidine (5-aza) and decitabine, constitute the first-line treatment for long-term management. HMA inhibit DNA methyltransferases, reduce abnormal DNA methylation, reactivate silenced genes, and promote differentiation of myeloid cells.[41] For patients that do not respond or develop resistance to HMA, Venetoclax, an inhibitor of the anti-apoptotic protein BCL-2, can be considered.[42] Chemotherapy regimens similar to those used in AML (such as an anthracycline in combination with cytarabine) can also be considered, especially for younger patients, usually before allo-HSCT.[43]

In 2006, the International Working Group (IWG) for treatment response in MDS has defined four clinical response categories (complete, partial response, stable disease and progression) based on complete blood count, blast count in BM and PB and presence of dysplasia.[44,45] A revised version of the IWG criteria was published in 2023 proposing flow cytometry and molecular testing as provisional categories.[46]

Take home

The remarkable article of the FAB group about the classification of MDS: *Proposals for the classification of the myelodysplastic syndromes.*[5] In just 11 pages and citing only 7 references, without any statistical analyses – only sharp observations and a concise morphologic description of *dyspoiesis* – their work laid the foundation for the MDS classification still in use today.

Flow Cytometry

⚠ Warning! Lasers! Technical terms! Difficult words!

Incident light: The light that touches an object or medium.

Solid-state laser: Lasers whose gain medium consists of a transparent solid material (e.g. crystal, glass or ceramics) doped with optically active ions. When the medium is exposed to an external energy source (optical pumping), usually from laser diodes, its dopant ions are excited to higher energy states. As these ions relax back to lower energy levels, they emit photons of a characteristic wavelength.

Intercalation: The insertion of a molecule into layered materials.

Flow cytometry (κυτταρομετρία ροής): A technique that uses the properties of light (incident and fluorescence) to analyze cells[a] aligned in single file, allowing measurement of multiple cellular characteristics.[b]

This section focuses on the technical components of FC, specifically the fluidic, optical, and electronic systems.[c] Although sample preparation and data analysis constitute equally important elements of FC, they fall outside the scope of this discussion.

Fluidics

Fluidics refer to the flow of cells in a single-cell alignment, which is the key for the successful analysis of individual cells.

The fluid dynamics behind this process, called *hydrodynamic focusing*, are fascinating: the cell suspension is injected into the center of a sheath fluid under higher pressure,

[a] In practice, the technique can be applied to both cells and particles; for the sake of simplicity, only the cells is used throughout this book.

[b] Nowadays, the term Multiparameter Flow Cytometry (MFC) is widely used to refer to FC, emphasizing the technique's ability to enable multiple simultaneous measurements. (M can also stand for multi-colored or multidimensional). However, since the aim of this section is to present the general principles of FC, it will be referred to simply as flow cytometry.

[c] This section was based mainly on textbooks[48–50], a few review articles[51–55] and the accessible and much appreciated education material that can be found on manufacturers' websites (Thermo Fisher Scientific, BD Biosciences).

and consequently, at higher velocity. Both the cell suspension and the surrounding sheath fluid enter a narrower channel resulting in their acceleration, but with different velocity. This difference of flow velocity prevents the two fluids from mixing with each other while simultaneously causing the cells to separate and align in single file, forming the characteristic flow needed for cell interrogation (Figure 2).

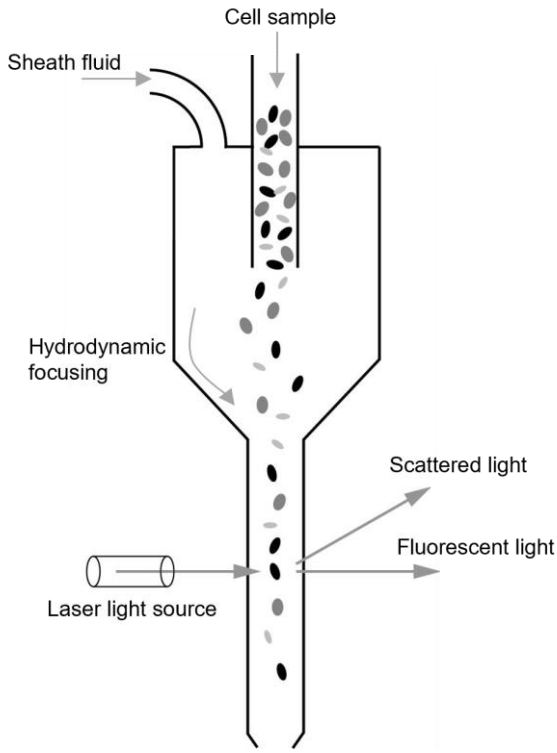

Figure 2. Fluidics in Flow cytometry.

Optics

The point where the flowing cells reach the light source is called the *interrogation point*. There, they are illuminated by *lasers* of different wavelengths, which serve both as optical light sources and excitation sources. The cells modify the properties of the incident light, while the *fluorescent probes* carried by the cells are excited by the light energy. The *scattered light* and the resulting *fluorescent emissions*, after being redirected through *filters*, are subsequently collected by *detectors*.

Lasers

Lasers are the mainstream light sources in FC. They provide a uniform, stable and energetic (high intensity) beam of a specific, very narrow wavelength.

The most common laser wavelengths used are 488 nm (blue), 405 nm (violet), 561 nm (green-yellow), 633 nm (red) and 355 nm (ultra-violet). Other laser wavelengths are available for specialized applications. Commercially available flow cytometers are typically equipped with blue and red lasers by default; a violet laser is often included in clinical applications. In research settings, many manufacturers offer the flexibility to replace existing lasers and/or add additional ones, in some instruments up to an impressive number of nine in total.

Solid-state lasers have to a great extent replaced the older gas lasers, thanks to their high-power output, precision and stability of the beam and their longer lifetime.

Light emitting diodes (*LEDs*) are gaining interest as excitation sources thanks to their lower cost and energy consumption and smaller size, but their broad spectral width is limiting their use in low-cost devices for research applications.

Fluorescence and fluorophores

Fluorescence is the process of energy transfer – *excitation* – to a fluorophore molecule and the subsequent energy release – *emission* – from it.

When a fluorophore absorbs light of sufficient energy, it enters a state of higher energy – the *excited* state – an unstable state lasting from 10^{-15} to 10^{-9} seconds. Still in the excited state, the fluorophore loses part of this energy, a process called *internal conversion* that is not accompanied by emission. Finally, the fluorophore releases the remaining of the remainder energy and returns to its initial, stable state – the *ground* state. Due to this partial loss of energy during excitation, the emitted energy is lower than the excitation energy, resulting in light of longer wavelength, a phenomenon called the *Stokes shift* (Figure 3).

Fluorescence has a broad range of applications across scientific disciplines, allowing for detection and visualization of various organic and non-organic targets. This detection is achieved either directly – by visual inspection of the emitted light, as in fluorescence microscopy – or indirectly, by collecting the emitted energy with detectors and converting it to electric signals, as in flow cytometry.

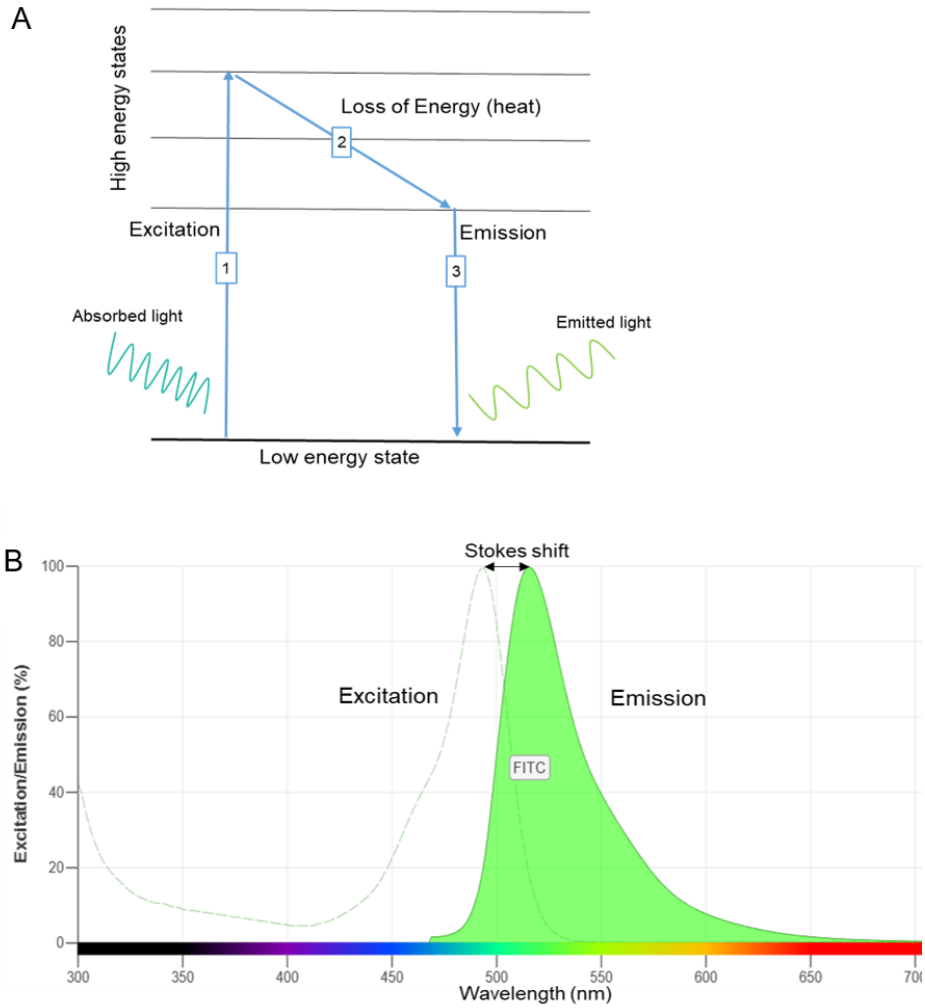

Figure 3. Process of fluorescence.
A. Jablonski diagram demonstrating the energy transition of the fluorophore: excitation leads the fluorophore to a higher energy state; partial loss of energy – internal conversion – follows; finally, the excess energy is emitted and the fluorophore returns to the low energy ground state. B. Excitation and emission spectra of FITC; the difference between the wavelengths of the excitation and emitted light is called the Stokes shift. Figure 3B created with the BD® Spectrum Viewer, with permssion from BD Biosciences.

Fluorophore molecules – or *fluorochromes* – are molecules with the ability to fluoresce, i.e. to undergo the process of fluorescence just described. When used to target biomolecules (proteins, nucleic acids, metabolites, lipids), either individually or combined with linking molecules, they are called *fluorescent probes*.

The most commonly used probes are *fluorochromes conjugated with synthetic antibodies*, which target mainly proteins, but also nucleic acids, polysaccharides and lipids. *Fluorescent stains* or *dyes* are fluorophore molecules that can directly bind to the target molecule, through chemical processes like intercalation, electrostatic or groove binding; these are commonly used for DNA and RNA targeting e.g. propidium iodide, DRAQ5, Hoechst). Fluorescent proteins (FP) – e.g. green fluorescent protein (GFP), isolated from the *Aequorea victoria* jellyfish – are genetically encoded by the target cells themselves through insertion of the *FP* gene with transgenic techniques. The *FP* gene can be fused with the gene of a protein of interest, which, when expressed, is already tagged with the FP.

Fluorophores have certain properties that are important to understand before exploring how they are used in FC.

Excitation range and excitation maximum: A fluorophore can be excited by a range of wavelengths, known as the excitation range; however, only a specific wavelength, called the excitation maximum, achieves optimal excitation.

Emission range and emission maximum: Similarly, a fluorophore molecule can emit light in a range of wavelengths. The same molecule can emit at a different wavelength with each excitation event, but each emission will be within this emission range. The wavelength at which the maximum emission intensity occurs is called the emission maximum.

Fluorescence intensity is the amount of light, i.e. the number of photons, emitted after the fluorophore gets excited. Intensity correlates linearly with the concentration of the excited fluorophore and therefore can be used for quantitative analyses.

These properties, inherent to fluorophores, need to be considered when designing an FC panel. The excitation ranges of the selected fluorochromes should match the available laser wavelengths. Excitation at the fluorophore's maximum however is not required, which allows a single laser to excite multiple fluorophores. The emission spectra should also be examined when selecting two or more fluorophores to be used simultaneously, to minimize overlap between their emission signals; if overlap occurs, it should be manageable through *compensation*. So, by combining a number of excitation lasers with fluorochromes of different excitation and emission ranges we get exciting results!

Light scatter

When light encounters a cell, three processes can occur: part of the light is scattered at various angles, part is absorbed by the cell, and the remaining portion passes through the cell and continues in its original direction with reduced energy.

In FC, two light scatter phenomena are useful for cellular measurements: i) diffraction = the light bends around the cell and continues slightly deviated at small angles, but forward (relative to the laser beam) nonetheless; this is called *forward scatter* (FSC), and ii) refraction = the light changes direction and speed after meeting obstacles *within* the cell (cytoplasmic granules, nucleus) and gets spread around at multiple angles; the light scattered at a ninety degree angle (relative to the laser beam) is collected, comprising *side scatter* (SSC). Forward scatter represents approximate cell size and side scatter represents cytoplasmic complexity (Figure 4).

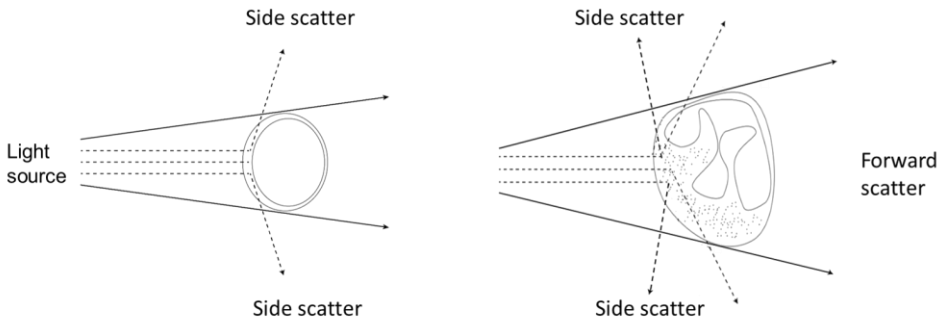

Figure 4. Light scatter properties of a lymphocyte and a neutrophile.
The neutrophil on the right is larger and has a more complex cytoplasm, due to its granules and segmented nucleus, compared with the lymphocyte on the left. Consequently, both the forward and side scatter values would be higher for the neutrophil.

The most common source of optical light in FC is the 488 nm (blue) laser, followed by the 405 nm (violet). Light scatter is unrelated to fluorescence, and its measurement can therefore be performed even on unstained cells.

Filters and detectors

So, incident light is scattered, and fluorescent light is emitted. Then what?

Mirrors and *filters* are used to decompose light to its constituent wavelengths and redirect it to detectors. First, light is redirected by *dichroic mirrors*; these allow only light above or below a specific wavelength to pass through, redirecting the remainder to other detectors. One more filtering level is the *bandpass filters;* these allow only a narrow range of wavelengths to pass through and reach its dedicated detector. The detectors convert photons to an electrical impulse and send it further to flow cytometry's electronic components.

Electronics

The electronics of flow cytometry start with analog-to-digital converters that change the electrical impulse into a digital signal, which can subsequently be processed by a computer equipped with an *acquisition software*. One of the processes performed in software, a sine qua non for the interpretability of multiparameter flow-cytometric data, is *compensation*.

Compensation is a mathematical process used to correct spectral overlap between fluorochromes. Because most fluorochromes emit light in a broad range of wavelengths, they can be detected not only in their primary detector but also in other detectors. This spillover can make populations appear positive for markers they do not truly express. By using information from single-stained controls, spillover to other detectors can be calculated and subsequently subtracted, i.e. compensated.[47] Compensation is a necessity for multiparameter flow cytometry where more than one, and up to 30 fluorochromes can be combined in the same panel. Today, many manufacturers and scientific societies (e.g. BD Biosciences, Thermo Fisher Scientific, FluoroFinder) provide online panel designer tools, which graphically visualize fluorochromes excitation and emission spectra for specific instrument configurations, flagging for spillover and facilitating fluorochrome selection depending on the available flow cytometer.

The compensated data are saved in the form of flow cytometry standard files (.fcs) and can be finally analyzed in the dedicated analysis software.

Cytometry

Cytometry means the measurement of cellular characteristics. Which?

Cell size, indicated by forward scatter.

Cellular granularity and complexity, traditionally measured by side scatter.

Protein expression: Flow cytometry is ideal for the detection of surface proteins, but, with appropriate procedures (permeabilization), intracellular proteins can also be detected. The emission signal provides both qualitative and quantitative information about the corresponding epitope: a positive signal indicates the presence of the epitope in the interrogated cell, while the signal intensity reflects the quantity of the epitope.

DNA content and cell cycle analysis, using DNA-binding dyes. The analysis is based on the principle that the amount of the DNA dye bound is proportional to the total DNA of the cell.

Apoptosis can be detected and measured using plasma membrane changes, detection of active caspase-3 and DNA fragmentation.

Cell function, including mitochondrial membrane potential, reactive oxygen species, calcium flux, intracellular pH, and proliferation using specific fluorescent probes.

Apart from cellular characterization, FC can be applied to the analysis of biomolecules such as protein or DNA, to assess responses of cells on specific drugs and to the study of microorganisms.

As such, FC has a wide range of applications in both clinical practice and research. Clinically, FC has a key role in the diagnosis, classification, and monitoring of hematologic malignancies and immune disorders. In research settings, flow cytometry contributes to tumor biology, stem cell and developmental biology, microbiology, and cell-based drug screening.

Take home

Howard Shapiro's *Practical Flow Cytometry.*[48] It is the most complete – and funniest – book on flow cytometry principles. For him, flow cytometry itself was a principle:

> It is more obvious than ever that flow can't bring world peace, but it can occasionally bring individual people from places or groups with opposing philosophies together in a common cause.

FC & MDS

Why FC in MDS?

Diagnosis of MDS in general, and of low-risk MDS in particular, is considered challenging, as reflected in its diagnostic work-up that requires integration of clinical, morphologic and genetic findings. None of these findings alone is diagnostic. Non-clonal cytopenias can manifest with the same clinical profile as MDS. Dysplastic features in one or more lineages are common in conditions like inflammation, infections, drug toxicity, nutrient deficiencies, ethanol or heavy metal poisoning; morphologic assessment is further complicated by interobserver variability. Before the molecular era, MDS diagnosis was based on cytogenetic studies including karyotype and FISH; however, these can detect abnormalities in only up to 50% of MDS cases.[28,29] Still, a considerable number of cases of cytopenia without blast increase or cytogenetic abnormalities remained a diagnostic challenge. In this background, the need for additional diagnostic tools was profound and multiparameter FC, given its short turnaround time, appeared well suited to fill this gap.

After the introduction of DNA sequencing methods, like next-generation sequencing (NGS), genetic abnormalities can be identified in as much as 90-95% of MDS cases.[10] Although DNA sequencing methods have nowadays become part of the standard of care for patients with suspected MDS, they remain expensive and time-consuming, and therefore their adoption by national health care systems for the investigation of unexplained cytopenias varies widely. Consequently, there remains an ongoing need for a fast and less costly screening tool to identify patients who could benefit from further molecular testing.

Furthermore, the increased sensitivity of molecular analyses has introduced new challenges: the myeloid precursor lesions, specifically clonal hematopoiesis of indeterminate potential (CHIP) and clonal cytopenias of undetermined significance (CCUS). The latter, by definition lacking significant morphologic dysplasia yet sharing common genetic aberrations with MDS, may be difficult to distinguish from low-risk MDS with subtle dysplasia;[56] even after a confident diagnosis of CCUS, a long-term monitoring for progression to MDS is warranted. These challenges highlight potential new applications for FC in the diagnosis and monitoring of myeloid precursor lesions.[57,58]

It should not be overlooked either that FC can rapidly and reliably rule out other hematolymphoid neoplasms or disorders that may mimic MDS or coexist with it, such as paroxysmal nocturnal hemoglobinuria, mastocytosis, large granular lymphocytic

leukemia or other lymphoproliferative disorders. And yes, we do receive the occasional bone marrow with the question *MDS?* that proves to be Waldenström macroglobulinemia.

Finally, as discussed in more detail below, several FC parameters have shown prognostic and predictive relevance, extending the method's use to risk assessment and treatment response prediction.

A (very) brief history of the relation between FC and MDS

The first article about FC in MDS is dated back in 1983, shortly after the first classification of MDS by the FAB and was a study of DNA ploidy and cell cycle analysis.[59] Studies on the aberrant expression of individual cell-surface markers in MDS appeared sporadically in the following years,[60–62] paralleling studies delineating normal hematopoietic maturation.[63–66]

The first comprehensive study of multiple myeloid, erythroid, and megakaryocytic markers in MDS was published by Stetler-Stevenson et al in *Blood* in 2001.[67] Using a three-color flow cytometer equipped with a 488 nm argon laser, they identified immunophenotypic and light-scatter aberrancies that today have recognized diagnostic value. A continuous flow of studies followed soon after, paralleling the advances in the technique; previous results were confirmed, insight into new diagnostic markers was provided and diagnostic scoring systems were suggested. Prognostic factors in MDS also started to be identified and predictive models began to be described.

In parallel, much of the flow cytometry community's effort was directed toward preanalytical issues. Indeed, the variations in sample quality and handling and in the fluorochromes and antibodies used can influence the final FC results. Method standardization became therefore as important as diagnosing MDS itself.

Since 2008, an international group of experts on the field, the international MDS Flow Working Group (*i*MDS Flow WG), under the umbrella of the European Leukemia Net (ELN), has gather their efforts to provide guidelines regarding both preanalytical and analytical issues and standardize FC in MDS, underscoring that FC should always be correlated with morphologic assessment.

Where are we now?

Diagnosis and diagnostic scoring systems

No single marker is diagnostic of MDS. A broad panel including granulocytic, monocytic and erythroid markers is therefore recommended. The megakaryocytic lineage is less accessible to FC, due to the small number of megakaryocytes and the contamination by platelets, and therefore this lineage is not included in the current recommendations. The FC aberrancies associated with MDS include altered population counts, altered scatter characteristics and immunophenotypic changes; the latter include altered intensity or loss of expression of normally expressed markers; asynchronous expression of differentiation markers (i.e. co-expression of mature and immature markers); and aberrant marker expression including lineage infidelity. Quite a few FC aberrancies associated with MDS have been described over the years;[68–76] among them, the ones with confirmed diagnostic value in MDS, validated through multicenter studies, are summarized in Table 5. The main principle for interpretation of these aberrancies as dictated by the ELN iMDS Flow WG is that at least three distinct aberrations in at least two lineages are associated with a high likelihood of MDS.

Table 5. FC aberrancies associated with MDS[a]

Lineage	FC aberrancies
Myeloid progenitors	**Increased percentage**
	Decreased/increased expression of **CD45**, CD34 and **CD117**
	Decreased/increased expression of **CD13**, CD33, **HLA-DR**
	Asynchronous expression of CD11b, CD15
	Aberrant expression of CD2, CD4, **CD5**, **CD7**, CD19, **CD56**
	Aberrant CD34/CD117 pattern
Granulopoiesis	**Decreased percentage (as ratio to percentage of lymphocytes)**
	Decreased SCC (as ratio to SSC of lymphocytes)
	Asynchronous expression of CD34
	Aberrant expression of CD5, CD7, CD56
	Aberrant pattern of CD15/CD10, **CD13/CD16** and CD13/CD11b
	Decreased expression of CD33
Monocytes	**Increased percenatge**
	Aberrant expression of CD2, **CD56**
	Decreased expression of **CD13**, CD15, CD64, CD11c
	Aberant pattern of CD36/CD14 and **HLA-DR/CD11b**
	Aberrant PB monocyte partition (in CMML)
	Decreased percentage of slan+ classical monocytes (in CMML)

Table 5 continued.

Lineage	FC aberrancies
Erythropoiesis	Increased percentage of erythropoiesis
	Increased percentage of CD117^{+} erythroid precursors
	Increased CV of CD36 and CD71
	Decreased expression of CD36 and **CD71**
	Decreased/increased expression of CD105
	Aberrant pattern of CD71/CD235a

[a] Aberrancies in bold have been further validated by a multicenter prospective study.[77]

The first widely used diagnostic scoring system, the so-called Ogata score, was introduced in 2009[71] and was validated by a multicenter study in 2012[78]. The scoring system was established in cohorts of patients with low-grade MDS, lacking blast increase, cytogenetic abnormalities and ring sideroblasts (i.e. the most challenging group diagnostically). It includes four parameters: increased CD34^{+} myeloid blasts, decreased CD34^{+} B-cell precursors, decreased CD45 expression by the myeloid blasts (calculated as the lymphocytes to myeloid blasts CD45 ratio) and decreased granularity of granulocytes (indicated by decreased SSC and calculated as the granulocytes to lymphocytes SSC ratio); one point is assigned for each abnormal parameter and a score of ≥ 2 is considered indicative of MDS. Sensitivity and specificity are 69% and 92% respectively for low-grade MDS. Its simplicity and the conservative antibody panel required have contributed to its wide adoption. The Ogata score is still routinely applied, but many studies have combined it with other markers suggestive of MDS to increase sensitivity and specificity.[68,79]

Other diagnostic scores have been proposed over time applying aberrant FC features in myelopoiesis and/or erythropoiesis.[69,70] In 2017, Cremers et al developed the integrated flow score (iFS), combining the Ogata score with confirmed FC aberrancies in myelopoiesis and erythropoiesis.[80] Using the four-parameter Ogata score as a base, additional points are added for FC aberrancies detected in the myeloid progenitors, the granulocytic/monocytic lineage, and/or the erythroid lineage. The final score defines three categories: A) no MDS-related features; B) limited number of changes associated with MDS, or C) features consistent with MDS. The iFS achieved 79% sensitivity and 86% specificity.

Two independent studies compared the different FC scoring systems, confirming that iFS can more accurately distinguish between MDS and non-clonal cytopenias, providing at the same time prognostic information about patient outcome.[81,82]A drawback of iFS, however, is that it is based on as many as 44 different FC parameters.

To address the overwhelming volume of diagnostic markers and scoring systems, the ELN iMDS Flow WG recently conducted a multicenter study that identified 17 FC aberrancies independently related to MDS and CMML.[77] Additional studies validating these results have started to appear in the literature.[83] These 17 aberrancies, presented in Table 5 with bold letters, are recommended as a mandatory core in every FC panel dedicated to diagnosis of MDS.

Prognostication and prediction

As our knowledge on immunophenotyping in MDS increased, some of the FC aberrancies associated with MDS have been shown to bear prognostic value as well.

The myeloid blasts count, as defined by morphology, is one of the cornerstones of risk stratification in MDS, included in the original IPSS as well as the subsequent IPSS-R and IPSS-M.[30,32] Equally, the count of myeloid progenitors (MyP), defined by FC, as well as phenotypical aberrancies in this compartment have been demonstrated to bear prognostic and predictive significance. In particular, high MyP counts or phenotypically aberrant MyP have been associated with worse response to 5-aza in high-risk MDS, worse response to treatment with growth factors, higher risk for relapse after allo-HSCT and worse overall survival (OS).[69,84–90]

Similar observations have been described for FC aberrancies involving cell populations other than MyP. Although there are reports about individual markers with prognostic significance,[91,92] the studies investigating prognosis and prediction in MDS consider various FC aberrancies collectively, usually as part of scoring systems. The cumulative number of phenotypic aberrancies has been shown to correlate with IPSS, to the number of mutations and cytogenetic risk, to response to treatment with 5-aza or lenalidomide, to the outcome after allo-HSCT and to overall survival.[69,92–97]

One of the most fascinating possibilities of FC is that it can reveal genotype-phenotype associations. Indeed, specific cytogenetic aberrations and mutations in MDS have been associated with specific phenotypes. A typical example is MDS del(5q) which has been associated with increased percentage and decreased CD45-MFI of MyP, decreased SSC in granulopoiesis along with increased maturation of the latter, manifested as more "normal" CD10 and CD71 expression compared to other MDS cases. This profile has been subsequently used for treatment monitoring of del(5q) MDS cases.[98,99] Increased expression of CD14 in granulopoiesis has been shown in cases with monosomy 7 or del(7q).[100] In *SF3B1*-mutant MDS, erythroid precursors showed more often aberrant CD71 expression and increased SSC; increased SSC was also observed in MyP; a constellation of phenotypical aberrancies (decreased CD11b, CD36 and CD64) has also been demonstrated in monocytes.[101] A distinct immunophenotypic profile was also

attributed to MDS carrying *SRFS2* mutations, characterized by abnormal CD11b/CD16 expression pattern in granulocytes combined with decreased CD45 expression in MyP.[102] CD7 expression in MyP has been demonstrated in *TP53*-mutated MDS and AML.[103,104]

Challenges in the implementation of FC in MDS

Standardization. Every step in the flow cytometric analysis, from sample storage to data analysis is prone to introducing bias. The medium in which the sample is preserved, sample handling (lysis, washing, staining, fixation); the antibody panels applied (clones, fluorochromes); the instrument setup; data analysis including gating strategies and interpretation can all affect the FC readout.[55,105]

How can results from different studies be compared then?

Indeed, standardization remains a major challenge in FC. Many international organizations and consortia, both in the fields of immunology and hematology, have addressed it. The International Clinical Cytometry Society (ICCS) provides educational modules and practical guides that identify and address common issues in FC analysis. The Clinical and Laboratory Standards Institute (CLSI) has published the H62 guideline (*Validation of Assays Performed by Flow Cytometry*) to offer strategies regarding instrument qualification, standardization, assay optimization, and regulatory compliance. The EuroFlow consortium provides protocols and standard operating procedures, validated through multicenter studies; these protocols include sample handling, instrument setup, as well as dedicated antibody panels designed for specific uses. Many other groups have provided harmonization strategies for interinstitutional studies of specific clinical or research questions; regardless of the purpose, the usual actions suggested are instrument harmonization, common antibody panels (lyophilized and desiccated reagent cocktails are often preferred) and software templates with standardized gating.[106–108] Likewise, in the field of MDS, the ELN iMDSFlow WG has published recommendations regarding preanalytical issues, essential antibodies to be applied and data interpretation.[109–112]

Phenotypic aberrancies in non-clonal disorders. A factor limiting the specificity of the proposed FC scoring systems is that many phenotypical aberrancies can be demonstrated in non-clonal conditions. The granulocyte/lymphocyte SSC ratio, one of the four Ogata score parameters, can be decreased in cases of cytopenia due to toxic effects and inflammation.[113] Patients with PNH and aplastic anemia can show expression of CD7 and CD56 in MyP and granulopoiesis.[82] Abnormal CD56 expression in monocytes and granulocytes can also be observed in regenerating BM, in inflammation and infection, as well as after G-CSF treatment.[69,114]

CMML

Yes, CMML did it! The "monocyte assay" is officially a diagnostic criterion in the 5th edition of WHO and the ICC for the diagnosis of chronic myelomonocytic leukemia.

CMML is a myelodysplastic/myeloproliferative neoplasm (MDS/MPN) characterized by persistent absolute ($\geq 0.5 \times 10^9$/L) and relative ($\geq 10\%$) PB monocytosis.[6,7] Dysplasia in any hematopoietic lineage can be present, but it can be minimal or even absent. In addition, persistent monocytosis can be observed in many reactive conditions.

Although many aberrant markers have been described in monocytes both in the context of MDS and CMML (Table 5), this assay is based only on two markers and biology. PB monocytes can be separated into three subsets according to their expression of CD14 and CD16: classical monocytes (cMo) $CD14^{++}CD16^{-}$, intermediate monocytes (iMo) $CD14^{++}CD16^{+}$ and non-classical (ncMo) $CD14^{-/low}CD16^{+}$. In normal individuals, all three subsets exist, while in inflammation, like autoimmune diseases and infection, the $CD16^{+}$ iMo and ncMo subsets may expand.[115]

The principle behind this assay is that in CMML, because of abnormal differentiation, the cMo predominate. The assay is based on the flow cytometric partitioning of PB monocytes, where a percentage of cMo ≥ 94% of total peripheral blood monocytes distinguishes CMML from reactive monocytosis (Figure 5). The "monocyte assay" has 94.1% specificity and 91.9% sensitivity.[116–118]

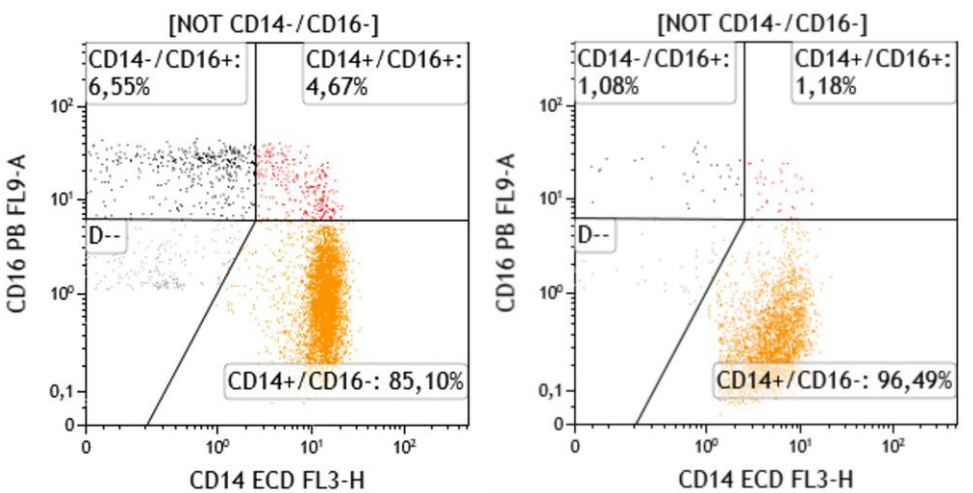

Figure 5. Monocyte assay for diagnosis of CMML.
In the left, normal PB with all three monocytic subsets present; cMO (CD14+/CD16-) below the 94% threshold. In the right, PB with CMML, with > 94% cMO.

Aims

The main goal of the studies presented in this thesis was to establish more firmly the role of flow-cytometric assessment of erythropoiesis in the diagnosis of MDS. To this end, different FC modalities were explored.

In Study I, a robust panel for the flow-cytometric analysis of erythropoiesis was developed, and its diagnostic value was evaluated.

In Study II, we explored the possibilities of Imaging Flow Cytometry, a unique technique combining conventional FC with single-cell imaging. Our focus was to translate the morphologic hallmarks of dyserythropoiesis into objective morpho-metric parameters, thereby integrating morphology into the diagnostic utility of FC.

In Studies III and IV, we applied an unsupervised clustering and visualization technique, the FlowSOM algorithm. Study III evaluated the algorithm's performance in identifying erythroid aberrancies in pathological bone marrow. Building on these insights, Study IV singled out MDS-associated aberrancies and investigated the potential of FlowSOM for monitoring MDS during treatment.

Methodology

This thesis was a joyful methodologic exploration of less common flow cytometric modalities. The common ground, however, of all the projects was an antibody panel designed for the study of erythropoiesis:

The ERY panel

Permeabilization: a chemical process in the preparation of FC samples, most often employing organic solvents or detergents, to create pores in the cell membrane and, thus, allow the entrance of antibodies in the cell. The chemicals used however are non-selective and can be harmful to other cell or fluorescent proteins, undermining the data quality. If you are curious about more permeabilization, there are many available articles or company protocols out there.

Ficoll (Ficoll-Paque) density gradient centrifugation: A method using the synthetic polysaccharide Ficoll to create, upon centrifugation, a gradient that separates cells by density. Commonly used in blood and BM samples to separate mononuclear cells from erythrocytes and granulocytes, though small quantities of the latter can be retained in the mononuclear layer.

The ERY panel is a more or less common antibody panel... but with a twist:

The DNA dye DRAQ5! (♪ superhero music ♪)

DRAQ5 is a fluorescent DNA dye with the ability to permeate the cell membrane and intercalate the double stranded DNA.[119–121] It can be applied to both living and fixed cells. It is a far-red stain, emitting at a broad spectrum from 665 nm to beyond 780 nm wavelengths with a peak at 681 nm, when bound to DNA; excitation is optimal at 647 nm (red laser), but it can be suboptimal excited at 488 nm (blue laser) (Figure 6).

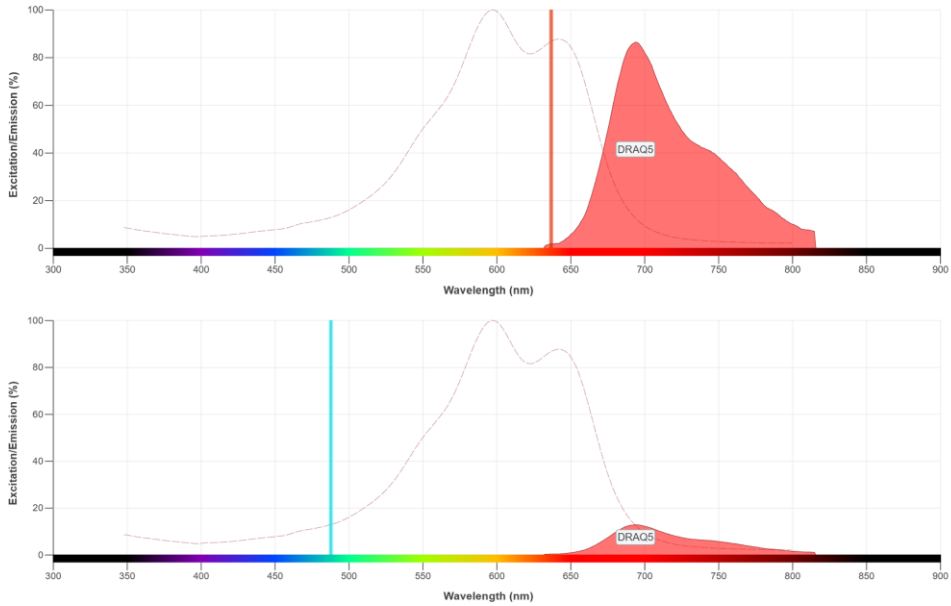

Figure 6. Excitation and emission spectrum of DRAQ5.
In both panels, the red dashed line represents the excitation spectrum and the red curve represents the emission spectrum of DRAQ5. The red vertical line in the upper panel represents the red laser (647 nm) and the blue vertical line in the lower panel is the blue laser (488 nm). Although the emission range is the same regardless of the laser used, the emission intensity is considerably lower when DRAQ5 gets excited by the blue laser. Figure created with the BD® Spectrum Viewer, with permssion from BD Biosciences.

Since this is a cell permeable stain, no permeabilization step is required as with other commonly used intracellular antibodies (e.g. TdT, MPO, intracellular immunoglobulin light chains). Permeabilization, although a necessary process for the study of intracellular markers, can adversely affect immunophenotypic and light scatter characteristics.[122,123]

And since DRAQ5 binds to DNA, it can be used to detect nucleated cells and successfully separate them from unwelcome noncellular material and enucleated cells, like debris, platelets and erythrocytes that dilute the FC samples. Erythrocytes in particular are usually removed by lysis. Lysis (λύσις), a Greek word meaning both "solution" (e.g. of a problem) and "dissolution" or "destruction", the latter being the most prevalent use in biology and FC. Although lysis is an important step in the processing of FC samples, avoiding it in specific FC applications can be advantageous. Identification and description of these advantages were one of the aims of our first study but also of others[70,124–126] and therefore these are thoroughly discussed in the Results section. But in short, lysis has been shown to affect erythropoiesis in both quantitative

(by destroying erythroblasts) and qualitative ways (by affecting FC parameters such as light scatter and immunophenotype). So, avoiding lysis can be beneficial in the study of erythropoiesis.

In addition, DRAQ5 binds to DNA *stoichiometrically*. This is another word of Greek origin (στοιχιομετρία), albeit a more intimidating one. With the help of a dictionary: *Stoichiometry*: the relationship between the quantities of reactants and products before, during, and following chemical reactions. In other words, the quantity of DRAQ5 used relates to the quantity of DNA it binds to. So, this is actually a great property because it allows quantification of the bound DNA. As such, it can be used for cell cycle analysis, detecting cells in S and G1/M phase through their duplicated DNA (Figure 7); and for DNA content analysis and detection of abnormal ploidy.[127–130]

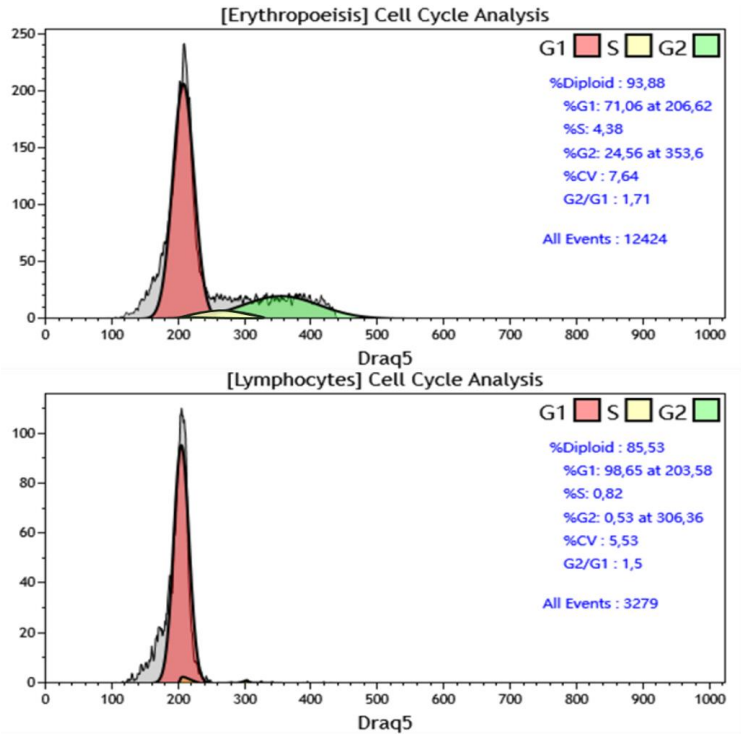

Figure 7. Cell cycle analysis with DRAQ5 created with the Michael H. Fox algorithm integrated in Kaluza analysis software (Beckman Coulter).
In the upper panel, the proliferating cluster of erythropoieisis (S/G2 phase) can be appreciated by the higher DRAQ5 values. By contrast, mature lymphocytes (lower panel) reside mainly in G1 phase.

By avoiding permeabilization and lysis and by allowing quantification of DNA, DRAQ5 sounds like the perfect stain to have in any FC panel where precise quantification of nucleated cells or DNA studies matter.

But nothing in life is perfect. Because of its strong signal, DRAQ5 emits considerable light in neighboring channels limiting the number of antibodies that can be used. When the ERY panel was designed by our team in 2017 it was meant to be applied to a 10-color Beckman Coulter Navios EX System, equipped with violet, blue and red solid-state lasers. Although the flow cytometer had the capacity for 10 (or more if combined) antibodies, the strong personality of DRAQ5 limited the number of antibodies used to six.

So now it's time to move the spotlight from the superhero to its six sidekicks and

The ERY panel itself!

Besides DRAQ5, this panel includes antibodies for the epitopes CD45, CD36, CD71, CD105, CD117 and CD13. Let's take a closer look at the profiles of these heroes:

CD45 is called "leukocyte common antigen" for a good reason. It is positive in *almost* all normal hematolymphoid cells. Erythropoiesis on the other hand is mostly negative, apart from the earlier precursors that show a dim expression. All hematolymphoid populations, no matter the sample (BM, blood, lymph node etc.) have a characteristic CD45 expression and show a characteristic pattern in the SSC/CD45 scatter plot, which can be considered the cells' ID card (Figure 8). CD45, is therefore a backbone marker in all FC panels that investigate hematolymphoid cells.

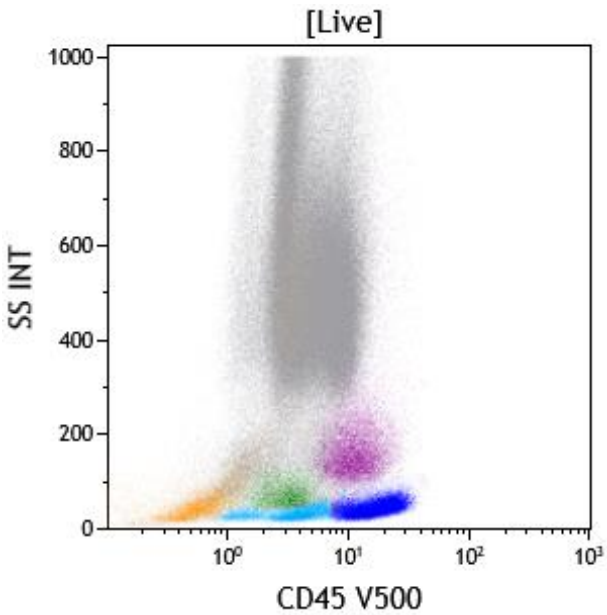

Figure 8. Normal BM in a SSC/CD45 scatter plot.
Each population has its characteristic position in this plot. Orange: erythropoiesis; grey: granulopoiesis; purple: monocytes; green: myeloid blasts; light blue: B-cell precursors; blue: lymphocytes.

CD36 is a glycoprotein expressed on the membrane of many cell types and having been known by different names. More often it is also referred to as a scavenger receptor, belonging to the class B scavenger receptor family. The function of CD36 has been well described in monocytes/macrophages as well as other, non-hematopoietic cells, like adipose and endothelial cells.[131]

CD71, or Transferrin receptor protein 1 (TfR1) is the protein responsible for the transport of iron from transferrin to cells. A function that is fundamental not only for hemoglobin producing cells (the erythroblasts) but for all cells, since iron-dependent enzymes are used throughout cell metabolism, DNA replication and repair and gene expression.[132,133]

CD105, or endoglin, is a glycoprotein that is part of the TGF beta receptor complex, strongly expressed in endothelial cells and associated with angiogenesis. It is also an established marker of erythropoiesis, expressed in the earlier stages of maturation. In BM it can also be expressed by lymphoblasts and activated monocytes.[134–136]

CD117, or c-kit is a tyrosine kinase receptor encoded by the *KIT* gene. Upon activation, it is involved in regulation of apoptosis, cell differentiation, proliferation, chemotaxis, and cell adhesion. In BM, CD117 is expressed in early myeloid and erythroid precursors and mast cells, but its expression expands in a wide range of cell types, both normal (germ cells, melanocytes, Cajal cells of the gastrointestinal tract) and neoplastic.[137]

And, finally, *CD13*. Also called aminopeptidase N, this glycoprotein functions mainly as a metallopeptidase on the surface of myelomonocytic cells, endothelium and certain epithelial cells (small intestine, liver, kidney); it has also been identified as an adhesion molecule.[138,139]

The choice of the markers above was based on the well described expression patterns of CD36, CD71, CD105 and CD117 in normal erythropoiesis, as well as in their well-documented contribution in diagnosis of MDS. A myeloid marker (CD13) was also necessary for a clear separation of erythropoiesis from myeloid cells with partially overlapping immunophenotype and for identification of myelopoiesis (Figure 9).

The channel distribution of the antibodies in the ERY panel are presented in Table 6. Of note, in the initial panel used between 2017 and 2018, DRAQ5 was on channel 7 and was excited by the red laser. Due to persistent challenges in compensating spill-over emission in neighboring channels (especially into channel 5), DRAQ5 was moved to channel 4, where it would be excited by the blue laser. Excitation by the blue laser resulted in lower emission intensity (Figure 6), the spill-over of which was easier to compensate. A detailed description of sample preparation, antibody quantities and

manufacturers for the ERY-panel, is presented in the Supplementary data of the 1st article.

Table 6. Channel distribution of the ERY panel fluorochromes-antibodies/dyes in Navios Ex.

	FL1 FITC	FL2 PE	FL3 ECD	FL4	FL5 PE-Cy7	FL6	FL7	FL8	FL9 PB	FL10 KO
	Blue laser					Red laser			Violet laser	
2017-2018	CD71	CD13	CD117	-	CD105	-	DRAQ5	-	CD36	CD45
2019	CD71	CD13	CD117	DRAQ5	CD105	-	-	-	CD36	CD45

A reviewer of the first article asked the reasonable question: Why not CD235a? Indeed, CD235a or Glycophorin A, a specific marker of the erythroid lineage, appears in many studies of erythropoiesis as an obvious choice.[70,72,124,126,140] CD235a however is not only positive in the mature erythroblasts, but also in erythrocytes. In a non-lysis protocol like the ERY panel, applied to whole bone marrow with hundreds of thousands of accompanying erythrocytes, CD235a would only cause problems in analysis and interpretation of data. Erythrocytes, inevitably, create aggregates with nucleated cells, which can lead to false positivity for CD235a. In the studies published by the group of Hematologics Inc in Seattle, Washington in 2014 and 2015, DRAQ5 has been used together with CD235a, but here lysis with NH_4Cl or Ficoll density gradient preparation preceded staining.[124,126] In the 2017 report of the iMDS Flow WG[141] about the immunophenotypic analysis of erythroid dysplasia in MDS, it is stated:

> The expression of CD235a largely depends on the success of removing mature erythrocytes from a sample. Moreover, membrane fragments of lysed erythrocytes may stick to other cells in the analysis sample, mimicking positivity. Hence, this parameter was considered too unreliable for evaluation when considered individually.

Ultimately, CD235a was not included in the group's most recent recommendations published in 2023.[112]

The heroes in action

Let's now watch these markers collaborate with each other to define erythropoiesis.

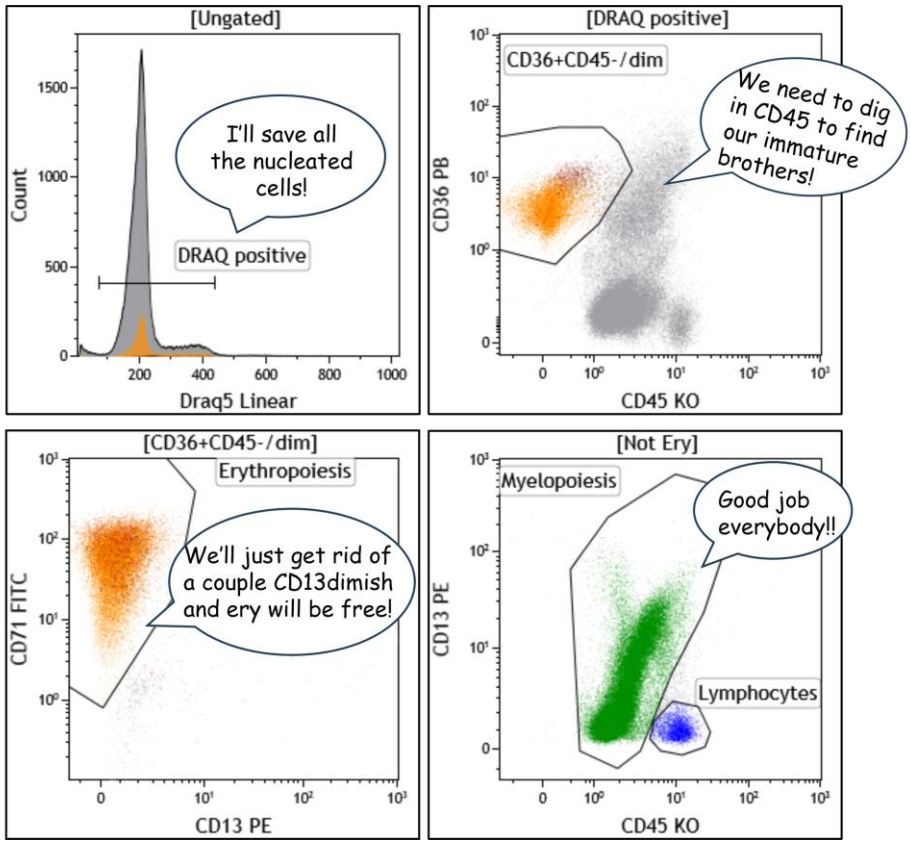

Figure 9. Gating strategy in ERY panel.

Acquistion of live, nucleated cells is based on DRAQ5 positivity. Retrieval of erythropoiesis begins in a CD36/CD45 plot; CD36 is used first since its expression precedes that of CD71. A broad gate including CD45dim is drawn, to include the immature erythroid precursors. Any admixed myeloid cells due to this broad gating are subsequently removed in the CD71/CD13 plot. Finally, myelopoiesis and lymphocytes are identified in the CD13/CD45 plot.

To assess maturation the two most useful plots were CD105/CD117 and CD71/CD36 (Figure 10). In the first, the two immature erythroid compartments, $CD117^{+}/CD105^{-}$ (proerythroblasts, ProEry) and $CD117^{-}/CD105^{+}$ (basophilic erythroblasts, Baso) and the Mature ($CD117^{-}/CD105^{-}$) are depicted. Disturbances in these three maturation patterns (left shift or decrease in the immature compartments) can be also roughly assessed. In the CD71/CD36 plot, the whole erythropoiesis is presented as a big, indistinct population; however, this plot gives a distinct pattern in cases with aberrant

erythroblasts with decreased CD36 and/or CD71 expression, as illustrated in Figure 18 in the Results section of Study I.

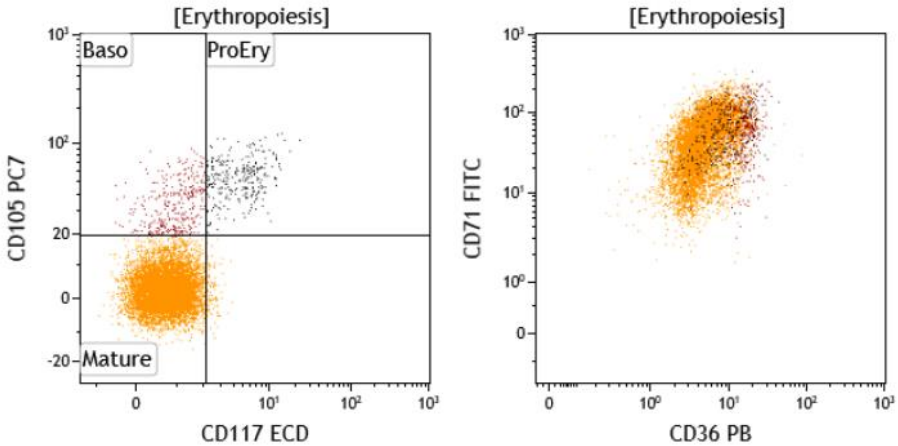

Figure 10. CD105/CD117 and CD17/CD36 scatter plots depicting normal maturation patterns.

Achilles heel

Like every hero, the ERY panel has its weaknesses. A drawback of retaining the non-lysed erythrocytes is that they can create aggregates with the nucleated cells. Although most of the cases were not affected, in some cases with considerable amounts of erythrocytes – yet not hemodiluted – these aggregates had a great impact on side scatter, prohibiting its interpretation (Figure 11).

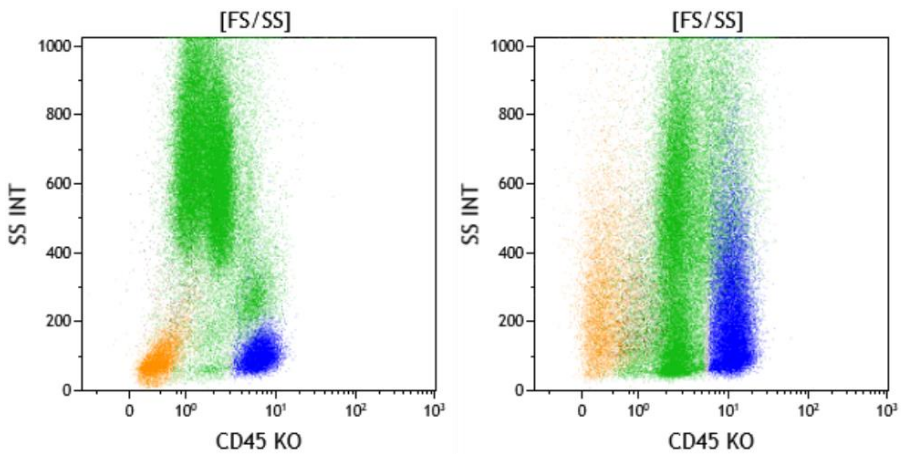

Figure 11. SSC/CD45 plots of two BM analyzed with the ERY panel.
In the left, the SSC properties are maintained; in the right the aggergates of unlysed eryhtrocytes with nucleated cells distort SSC, rendering it difficult to interpret.

Stay tuned with us: in Results section you can marvel the achievements of ERY panel and DRAQ5 in their fight against lysis.

In short

The ERY panel was designed by our team for the study of erythropoiesis based on a non-lysis protocol - an approach that is underexplored and less commonly applied.

This antibody panel forms the backbone of all studies presented in this thesis.

Advantages: No lysis = no loss of erythroid precursors and thus the whole erythroid compartment is available for assessment. DNA analysis is possible.

Weaknesses: The strong DRAQ5 emission limits the number of antibodies to be used and can make compensation of spill-over emission to neighboring channels challenging. The non-lysed erythrocytes, if in considerable quantities, aggregate with nucleated cells affecting the light scatter parameters.

Imaging Flow Cytometry

Brightfield (BF) microscopy: A basic light microscopy technique that uses white light to illuminate the sample. The sample partially absorbs light, and the transmitted light produces a shadow-like image against a bright background.

Transmitted light: The light that passes though the illuminated sample (i.e. not absorbed or scattered) and gets collected by an optical lens.

Charge-coupled device (CCD) camera: Camera equipped with an electrical device that can convert light input into electronic signals.

Light decomposition: Separation of light into its component wavelengths

Morphometry (μορφομετρία): Quantitative measurement of parameters related to form, like shape and size.

Imaging flow cytometry was the method explored in Study II. It can be summarized as the combination of conventional MFC and single-cell imaging based on the principles of brightfield (BF) microscopy. The method is intended for research use only.

The technique has been available since the early 2000s (Amnis® ImageStream became the first commercially available imaging flow cytometer in 2004) and has been widely used in basic and applied research, with applications in biology, microbiology, immunology;[142–148] it has been shown to be particularly useful in the study of particles and extracellular vesicles.[149,150] In the field of hematology, it proved popular in the study of erythrocytes (especially in red cell membrane disorders) and erythropoiesis[151–154] as well as of platelets and megakaryocytes;[155,156] a fascinating application is the detection of cytogenetic abnormalities in chronic lymphocytic leukemia and multiple myeloma, an alternative to conventional FISH analysis.[157,158]

The flow cytometer used in our study was an Amnis® ImageStream®^{X} Mk II imaging flow cytometer (Cytek® Biosciences Inc., Fremont, CA, USA), equipped with three lasers (violet, blue and red) and two charge-coupled device (CCD) cameras. Images and fluorescence emission were collected through a 12-channel system. Data analysis was performed with the dedicated IDEAS® software.

The flow cytometer function of ImageStream follows the common principles of multiparameter flow cytometry as presented in the introduction of this thesis. However,

it is its unique imaging function and the subsequent morphologic assessment of the generated images that deserve (and require) further examination.

Image generation system

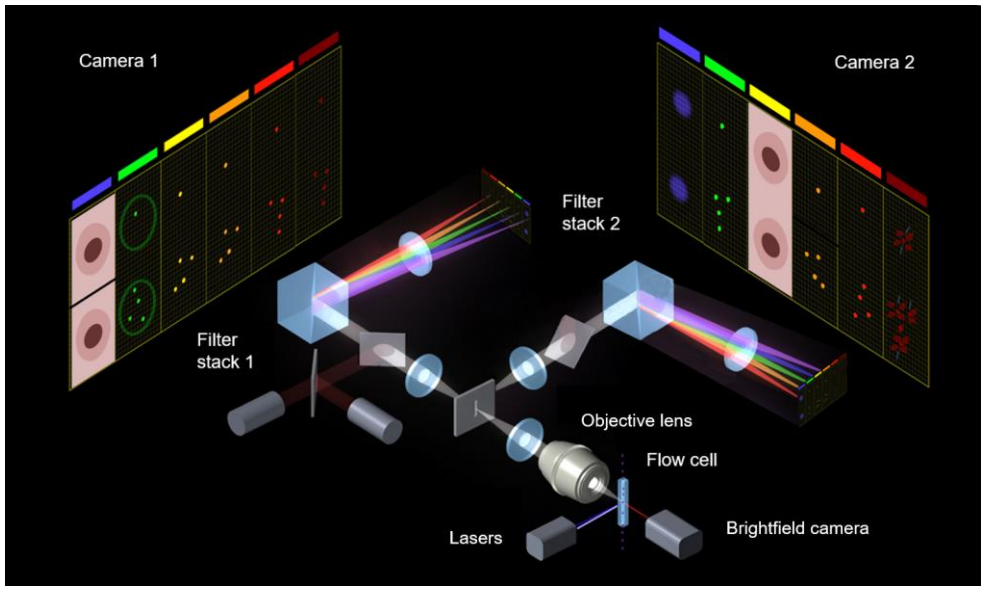

Figure 12. Optics of ImageStream. Figure adapted with permission from Cytek Biosciences.

The flowing cells are illuminated simultaneously by a BF light source and the lasers. The derived transmitted and scattered light and fluorescence emissions are first received by an objective lens (adjustable to x20, x40 and x60 magnification) and then are filtered by filter stacks that decompose light into six beams of different wavelengths. These are collected by six spatially separated channels in each CCD camera. So, with a two-camera system, up to 12 images can be collected per cell, including side scatter, BF and fluorescence images. In a typical 12-channel setup, two channels are dedicated to BF images, one to side scatter and up to nine channels can collect fluorescence information. This information concerns both the intensity and spatial localization of the emitting signal. In other words, one can directly visualize the localization of fluorochrome-conjugated antibodies in relation to the cell, a property extremely useful for assessing particle internalization or epitope colocalization.

Images are collected in pixels, whose size depends on the chosen optical magnification and the flow speed (ranging from 0.1 to 2 μm^2).

Morphologic assessment

So, we have our images. Up to 12 images per cell, tens of thousands of cells analyzed. How can we handle this large volume of information?

We launch the analysis software IDEAS®. Impatiently, we start browsing the brightfield images to see how our cells look like. And here comes the first disappointment. These cells have nothing to do with the beautiful May-Grünwald Giemsa-stained cells on our smears! These are just gray shadows, the nucleus hardly being discerned. But the information is there, and we just need to learn how to retrieve it. And here comes the second disappointment: RTFM (Read The "Factory" Manual) – no shortcuts. The instruction manual and not the manual assessment is your friend in the land of Imaging flow cytometry. Here, morphologic assessment is performed through morphometric analyses, i.e. precise mathematical measurements of form, shape, and size.

In the IDEAS® software, these mathematical measurements of morphology and fluorescence intensity are called *features* and are grouped into six categories: size, location, shape, texture, signal strength and comparison. Users can also create new features by combining existing ones. The area of a cell (literally, the group of pixels) in which these measurements are performed is called a *mask*. If, for example, we are interested in the size of a nucleus, then we apply the size feature *Area* to the mask covering the nucleus. Even fluorescence intensity calculations are performed on the mask corresponding to the emission area. Figure 13 shows some examples of masks used in our study for morphometric and fluorescence intensity measurements.

The wide range of available features allows for versatility and flexibility; the translation of images to measurable parameters ensures objectivity. Morphometric assessment can be applied to tens of thousands of cells, in contrast to the few hundred that are typically evaluated manually. At the same time, this abundance of possibilities can prove overwhelming; selecting appropriate features is not always straightforward or feasible and is subject to operator variability. In addition, there is growing interest in label-free (BF) images, which contain more information than the eye can appreciate. In this context, many artificial intelligence applications, in particular machine learning algorithms, have emerged to facilitate identification of discriminating features, cell classification and to handle the complexity of imagery data.[159–166]

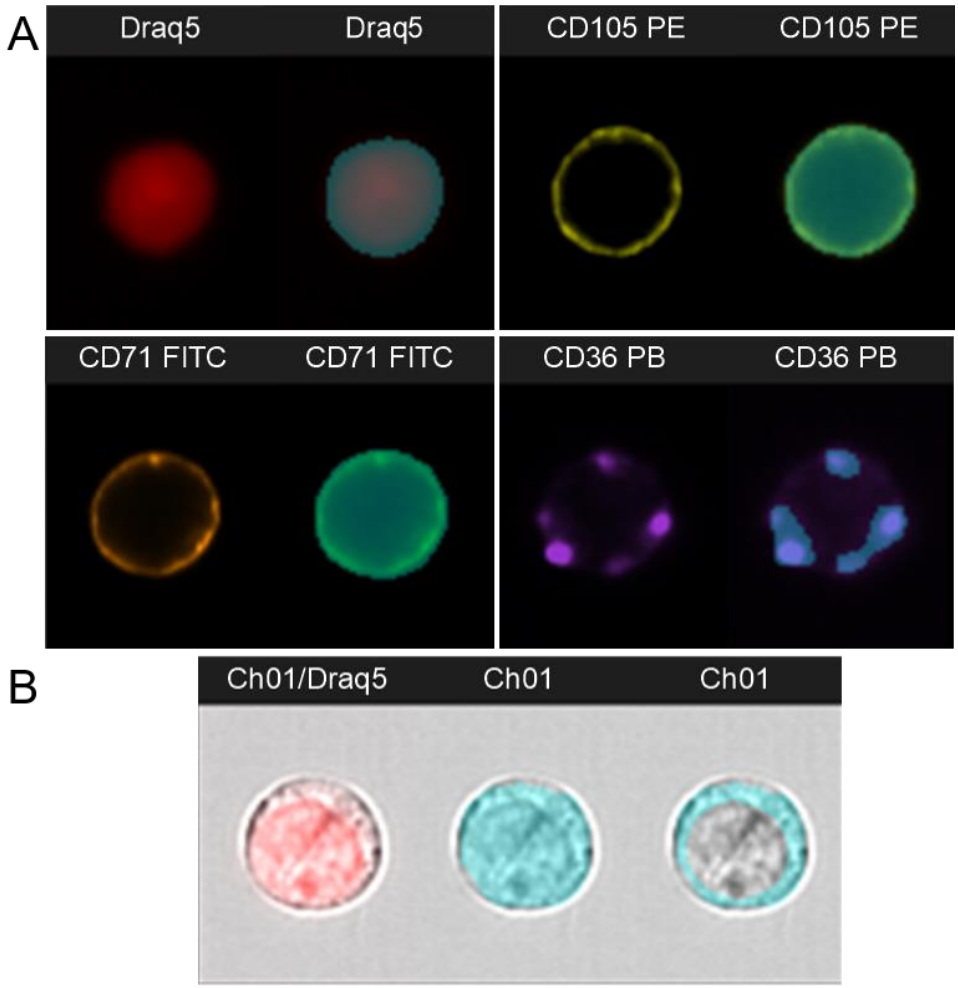

Figure 13. Masks used in Study II for morphometric and fluorescence intensity measurements. A. Images of four fluorescent markers (DRAQ5, CD105, CD71 and CD36) without maks (left) and with their corresponding mask in turqoise (right). B. From left to right, the combined BF/DRAQ5 image without mask, the BF image with the mask corresponding to Cell Area and the BF image with the cytoplasmic mask.

In our hands

In our study, the antibody panel used was, of course, the ERY panel; channel distribution of BF images, side scatter and antibodies used are presented in Table 7 and Figure 14. An adjustment in the instrument setup that was particular to our study due to the non-lysis protocol applied was the reduction of flow velocity to the lowest

possible, i.e. 44mm/sec. That was necessary to avoid erythrocyte aggregation but, as expected, it led to a prolonged acquisition time (typically one to two hours).

Table 7. Channel distribution of fluorochromes-antibodies/dyes and BF in ImageStream.

FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10	FL11	FL12
	Blue laser					Violet laser			Red laser		
BF	CD71 FITC	CD105 PE-Cy7	CD117 ECD	-	SSC	CD36 PB	CD45 KO	BF	-	-	DRAQ5

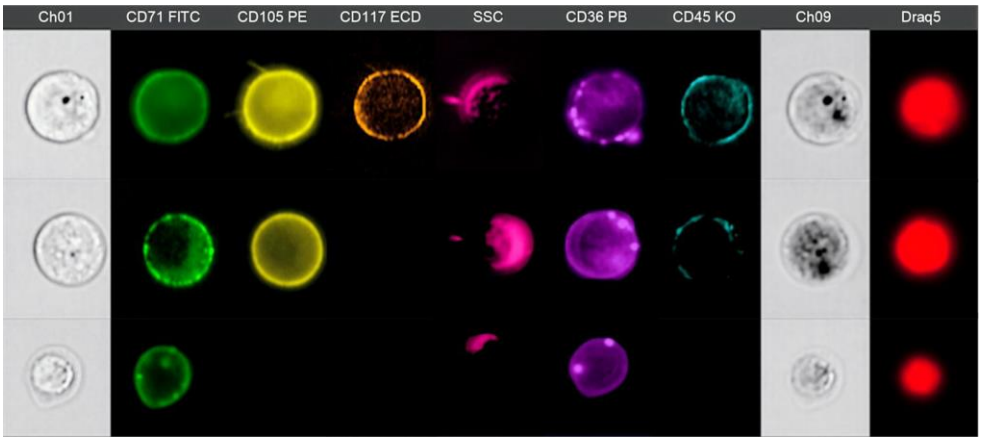

Figure 14. Image gallery of BF images and markers used in ImageStream.
From top to bottom, a proerythroblast, a basophilic erythroblast and a mature erythroblast are shown.

One of the most interesting methodological aspects of IFC are the strategies – i.e. the combinations of features and masks – developed by the researchers to describe and calculate the parameters of interest. Although I would like to elaborate on our strategies, such a level of technical detail does not seem appropriate for a thesis. This information, however, is available in the Supplementary data of the 2nd article, where an explanatory list of the features used in the study is also included. Also, a comparative discussion about our and other researchers' strategies follows in the Discussion section of Study II.

Here, I will summarize the parameters included in our study. The cohort of NBM was used to define normal references for the studied parameters. First, we defined megaloblastoid changes (i.e. the combination of cell enlargement with asynchronous maturation of cytoplasm and nucleus) in IFC terms. To calculate cell size, we used the feature *Cell Area* applied on the BF mask (Figure 13). Since nuclear maturation involves chromatin condensation, we required a quantitative measure of this process. We

calculated therefore the ratio of the feature *Bright Detail Intensity* of the DRAQ5 mask to the *Nuclear Area* (defined by the DRAQ5 mask, Figure 13); our rationale was that more condensed DNA would result in brighter DRAQ5 intensity within a smaller area and that, accordingly, an increasing ratio would correspond to increasing condensation. Next, we defined nuclear abnormalities using the feature *Aspect Ratio Intensity* of the DRAQ5 nuclear mask, applying a cutoff value of 0.8; the performance of the selected feature and cutoff value in detecting asymmetrical, abnormal nuclei was confirmed by manual inspection. The identification of binucleated cells was more complex and required a combination of shape features, including *Lobe Count, Circularity, Symmetry, Aspect Ratio Intensity* and *Compactness*. To explore the cytoplasmic compartment, the *Haralick texture* features were exploited.[167] Finally, to investigate the CD36 "dot-like" expression pattern, the *Spot Count* feature was applied on the CD36 mask.

In short

Imaging flow cytometry: A method combining MFC and imaging. Image analysis is based on morphometric measurements rather than manual assessment.

Advantages: Unique method. Flexibility thanks to the large number of features. Allows for image analysis of a larger number of cells compared to traditional light microscopy. Both built-in and external machine learning modules are available, allowing for automatization.

Weaknesses: In general, a time-consuming method. Data analysis involves a considerable learning curve including an inevitable trial and error process. Analysis software has a complex user interface (compared with the ones most commonly used in clinical and research laboratories).

FlowSOM

Euclidean distance: The straight-line distance between two points in space – like measuring with a ruler.

Iteration: One complete repetition of a process or set of steps, where the output of one step can be used to refine the next.

Arcsinh (inverse hyperbolic sine): A mathematical function for data transformation. In FC, it is used for transforming fluorescence intensity values (which can span several orders of magnitude, including negative values) in a linear way for lower values (weak expression) and a logarithmic way for higher values (strong expression).

Acyclic graph: A graph connecting nodes by branches, in which there is no way to start at one node, follow a continuous path along the branches, and return to the same node without retracing any branch. In simpler terms, you can never loop back to where you started.

FlowSOM is the computational analysis method applied in studies III and IV. It is an algorithm performing unsupervised clustering and visualization of high-dimensional flow cytometry data. In practice, this allows for the distinction of cell populations that manual – supervised – flow cytometric analysis cannot discern. Deeper understanding of rare or "concealed" populations may be beneficial to disciplines such as immunology and oncology.

FlowSOM is based on *self-organizing maps* (SOMs). The term SOM refers to a computing system that creates a two-dimensional representation (a *map*) of multidimensional data, allowing the visualization and interpretation of complex data.[168] I will try to walk you through this process step-by-step, to make sure that both you and I understand. I will use an example related to flow cytometry, where input data represents the phenotype of cells, based on multiple marker expression, e.g. CD36, CD71, CD105 and CD117. Each cell's phenotype is called an *input vector*. In our example, an input vector will be a string of numbers representing the fluorescence intensity of the four applied markers.

- The first step, *initialization*, is the random creation of a network or grid of *neurons* or *nodes*, each of them with a prototype profile (in our case a prototype phenotype), which is called *weight vector*. Importantly, this weight vector is randomly defined, not necessarily corresponding to an existing one, but still

based on the defining parameters (in our example, the expressed markers). So, it will be a random string of intensity values for CD36, CD71, CD105 and CD117.

- The next step is *competition*. Each cell's input vector is compared to the predefined weight vectors and gets assigned to the neuron whose weight vector is closest to that cell's vector. This "winner" neuron is called the *Best Matching Unit* (BMU). Comparison of vectors is based on distance metrics; in FlowSOM, Euclidean distance is applied.
- *Adaptation*. This is the training step of the process. The BMU updates its weight vector to better match the input vector (remember, the weight vector is an artificial one). The neurons neighboring the BMU will also adjust their weight vectors accordingly.
- *Iteration*: This process is repeated for each cell of the input data and with each repeat the distance between the neurons is adjusted. With ongoing training, the organization of neurons becomes increasingly refined, eventually stabilizing as all cells are assigned to their best-matching neuron.

Finally, a map is generated in which all input cells are clustered to neurons whose relative positions visualize their similarities: nearby neurons represent similar data, whereas distant ones reflect greater dissimilarity. The whole learning and organizing process takes place by itself and is completely unsupervised, justifying the term *self-organizing*.

FlowSOM was developed at Ghent University, Belgium and was launched in 2014,[169] as an answer to the increasing complexity of flow cytometry data, particularly following the introduction of mass cytometry in research. The algorithm became available as an open-source R-based script, which allowed for free customization and development by other users. Besides R, FlowSOM can be run across multiple platforms, either through integration with the R environment, such as OMIQ, CytoExploreR or the cloud-based Cytobank, or via dedicated plugins in software such as FlowJo and Kaluza.

Regardless of the platform used, FlowSOM analysis is performed on flow cytometry standard (.fcs) files and the workflow consists of the same four steps:

i) The indispensable quality control and data transformation. This includes compensation check; checking for flow rate abnormalities; removing doublets and debris; scale transformation (logicle or arcsinh are preferred for fluorescence intensity and linear for scatter). Importantly, to ensure comparable measurements, the pre-analytical steps should also be standardized as much as possible. Although these controls are crucial in all kinds of FC analysis, they are of particular importance in FlowSOM

analysis: due to the method's high sensitivity, technical deviations can interfere with the biological ones, leading to subpopulations that are not biologically meaningful.

ii) Creation of SOMs as explained above and visual transformation of the derived grid of clusters to Minimal Spanning Trees (MSTs). MSTs are weighted, acyclic graphs in which nodes are connected by branches, such that the total sum of branch weights will be minimal (Figure 15). Consequently, each node is connected to the most similar ones.

iii) Outcome inspection. This is a manual step, in which the generated nodes are plotted against classical bivariate plots to ensure that they correspond to meaningful populations and verify their phenotypic characteristics.

iv) Interpretation of results in a biological context.

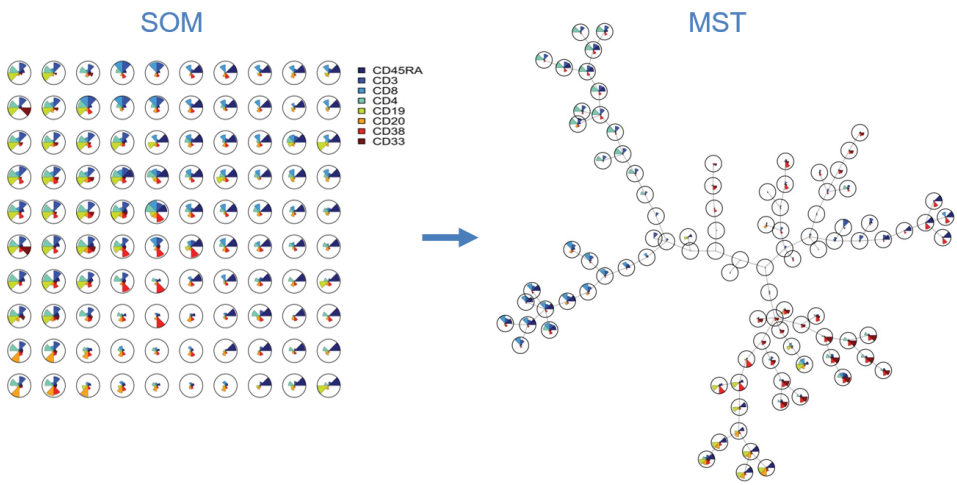

Figure 15. Visual transformation of SOM to MST.
A self-organizing map (SOM) consisting of a 10 x 10 grid is converted to a Minimal Spanning Tree (MST) of 100 nodes. Nodes in both the SOM and the MST are presented as star charts, indicating the mean intensities of the markers for cells assigned to that node. This is a case of a BM and the markers used are listed next to the SOM. Adapted from Van Gassen et al. (2015) with permission from John Wiley & Sons.

Although the clustering process is unsupervised, there are parameters that can be customized by the user: the number of nodes created, the phenotypical markers to be used for training the SOMs and the population to be analyzed. As a rule of thumb, if we are interested in rare populations or population purity, overclustering is encouraged. On the other hand, large numbers of clusters can lead to unnecessary segmentation of populations. During the study design phase, it is recommended to test different numbers of clusters to determine the most informative one. Careful choice of the

defining markers is also crucial to ensure clustering to biologically meaningful populations. Finally, there is also the option to apply the algorithm in a specific subpopulation, e.g. only in erythropoiesis instead of the whole bone marrow.

Another advantage of the algorithm is the wide range of visualization possibilities. The MSTs themselves convey the following information: node size represents population size, while the relative location of the nodes reflects the phenotypic similarity – or the lack of it – between populations. In addition, each node can be visualized as a star chart depicting the mean intensity of all markers applied, allowing for a general inspection of the outcome (Figure 15).

The number of studies applying FlowSOM is ever-expanding. The original article introducing FlowSOM in 2015 – *FlowSOM: Using self-organizing maps for visualization and interpretation of cytometry data* – counts already 1385 citations![169] The main applications are found in the fields of immunology and oncology and many of the studies employ mass cytometry; managing the complexity of mass cytometry data was after all one of the primary objectives for the development of the method. In comparative studies of clustering and computer-assisted algorithms, FlowSOM proved superior owing to its performance, speed, and visualization capabilities.[170–173]

In our hands

We were introduced to FlowSOM by Marie Christine Béné, whose group has developed a protocol for studying normal and neoplastic bone marrow.[174,175] The protocol was built in R, which can be integrated as a plug-in into the Beckman Coulter Kaluza analysis software. Subsequently, the protocol was adjusted to our ERY-panel.

An initial study was conducted on 11 normal BM samples to characterize normal erythroid populations, which were then used as normal reference.[176] In that study, the 11 samples were merged before analysis, whereas in studies III and IV, the BM samples were analyzed independently. In all studies, MSTs of 100 nodes were generated for each sample, representing the whole bone marrow. The nodes belonging to erythropoiesis, designated *erythroid nodes*, were then identified by back gating in a CD45/SSC bivariate plot, further refined using CD36 and CD71; in Study IV we followed the strategy described in the section about the ERY panel (briefly, sequential gating first in a CD36/CD45 and then in a CD71/CD13 bivariate plot). For each erythroid node, the following parameters were exported: population size – calculated as percentage of erythropoiesis; MFI of DRAQ5, CD45, CD36, CD71, CD105, CD117; SSC and FSC intensity; in Study III, the percentage of the $DRAQ5^{high}$ fraction for each node was also defined.

To characterize the erythroid nodes and identify the abnormal ones, the normal references defined in the initial study on normal erythropoiesis were used in Study III. In Study IV, however, a new cohort of normal BM was analyzed, and new normal reference values were calculated. The reason was that the ERY panel applied in study

IV had a slightly different set-up compared to the one used in Study III (Table 6). As explained above, due to the high sensitivity of FlowSOM, technical deviations need to be minimized to avoid noise in data interpretation. Therefore, avoiding mixing cases analyzed with different set-ups was indispensable.

Based on the corresponding reference values, each erythroid node was characterized. A metaclustering process followed, grouping the identified erythroid nodes into larger subsets, the *erythroid clusters* (Figure 16).

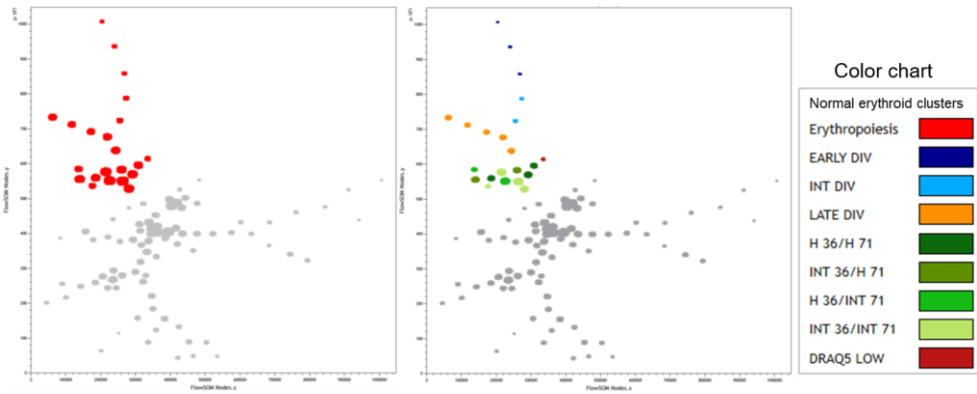

Figure 16. FlowSOM analysis of NBM in Study IV.
MST of NBM consisting of 100 nodes. In the left MST, the erythroid nodes are highlighted with red. After metaclustering, eight normal erythroid clusters were ideniftifed; their designation and color coding are shown in the color chart (detailed characterization of the clusters is presented in Table 9).

The approaches used to define the aberrant erythroid nodes and clusters also differed between the two studies. As Study III aimed to detect as many aberrancies as possible, a wide range of parameters was applied to characterize the erythroid populations. Besides DRAQ5, CD36, CD71, CD105 and CD117 expression – the backbone markers in both studies – side scatter and the size of the dividing ($DRAQ5^{high}$) fraction in each node were also used as characterizing features. After gaining insight from Study III, a simpler approach was applied in Study IV, using only the backbone markers described above. Side scatter was omitted for the reasons explained in the Methodology of the ERY panel. Shortly, in the non-lysed samples, erythrocytes can create aggregates with nucleated cells, which, if present in large numbers, can interfere with the light scatter parameters.

In short

FlowSOM is an automated method performing unsupervised cluster analysis and visualization of complex flow cytometry data.

Advantages: Faster but equally reliable in population identification compared to similar methods. Being an open-source algorithm allows for continuous development and adaptation. A range of visualization options is available.

Weaknesses: Technical deviations can interfere with biological ones, affecting the outcomes. Strict preanalytical quality control is required. The R software environment can require training, but alternatives offering simpler interfaces (e.g. Cytobank) exist.

Samples

All samples used in our studies were fresh, heparinized bone marrow aspirates, analyzed within 48 hours upon arrival in the flow cytometry unit, department of Clinical Genetics, Pathology and Molecular diagnostics, Lund University Hospital, Lund, Sweden.

Criteria for inclusion in the normal cohorts used throughout the studies were: normal blood count, morphologically normal hematopoiesis, no BM involvement by lymphoproliferative or other neoplasia and no previous history of treatment for malignancy.

Ethics Statement

All studies included in this thesis were conducted according to the guidelines of the Declaration of Helsinki and approved by the Regional Ethical Review Board of Lund, Sweden (Nr 223/2017).

Results and discussion

Study I

Participants

The study was conducted in two steps: i) validation of our non-lysis ERY panel; and ii) identification of quantitative and phenotypical aberrancies associated with MDS.

The validation cohort consisted of 80 BM samples, referred to our department during spring 2017 for various investigational reasons. Inclusion criteria was representative FC samples as well as representative BM smears for comparison with morphological differential count.

To detect aberrancies characteristic of MDS we compared the following three cohorts: 68 patients with untreated MDS; 43 patients with non-clonal cytopenias, designated hospital controls (HC); and 29 normal controls (NC). The MDS cohort was further subdivided into two cytomorphologic risk groups – i.e. based on the blast count: a low-risk (n= 37) and a high-risk group (n = 31). The characteristics of the participants in this phase of the study are presented in detail in Table 1 of the 1st article.

Results

Validation of non-lysis protocol

Eighty BM samples with various diagnoses were analyzed with the ERY panel. In addition, FC analysis with other panels using ammonium chloride lysis was routinely applied for diagnostic purposes. Quantification of erythropoiesis, granulopoiesis and $CD117^+$ myeloid precursors was performed with three methods: FC analysis with the ERY-panel, FC analysis with lysis-based panels and differential count on BM smears – the latter was considered the gold standard. The population frequencies retrieved were subsequently compared to each other.

- No significant difference was observed in the percentage of erythropoiesis and of $CD117^+$ myeloid precursors between the ERY-panel and morphology.

- Significantly decreased erythropoiesis was observed in FC panels using lysis compared to both ERY-panel and morphology (p <0.05 for both comparisons).
- Conversely, significantly increased granulopoiesis and CD117^{+} myeloid precursors were observed in FC panels using lysis compared to both ERY-panel and morphology (p <0.05 for both comparisons).
- Bland Altman plots confirmed a better agreement between the ERY-panel and morphology, regarding all three examined populations.

In fifteen of the above-mentioned samples, we applied both the original non-lysis protocol and a lysis step before staining with the ERY-panel. Subsequent comparison revealed in the lysis-treated samples a significant decrease in erythropoiesis along with a significant increase in granulopoiesis (p <0.001 for both comparisons); no difference was observed in the percentage of CD117^{+} myeloid precursors or lymphocytes. Light scatter was also greatly affected (Figure 17).

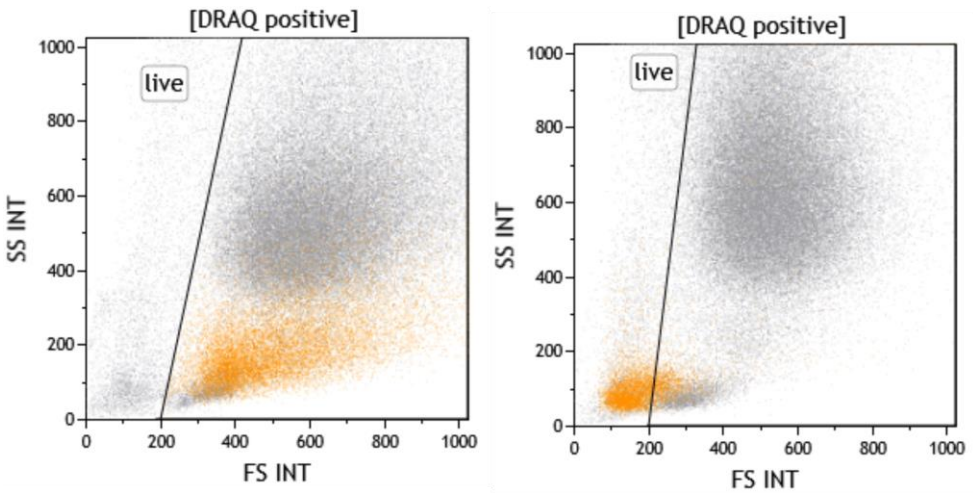

Figure 17. Effect of lysis in light scatter.
The same BM sample analyzed with the original ERY panel (left) and with an additional lysis step before staining (right). Both side and forward scatter of the erythroid precursors (orange) decrease so markedly that the population shifts outside the live-cell gating boundary.

Regarding marker expression, the lysed samples showed decreased MFI and CV of CD36 (p <0.001); decreased MFI and CV of CD71 (MFI p = 0.031; CV p <0.001); decreased MFI and CV of CD117 (MFI p = 0.003; CV p <0.001); and decreased MFI – but not CV – of CD105 MFI (p = 0.007).

Quantitative and phenotypic aberrancies in erythropoiesis discriminating MDS from non-clonal cytopenia

To identify the features that better distinguish MDS from non-clonal cytopenias, we compared 68 BM samples from MDS patients with 43 patients with non-clonal cytopenias (HC) and 29 normal controls (NC). The latter were also used to define normal reference ranges.

In MDS, the overall percentage of erythropoiesis was higher than in NC ($p = 0.044$), while the proportion of early $CD117^{+}$ erythroid precursors was significantly reduced compared to NC ($p = 0.001$). However, no differences were observed in these populations when MDS was compared to HC.

Among the evaluated markers, the aberrancies which significantly differentiated MDS from HC were increased CV of CD36 ($p <0.001$); reduced MFI of CD36 ($p = 0.004$); and increased CV of CD105 ($p <0.001$). Of note, when we compared HC with the low- and high-risk MDS subgroups respectively, these changes were statistically significant only for the high-risk group.

The CV and MFI of CD71 as well as the MFI of CD105 did not discriminate between MDS and HC.

An aberrant $CD36^{-/dim} \pm CD71^{-/dim}$ erythroid subpopulation was detected in 66% of MDS samples and only 11% of non-clonal cytopenias ($p <0.001$), yielding a sensitivity of 81.7% and specificity of 85.3% for MDS diagnosis. This population is beautifully illustrated in a CD71/CD36 bivariate plot assuming a pattern that in our lab is called "the elephant trunk" (Figure 18). The expression pattern of this subpopulation parallels that of reticulocytes, leading us to the assumption that it represents a genuine manifestation of asynchronous maturation.

A second aberration involved diminished CD105 expression in early CD117+ erythroid precursors, seen in 25% of MDS cases and only 4% of hospital controls ($p <0.001$). Complete loss of CD105 in CD117+ precursors was observed in a subset of cases (Figure 18).

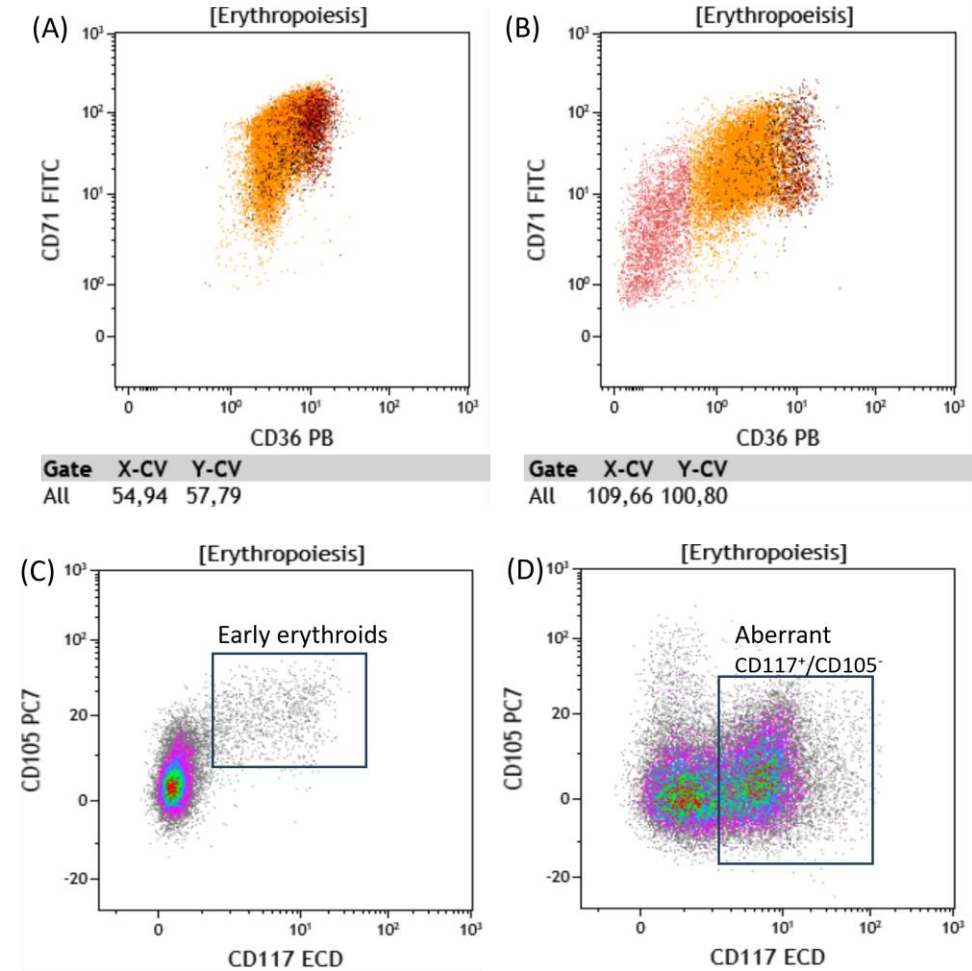

Figure 18. Phenotypical aberrancies detected in MDS with the ERY panel.
(A) NBM for comparison;CD71/CD36 plot depicting normal erythropoiesis. (B) MDS case with an aberrant population (pink) with decreased CD36 and CD71 expression ("elephant trunk"). The CV values of the two markers are markedly increased compared to NBM in the left. (C) Early erythroid precursors with aberrantly decreased CD105 expression. (D) MDS with an aberrant $CD117^{+}/CD105^{-}$ population.

Discussion

Studies of dyserythropoiesis

Back in 2018-2019, when we were working on this study, the articles on FC assessment of erythropoiesis in the clinical context of MDS were few, at least compared to the more popular myelopoiesis.[67,70,72,80,124,140,177,178] The main reason, as explained in the majority

of these works, was the paucity of available markers defining erythropoiesis. The common markers applied in these studies usually included CD45, CD36, CD71, CD105, CD117 and CD235a, as well as less common ones, such as cytosolic H-ferritin (HF), cytosolic L-ferritin (LF) and mitochondrial ferritin (MtF),[177] and were used to identify aberrant expression patterns suggestive of MDS. In addition, quantitative changes in erythroid subpopulations have been identified. The most important FC aberrancies of the erythroid line associated with MDS are summarized in Table 8.

Table 8. MDS-related aberrancies and comparison across non-lysis and lysis protocols[a]

Erythroid aberrancies in MDS	Violidaki et al	Studies using non-lysis	Studies using lysis
Increased erythropoiesis	No	Not tested	Yes[72,178]
Altered CD117$^+$ erythroid precursors[b]	No	Observed[124]	Yes[80,178]
Increased CD105$^+$ erythroid precursors	No	Observed[124]	No
Decreased CD36 intensity[c]	Yes	Yes[70,124]	Yes[80,178]
Increased CD36 CV	Yes	Yes[70]	Yes[80,178]
Decreased CD71 intensity[c]	Yes	Yes[70,124]	Yes[67,77,80,140,177,178]
Increased CD71 CV	Yes	Yes[70]	Yes[80,178]
Increased/decreased CD105 intensity[c]	No	Observed[124]	Yes[140,177,178]
Increased CD105 CV	Yes	Not observed[124]	Yes[177]
Asynchronous CD71/CD235a pattern	Not tested	Observed[124]	Yes[67,72,178]

[a] Aberrancies reported to distinguish MDS from non-clonal cytopenias. Data are pooled, not distinguishing between risk groups or specific MDS diagnoses. Findings that were observed but not confirmed with statistical tests are presented as observed/not observed.
[b] Both increase and decrease is reported.
[c] Various measurements applied (MFI, GMFI).

Among these, the parameters validated through multicenter studies and integrated in the ELN recommendations as well as in the iFS score are increased CV of CD36 and CD71, decreased MFI of CD71 and the altered percentage of CD117$^+$ erythroid precursors.[80,81,112,179] Regarding CD105, in the multicenter study performed in 2017[178] it is stated

> Besides, irrespective of the finding that data for CD105 significantly discriminated between subgroups, this marker was not included. Entering CD105 data would have reduced the power of the (multicenter) analysis, since data on CD105 were only available in a limited number of centers (5/18) and cases.

It is therefore concluded that its diagnostic value in MDS remains to be elucidated.

Of note, in a recent multicenter prospective study performed on an impressive number of 1682 patients with suspected MDS/CMML aiming to validate the diagnostic potential of the various suggested FC aberrancies, only the percentage of erythropoiesis and CD71 expression were evaluated; of these, only CD71 was proven to independently relate to MDS.[77] However, the criterion for choosing the parameters to be validated was that they should have been analyzed in at least 50% of the study cohort cases, and therefore, several aberrancies recommended by ELN recommendations have not been analysed.

Studies addressing the "lysis vs non lysis" issue

Studies addressing the "lysis vs non-lysis" issue are even fewer.[70,124–126] And all of them suggest different approaches!

In 2013, the French group, who also stands behind the RED score, used CyTRAK orange as a DNA dye.[70] In their study they applied both a lysis (VersaLyse, Beckman Coulter) and a non-lysis protocol to BM samples from patients with MDS and non-clonal cytopenias. They compared light scatter between the two protocols and confirmed the effect of lysis on scatter properties and cell size. They even prepared smears from the BM samples after staining with the two protocols visualizing the drastic effect of lysis in cell size. However, they did not compare marker expression between the two protocols.

DRAQ5 or CyTRAK orange?

Both stains have similar properties; they are both permeable and bind to DNA. CyTRAK can also bind weakly to the cytoplasm, a property useful in fluorescence microscopy. However, due to this binding, its emissison peak is broader, preventing a clear distinction between diploid and tetraploid DNA, unlike DRAQ5. Therefore, CyTRAK cannot be used for DNA cell cycle analysis. Another aspect is their different excitation and emission spectra. CyTRAK is optimally excited from 488 to 550 nm (blue and green-yellow lasers), and has a peak emission of 610 nm (orange). The choice between the two stains therefore depends on the instrument's configurations and the overall fluorochrome panel to be applied.[180]

The Hematologics group used DRAQ5 combined with density gradient separation (Ficoll) and compared it with a lysis protocol (based on ammonium chloride). They

subsequently reported on the effect of the two protocols on light scatter and immunophenotypic parameters, both in normal BM and MDS.[124,126] Again, a drastic effect of lysis on the scatter properties of mature erythroid precursors was observed, but not in the immature $CD105^+$ ones, leading to the assumption that the appearance of carbonic anhydrase (an enzyme required for the enzymatic process of NH_4-based lysis) takes place after loss of CD105 expression. They even compared cells affected by lysis – depleted of their cytoplasm – with a deflated balloon containing a tennis ball! They also reported lower CV of the intensity of the applied markers (CD36, CD71, CD105, CD235a) with the lysis method, both in the normal cohort and in MDS, a finding which they interpreted as a "more consistent quantitative antigen expression" when lysis is used. They did not however compare the CV values between normal controls and MDS. Now that it is well established that the CV of CD36 and CD71 increases in MDS, an alternative interpretation of their results is that lysis treatment may underestimate these CV values. Ultimately, their final conclusion was in favor of a lysis approach.

Why not DRAQ5 combined with Ficoll?

Ficoll density gradient centrifugation leads to loss of granulocytes, besides erythrocytes and therefore, its use has been discouraged.[110] In the 2024 meeting of the ELN iMDSFlow WG, Dr Brodersen, upon a discussion on lysis vs non-lysis admitted: "Ficoll, no I would not recommend it".

Much earlier, in 2008, Allan et al performed a study of hematopoiesis based on DRAQ5 with a very similar design as ours;[125] with a four-color flow cytometer, however, the erythroid antibody panel was limited to CD45 and CD71. BM samples with various diagnoses were analyzed in parallel with a non-lysis and a lysis protocol (ammonium chloride). The study focused on quantification of cell lines only; therefore, percentages of various cell populations retrieved with the two methods were compared. They also compared the population percentages with the differential count obtained by morphologic assessment of BM smears. Higher percentages of myelopoiesis and MyP as well as significantly lower percentages of erythropoiesis were observed in the lysis protocols, compared to both the non-lysis approach and to morphology.

Regardless of the approach applied, the common and consistent findings in all these studies were loss of erythroid precursors – the mature ones in particular – and significantly decreased light scatter. Impact on intensity variables of the examined markers,

mainly CD36, CD71 and CD105, was also observed, but not always at a statistically significant level.

Are our results consistent with the aforementioned studies?

We did confirm the effect of lysis in population sizes, resulting in significant underestimation of erythropoiesis and, conversely, in significantly higher percentages of granulopoiesis and of MyP. We also confirmed the impact on scatter properties and cell size. Effect on phenotypical parameters was also shown and proved to be statistically significant for all the interrogated markers: CD36, CD71, CD105 and CD117.

Regarding identification of MDS-defining aberrancies, the reported increase in the percentage of erythropoiesis as well as the altered percentages of $CD117^+$ and of $CD105^+$ erythroid precursors in MDS could be observed but did not prove statistically significant when we compared MDS with non-clonal cytopenias. Neither could we prove statistical significance in one of the most consistently reported findings in the literature, namely the altered CD71 intensity. Finally, we demonstrated statistically significant increase of CV of CD105 in MDS, but not of MFI of CD105, as shown by others.[124,140,177,178] This comparison is summarised in Table 8.

From the comparison above, the finding that stands out is our reported lack of significance in CD71 expression. Is this result discrepant from what has been reported in other studies? Although decreased CD71 expression in MDS has been consistently described in the majority of the aforementioned studies, some could not prove significance,[70] and yet some lacked statistical analysis whatsoever[67,124]. Could there be some additional biological explanation for this heterogeneity of observations? CD71 expression is dynamic, not only dependent on the stage of maturation but also regulated by erythropoietin and upregulated in anemia.[181] I do not claim that CD71 is not informative in terms of dyserythropoiesis, but I strongly believe that in the FC study of erythropoiesis, at least one additional marker, preferably CD36, should be included. I express this opinion under the light of the recent multicenter study for the validation of MDS associated FC aberrancies, in which only CD71 was validated; markers analyzed in less than 50% of the cases were excluded.[77]

Additional applications of the ERY panel and technical considerations

Erythroid destruction can lead to a dramatic increase in the relative proportion of other cell populations. A not uncommon phenomenon in lysis-treated BM samples is the overestimation of $CD34^+$ blasts in MDS cases, sometimes reaching levels above 20%; this is particularly true in cases with markedly expanded erythropoiesis (Figure 19).

Another complication of lysis effects on scatter properties and cell size is that the "shrunk" erythroid precursors often gather outside the viability gate, commonly based on FSC/SSC (Figure 17). This fact can have a disturbing effect on live gating, because one has to either gate broadly including debris or gate tightly sacrificing the erythroid precursors.

Finally, since DRAQ5 can separate all nucleated cells from erythrocytes, the panel can be used to assess hemodilution. The subsequent presence of erythroid precursors, CD117^{+} myeloid precursors (provided there is no erythroblastosis in PB) and CD117^{+} mast cells can confirm representativeness.

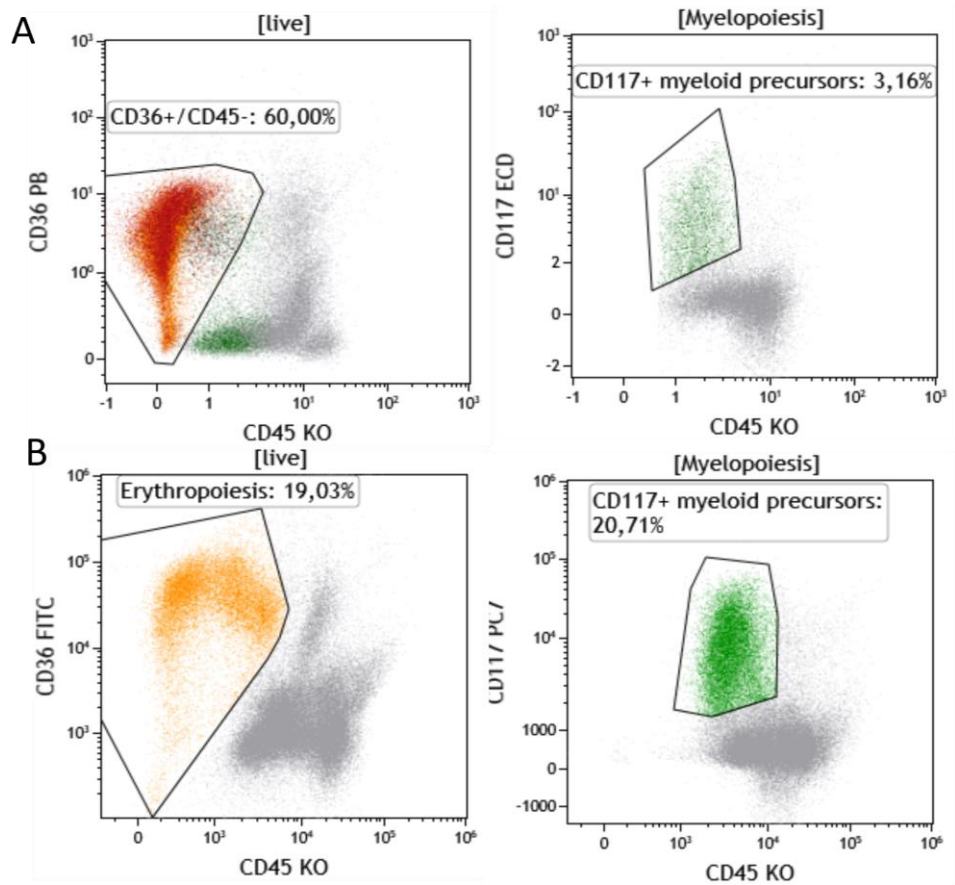

Figure 19. MDS case with discrepancy in blast count between the ERY and a lysis- based panel. (A) In the ERY panel, 60% erythroid precursors and 3% CD117^{+} myeloid precursors were measured. (B) In a lysis-based panel the erythroid precursors were 19% and the CD117^{+} myeloid precursors almost 21%; the CD34^{+}/CD117^{+} blasts were 17% (data not shown). This finding would motivate a diagnosis of MDS with blast increase type 2 (WHO 5th edition) or MDS/AML (ICC). By morphology, the blast count was 4%!

What are the current views regarding FC study of erythropoiesis in MDS?

Today, analysis of erythropoiesis is well integrated into the flow-cytometric diagnostic work-up of MDS, but it seems that the choice of optimal markers has not been consolidated yet. At the same time, the focus appears to be shifting from adding new markers to validating those already proposed.[77,83] Paradoxically, however, markers with proven value are being excluded from validation studies because the available data remain limited, as discussed above.[77,178] A shift of interest in the study of erythropoiesis in the context MDS is also reflected in the literature, where the most recent publications attempt to identify prognostic and predictive factors[29–31] or explore alternative FC modalities, especially computational methods[26–28].

Regarding the "lysis vs non-lysis" issue, the ELN iMDS Flow WG has made some attempts to address it. In the 2023 recommendations on pre-analytical and technical issues,[111] the conclusion, mainly based on our data, is that

> non-lysed whole samples (optionally diluted in phosphate-buffered saline [PBS]) with an adequate gating strategy thus seem a reasonable and feasible option for an accurate assessment of the erythroid compartment in suspected MDS.

Still, no concrete recommendations were provided.

In all sincerity, could non-lysis protocols for the study of erythropoiesis be established in the diagnosis of MDS?

They could. Personally, I think they should. The non-lysis approach is superior in terms of quantification; and studies utilizing intact erythropoiesis are likely to generate more robust and reliable results.

That said, despite the demonstrated effect of lysis on the phenotype, lysis-based studies have adequately identified phenotypical aberrancies associated with dyserythropoiesis. I can also acknowledge the practical advantages of lysis-based protocols: they allow the combination of erythroid and myeloid markers within the same panel – particularly relevant as the complexity of FC panels increases. Also, a common sample processing approach for many/all assays used in a laboratory can facilitate standardization and prove time saving, especially for high-throughput clinical laboratories. Finally,

I had the good fortune to attend the iMDS Flow WG meetings in 2023 and 2024, where the "lysis vs non-lysis" issue has been discussed. Although the drawbacks of lysis protocols are now better understood, their practical advantages seem to outweigh these limitations. I would also dare say that the institutes applying non-lysis protocols are too few and – admittedly – too small to strongly influence the field.

Final thoughts

Finally, the great diversity in methodology applied in the aforementioned studies should be discussed. The methodological differences in all possible aspects and steps of study designs (definition of normal references, measured parameters, statistical tests selected) render the comparison of results between studies difficult, if not impossible. Some examples: Brodersen et al[124] define as abnormal CV and MFI values those that deviate a half log from normal; Mathis et al[70] use Geometric Mean Fluorescence Intensity, while all the others – including us – apply Mean Fluorescence Intensity. This diversity underscores one of the most vexing issues in Flow Cytometry: lack of standardization. Multicenter studies combining data from institutes applying different protocols try to circumvent diversity through data normalization and harmonization, an approach that is considered acceptable but can introduce additional bias.

Study II

Participants

In this study, we compared BM from patients with untreated MDS and MDS/MPN (MDS group, n = 26) with normal BM (NBM group, n = 12). All samples were collected and analyzed between 2018 and 2020.

The NBM group consisted of ten BM samples retrieved from patients who underwent BM investigation for lymphoma staging; one patient was referred with clinically suspected mastocytosis, but no evidence of such after completed medical investigation; and one patient was investigated because of a focal lesion in the spleen, initially suspicious for lymphoma, which proved to be hemangioma.

The MDS group consisted of 21 patients with MDS and five patients with MDS/MPN, all classified according to the revised 4th edition of the *WHO Classification of Haematolymphoid Tumours.*[182] Detailed presentation of the MDS diagnoses and the patients' characteristics is provided in the Supplementary Table S1, in the Supplementary Data of the 2nd article.

Results

In this study, we evaluated the utility of Imaging flow cytometry for the analysis of erythropoiesis in MDS. The ERY panel, slightly modified, was applied to BM samples from 26 patients with untreated MDS and 12 normal bone marrow (NBM) controls. The following MDS-defining changes were explored:

Cytoplasmic changes

In MDS, erythropoiesis demonstrated significantly higher cytoplasmic texture values compared with NBM, particularly for the Haralick features *Contrast* and *Variance* (p <0.001 for both features). The finding, however, was not correlated with cases with increased counts of ring sideroblasts. As control, we compared cytoplasmic complexity of granulocytes and lymphocytes between MDS and NBM; in these two populations no differences were observed.

Nuclear abnormalities and binucleation

Cells with abnormal nuclear morphology were increased in MDS (mean 11.5%) compared to NBM (mean 9.1%, p = 0.017). This increase was predominantly confined to the mature erythroid compartment (66.6% of abnormal nuclei in MDS vs. 54% in

NBM, p = 0.001), while early stages showed no significant difference. Binucleated erythroblasts were also more frequent in MDS (mean 0.9% vs. 0.4% in NBM, p <0.001), again primarily within mature erythropoiesis (73% vs. 57%, p <0.001). Analysis of DRAQ5 intensity revealed that most binucleated cells in MDS were diploid and in G1 phase, suggesting non-proliferative "bilobated" erythroblasts, whereas NBM contained proportionally more proliferating binucleated forms (G2/M phase).

Megaloblastoid changes

Across all maturation stages, erythroblasts exhibited significantly larger cell size in MDS compared to NBM (p <0.001) (Figure 20). Nuclear condensation, quantified as the ratio of *Bright Detail Intensity* of DRAQ5 to nuclear area (BDI_DRAQ5/Nuclear Area), increased with maturation in both groups. When all erythroblasts were analyzed, nuclear condensation did not differ significantly between MDS and NBM. However, when restricted to erythroblasts with enlarged cell size (> mean + 2SD of normal), nuclear condensation was significantly lower in MDS compared to NBM, a finding observed across all maturation stages (p <0.001), demonstrating objectively that megaloblastoid change is characterized by enlarged cells with less condensed chromatin.

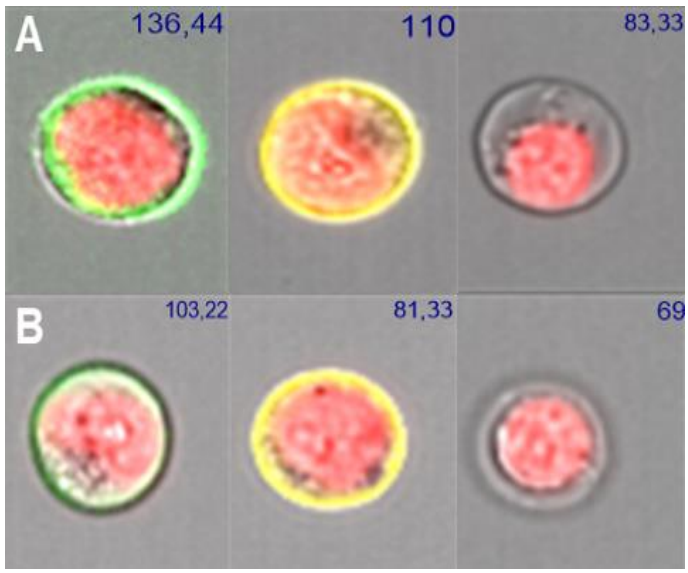

Figure 20. Increase of cell size in MDS.
A. An MDS case with increased cell size in all maturation stages; from left to right: proerythroblast (green, CD117), basophilic erythroblast (yellow, CD105) and a mature erythroblast. B. A normal case for comparison. The numbers in blue font represent cell size in μm^2.

CD36 dot-like expression pattern

One finding that drew our attention was the dot-like expression pattern of CD36. To further investigate this, we applied the *Spot Count* feature and identified three groups: with >5, 1-4 and zero spots. We observed decreasing spot count across maturation stages both in NBM and MDS (Figure 21A). In MDS, cells without any CD36 spots were significantly increased compared to NBM, both in the mature compartment (p = 0.03) and for the CD117$^-$/CD105$^+$ Baso (p = 0.02); accordingly, mature erythroid cells and Baso with >5 spots were decreased in MDS (p = 0.001 for both maturation stages). No difference was observed in the CD117$^+$/CD105$^+$ ProEry stage (Figure 21A). As expected, CD36 spot count correlated with the CD36 MFI per maturation stage.

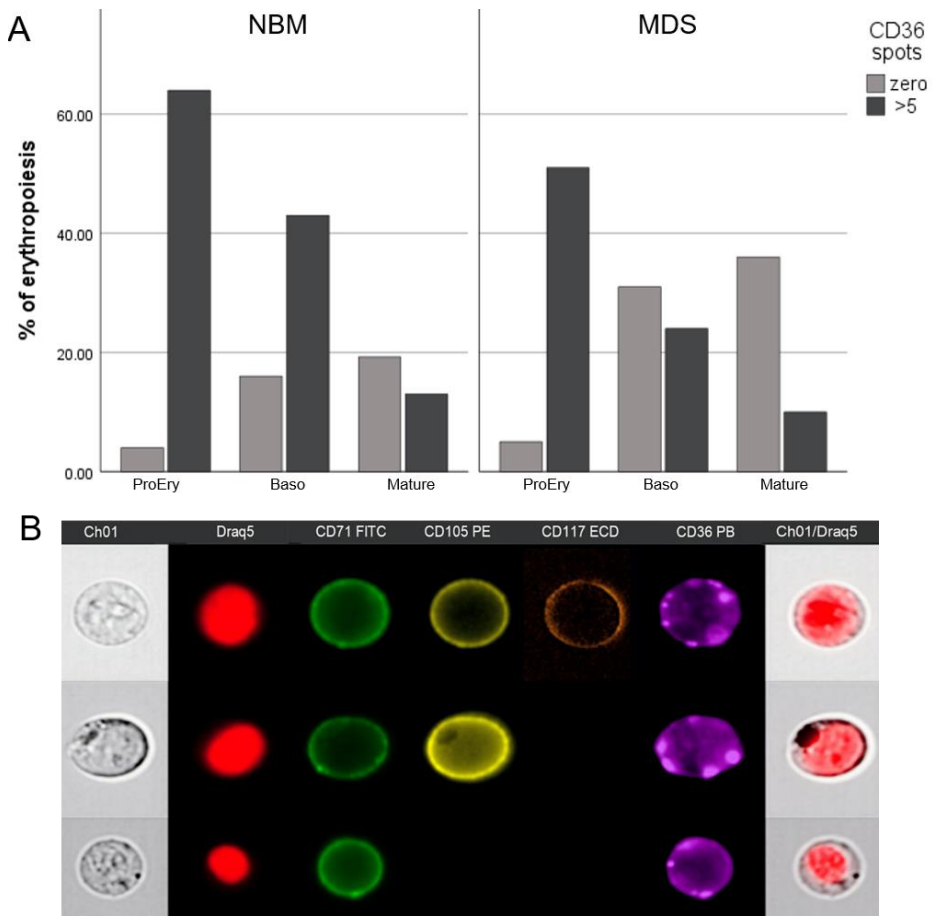

Figure 21. CD36 "dot-like" expression pattern.

A. CD36 spot count across maturation stages in NBM and MDS. B. Image gallery of a proerythroblast, a basophilic erythroblast and a mature erythroblast, showing decreasing CD36 spots with maturation.

Discussion

The initial aim of this study was to explore the diagnostic value of IFS in MDS, by comparing MDS with both normal BM – and, most importantly – with cases of non-clonal cytopenia. In other words, can IFC be used in the diagnosis of MDS?

The samples used in this study were collected and analyzed between 2018 and 2020, but due to two consecutive periods of maternity leave, data analysis started only in 2023. In the meantime, many things happened. Less experienced personnel were involved in the FC analysis, which led to suboptimal sample processing; consequently, a considerable amount of data, especially from non-clonal cytopenias, could not be used. And, in 2020, Rosenberg et al published an article with very similar design: *Exploring dyserythropoiesis in patients with myelodysplastic syndrome by imaging flow cytometry and machine-learning assisted morphometrics.*[183] Even their antibody panel was similar to ours, including DRAQ5, Thiazole Orange, CD45, CD235a, CD71, CD105, and CD117. Their main results concerned cell size and binuclearity, the two most common morphologic findings of dyserythropoiesis that were also objectives in our study.

Still, until today, no other studies applying IFC in MDS exist. The scientific gap in this field was evident. In addition, some questions remained unexplored in Rosenberg's work, for example, regarding the cytoplasmic compartment. Therefore, conducting this study, even just to confirm previous results and support the reproducibility of the method, would already be a valuable contribution. However, without an adequate number of cases of non-clonal cytopenias, we needed to redefine the aim of the study.

Interobserver variability and subjectivity are known limitations of the morphologic assessment of dysplasia in BM and PB smears. There is a limited number of studies that have attempted to define and even quantify the dysplastic features in MDS – how large is indeed a megaloblast?[26,184] Still, assessment is commonly based on experience, while some findings – like the megaloblastoid changes in the earlier maturation stages – are genuinely difficult to assess. The final aim of this study therefore became the identification of morphologic findings of dyserythropoiesis that are accessible to IFC and their translation into objective morphometric parameters.

In this context, the most important results of our study were:

- the quantification of nuclear condensation and, thus, of nuclear maturation, which allowed for a more precise definition of the phenomenon of asynchronous maturation and of megaloblastoid changes, and

- the definition of cytoplasmic complexity as a sign of erythroid dysplasia, which allowed the use of this less recognized cellular compartment as a reliable diagnostic parameter in MDS.

This last finding could be further discussed. In morphologic assessment, the changes observed in the cytoplasmic compartment are ring sideroblasts, cytoplasmic vacuoles and basophilic stippling.[26] Although ring sideroblasts and vacuoles could be visualized in the BF images (Figure 22C), we could not elaborate appropriate features to measure them.

Therefore, we employed the texture features, assessing the general texture variation of the cytoplasm. However, cases with higher cytoplasmic texture were not correlated with MDS with ring sideroblasts. This observation prompted us to explore other explanations for the MDS-associated cytoplasmic complexity. In MDS, impaired mitochondrial gene expression and accumulation of mitochondria have been described.[185] In addition, it has been shown that erythroid maturation depends on mitochondrial clearance.[186,187] These observations led to the hypothesis that disturbances in mitochondrial clearance could be an MDS-associated event responsible for higher cytoplasmic complexity. Since the Fc study of mitochondria is already well established,[188] confirmation of our hypothesis could be feasible.

Furthermore, IFC allowed for better insight into the biology of MDS. The well-known binucleated erythroblasts were shown to be diploid, best fitting with a single but "broken in two" – aka bilobated – nucleus, a finding supporting their abnormal nature.

Examples of erythroblasts with nuclear abnormalities and ring sideroblasts are shown in Figure 22.

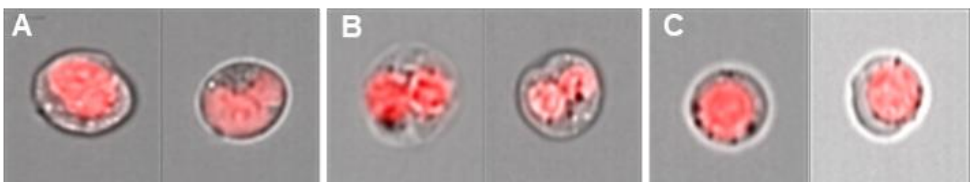

Figure 22. Morphologic features of dyserythropoieisis visualised with IFC.
A. Erythroblasts with abrormally shaped nuclei. B. Binucleated cells. C. Ring sideroblasts.

Analyzing data with Imaging flow cytometry can be fascinating, like intellectual popcorn – small findings capture attention, and new scientific questions emerge all the time. For example, our finding of "dot-like" expression of CD36 prompted me to search more about CD36. This marker was not included in McGrath's or Rosenberg's IFC studies, but I found studies using fluorescence microscopy to visualize CD36 in cell cultures; in these, a fine dot-like pattern can be discerned.[189,190] Searching further,

I found studies providing evidence that CD36 is located in membrane lipid rafts, something that could nicely explain the dot-like pattern we observed.[190–192]

A comparison with Rosenberg's study from 2020 was strongly encouraged by the reviewers when we submitted our article for publication.[183] In their study, an increase in cell size is used as synonymous with megaloblastoid changes. However, megaloblastoid changes are defined as the combination of increased cell size and asynchronous maturation. By measuring both cell size and nuclear condensation, we provided a more exact definition of the phenomenon. Their strategy to identify binucleated erythroblasts can also be discussed. Although we followed the detailed steps they describe in their supplementary data, the watershed mask they applied did not show adequate sensitivity or specificity in our hands. Neither did we manage to find another strategy exclusively identifying binucleated cells. Instead, we applied the Feature Finder Wizard of the IDEAS® software to identify the most distinguishing features (*Lobe Count, Symmetry, Circularity, Compactness*) and, by combining them, we reached the narrowest population of cells with abnormal nuclei including the binucleated cells. Manual inspection and "hand-picking" were subsequently performed to collect the real binucleated cells in order to study them more closely. We did, however, provide a sound strategy for the identification of abnormal nuclei in general, which were shown to be significantly increased in MDS compared to NBM, whereas in Rosenberg's study

> the applied strategy did not support distinction between dysplastic MDS BM and control samples, emphasizing the difficulties associated with quantification of nuclear irregularities.

In addition, to distinguish binucleated erythroblasts from doublets (i.e. cells clustered together), Rosenberg's group used only CD235a; however, this method does not consider doublets between erythroblasts and other cell types, e.g. lymphocytes. Finally, their material consisted of cryopreserved BM mononucleated cells isolated with a density gradient medium. Studies on the effect of cryopreservation focus mainly on immune PB cells or mesenchymal BM cells and the effects on their quantities and functionality,[193,194] data regarding potential effects on marker expression are controversial.[195–197] Although there is not enough evidence supporting the inferiority of cryopreserved BM cells, Rosenberg's group suggested themselves that further investigations be performed on fresh, non-lysed bone marrow.

Besides Rosenberg and her group, erythropoiesis has been earlier studied with IFC by Kathleen McGrath; her works aimed mainly to delineate normal stages of the cell line in human and mice.[152,198] She designed a protocol based on DRAQ5 and identified some of the technical issues to be considered. It is intriguing why erythropoiesis, which

has been less explored by conventional FC compared to myelopoiesis, attracted so much interest in IFC. Probably, the nuclear uniformity of normal erythroblasts – i.e. round forms – compared to the greater nuclear variation of the granulopoietic maturation stages as well as the nature of dyserythropoietic changes (e.g. cell size), are more accessible to measurement by IFC.

Some technical considerations regarding the technique have already been discussed in the Methodology section. Here, it can be concluded that in IFC, morphologic assessment does not mean "eyeballing" the cells but using mathematical expressions, the *features*, to describe them. This allows for a large number of cells to be assessed, and, importantly, in an objective manner. However, although the use of these features is indispensable for translating morphologic findings into measurable morphometric parameters, it can also prove challenging. Mastering their wide range requires time as well as a trial-and-error process; and, as highlighted by the comparison between our and Rosenberg's study, finding the most suitable strategies is not always straightforward or even feasible. In this context, IFC is a technique that could substantially benefit from AI-applications.[159–165]

In all sincerity, could IFC be used in the diagnosis of MDS?

I think yes. The method is currently used only in research and data analysis can be time-consuming, but considerable development is ongoing. The possibility of integrating artificial intelligence applications, could further improve the method's effectiveness. For application in MDS, well-structured studies, including a large number of MDS cases and controls, preferably investigating all hematopoietic cell lines, could validate diagnostic morphometric parameters that may surpass traditional morphology in objectivity.

Rephrasing the original question: *In all sincerity, could IFC replace a century's tradition of using Romanowsky-type stains for blood cells?*

Well, now the answer is less straightforward. As an old-school morphologist myself, I would have a hard time parting from my beautifully MGM-stained smears and replacing them with histograms.

Studies III and IV

Participants in Study III

In this study, five BM samples from patients with non-clonal cytopenia and 11 samples from MDS patients at diagnosis were analyzed with FlowSOM; both low and high-risk MDS cases were included. For one MDS patient, four follow-up samples (during treatment with 5-aza and after allo-HSCT) were also examined. Samples were collected during 2017 and 2018, and therefore the revised 4th edition of the *WHO Classification of Haematolymphoid Tumours*[182] applies to the MDS diagnoses.

The diagnosis and characteristics of the 16 patients included in this study are summarized in Table 1 of the 3rd article.

Results of Study III

In this study, pathological BM samples (n=16), clonal and non-clonal, were analyzed with FlowSOM, aiming to evaluate the contribution of the technique to identify FC aberrancies in disease. Identification of abnormal erythroid nodes was based on the normal reference values defined in a previous FlowSOM study on 11 NBM.[176]

In all pathological BM samples, phenotypical changes were observed. FlowSOM identified in total 18 erythroid clusters that were absent in NBM; eleven of these were present only in MDS. These abnormalities mainly involved reduced or asynchronous expression of CD36 and/or CD71, as well as alterations in side scatter properties. In addition, quantitative changes were observed, such as increase, decrease or disappearance of normal erythroid clusters.

Based on proliferative activity and phenotypic aberrancies, three main patterns were observed in MDS: i) reduced proliferation combined with phenotypically abnormal dividing precursors, ii) preserved proliferation with maturation defects in late precursors, and iii) relatively preserved erythropoiesis with minimal phenotypic alterations. Late-stage erythroid precursors with increased side scatter were mainly encountered in cases with excess blasts.

Participants in Study IV

In this study a difference from normal approach was applied to identify aberrancies in MDS. To this end, a cohort of patients was higher risk MDS was compared to a cohort of NBM.

The NBM group consisted of 11 samples; two from healthy donors and nine from patients who underwent BM investigation for lymphoma staging that was concluded to be negative.

The MDS group included 34 patients with higher risk MDS (as defined by IPSS-R and IPSS-M), all of whom had diagnostic samples and available follow-up data during treatment with 5-aza, at different time points. Six of them had additional data after allo-HSCT.

All samples were collected and analyzed between 2019 and 2022; the revised 4th edition of the *WHO Classification of Haematolymphoid Tumours*[182] also applies here.

For the MDS patients included in this study, the diagnoses, clinical and genetic profiles and times of collection of follow-up samples are summarized in Supplemental Table S1 of the 4th article.

Results of Study IV

This is an ongoing study, and the reported results are preliminary.

The main aim of the study was to evaluate the potential of FlowSOM as a monitoring tool for MDS patients during treatment. However, since this study included a larger number of MDS cases compared to the previous one (n = 34 vs 11), we also attempted to expand our observations about FC changes in diagnostic MDS samples and explore possible associations with genetic data.

As a first step however, it was necessary to redefine the normal reference values. As explained in the Methodology, due to the slightly different panel setup compared to Study III, a new cohort of NBM was analyzed, to serve as reference for comparison with MDS samples. In this new NBM cohort, eight erythroid clusters were identified: six of them were already described in the previous FlowSOM study of normal erythropoiesis.[176]

When the same clustering approach was applied to diagnostic samples from 34 high-risk MDS patients, nine additional, phenotypically abnormal erythroid clusters were detected. These included aberrant early, intermediate, and late dividing clusters, as well as mature, non-dividing ones, with decreased CD36 and/or CD71 expression. In 10 of the 34 diagnostic MDS samples (29.4%) an abnormal immature $CD117^{+}/CD105^{-}$ population was also observed. Quantitatively, MDS cases showed mainly a decrease of normal clusters, with the immature ones being most affected.

Both normal and abnormal erythroid clusters described in this study are summarized in Table 9.

Table 9. Normal and abnormal erythroid clusters and their characteristics

Normal clusters	
EARLY DIV	DRAQ5^{HIGH} CD117^{+} CD105^{+} CD36^{HIGH} CD71^{HIGH} CD45^{LOW}
INT DIV	DRAQ5^{HIGH} CD117^{-} CD105^{+} CD36^{HIGH} CD71^{HIGH} CD45^{LOW}
LATE DIV	DRAQ5^{HIGH} CD117^{-} CD105^{+} CD36^{HIGH} CD71$^{HIGH/INT}$ CD45$^{-/LOW}$
HIGH 36/HIGH 71	DRAQ5^{+} CD117^{-} CD105^{-} CD36^{HIGH} CD71^{HIGH} CD45^{-}
HIGH 36/INT 71	DRAQ5^{+} CD117^{-} CD105^{-} CD36^{HIGH} CD71^{INT} CD45^{-}
INT 36/HIGH 71	DRAQ5^{+} CD117^{-} CD105^{-} CD36^{INT} CD71^{HIGH} CD45^{-}
INT 36/INT 71	DRAQ5^{+} CD117^{-} CD105^{-} CD36^{INT} CD71^{INT} CD45^{-}
DRAQ5 LOW	DRAQ5^{LOW} CD117^{-} CD105^{-} CD36^{INT} CD71^{INT} CD45^{-}
Abnormal clusters	
EARLY DIV A	DRAQ5^{HIGH} CD117^{+} CD105^{+} CD36≠HIGH and/or CD71≠HIGH CD45$^{-/LOW}$
INT DIV A	DRAQ5^{HIGH} CD117^{-} CD105^{+} CD36≠HIGH and/or CD71≠HIGH CD45$^{-/LOW}$
LATE DIV A	DRAQ5^{HIGH} CD117^{-} CD105^{+} CD36≠HIGH and/or CD71^{LOW}
CD117+/CD105-	DRAQ5$^{+/HIGH}$ CD117^{+} CD105^{-}
HIGH 36/LOW 71	DRAQ5^{+} CD117^{-} CD105^{-} CD36^{HIGH} CD71^{LOW}
INT 36/LOW 71	DRAQ5^{+} CD117^{-} CD105^{-} CD36^{INT} CD71^{LOW}
LOW 36/HIGH 71	DRAQ5^{+} CD117^{-} CD105^{-} CD36^{LOW} CD71^{HIGH}
LOW 36/INT 71	DRAQ5^{+} CD117^{-} CD105^{-} CD36^{LOW} CD71^{INT}
LOW 36/LOW 71	DRAQ5^{+} CD117^{-} CD105^{-} CD36^{LOW} CD71^{LOW}

The changes that significantly differentiated MDS from NBM were i) decrease of the normal immature clusters (EARLY DIV and INT DIV; p = 0.002 and p = 0.001, respectively), ii) the emergence of an abnormal LATE DIV A cluster (p = 0.002), and iii) appearance of an abnormal mature LOW 36/INT 71 cluster (p = 0.0001).

These three key aberrancies defined five distinct FC patterns ranging from normal-like to highly abnormal profiles: i) pattern 1: no change compared to NBM, ii) pattern 2, decrease of immature subsets only, iii) pattern 3: presence of abnormal subsets only, iv) pattern 4: decrease of the immature subsets + one abnormal subset, and v) pattern 5: all three changes present (Figure 23).

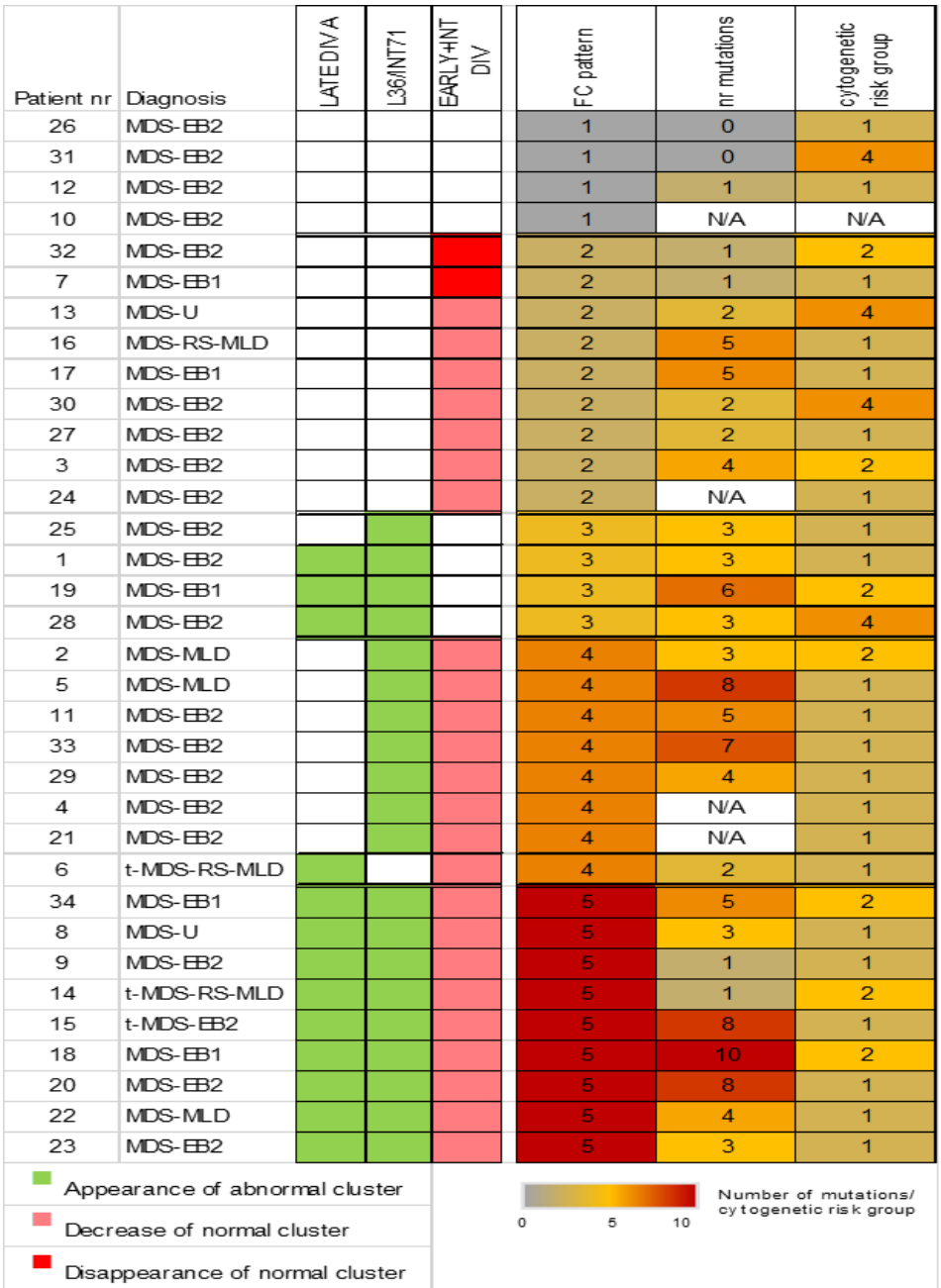

Figure 23. Left panel: Distribution of the three FC changes indicative of MDS per patient at diagnosis. Right panel: Derived FC patterns adjacent to the number of mutated genes and cytogenetic risk group per patient at diagnosis.

The number of FC aberrancies correlated positively with the number of mutated gene variants per patient (*eta* = 0.650; p = 0.040), while no association was observed with cytogenetic risk group, gene function categories or the most recurrent mutations (RUNX1, ASXL1, STAG2).

During treatment with 5-aza, FC patterns evolved dynamically. At 6 months (n = 20), 25% of patients showed FC improvement, 45% remained stable, and 30% worsened. By 12–18 months (n = 12), improvement increased to 58% (p = 0.024). These data are shown in Figure 24. Improvement was defined mainly by disappearance of abnormal erythroid clusters, while full normalization – including restoration of early dividing subsets – was less common in general, but could be observed more frequently after prolonged therapy.

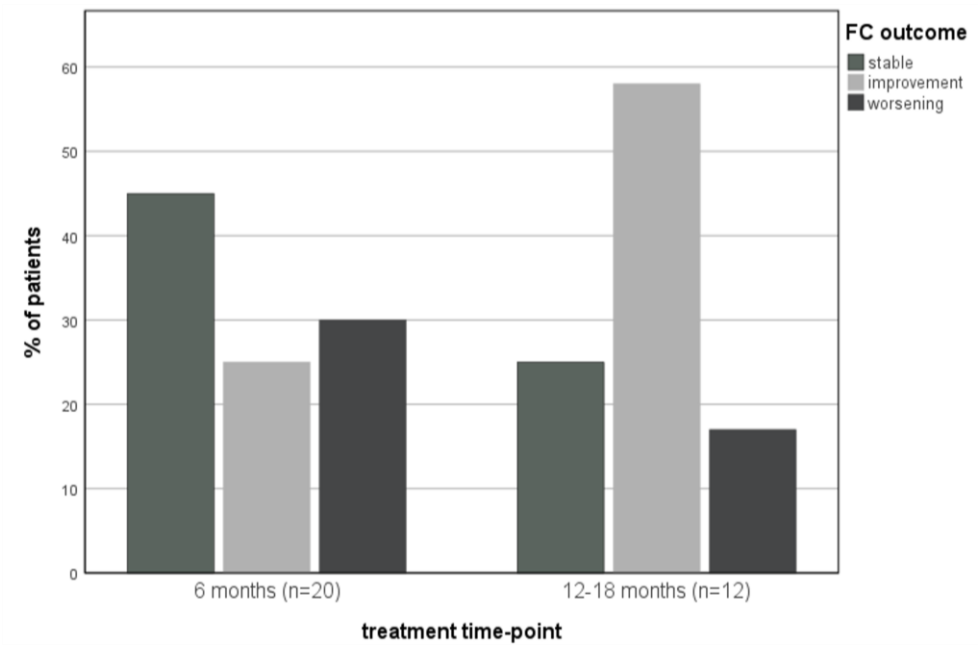

Figure 24. Distribution of patients across the three FC outcomes (stable, improved, worsened) at 6 and 12–18 months of treatment with 5-azacitidine.

At 6 months, 5/20 patients had available genetic data; these patients showed either improved or stable FC patterns along with an improved genetic profile (defined as reduction of the number of mutations and/or of VAF and/or of the cytogenetic abnormalities). By 12-18 months, 4/12 patients with FC improvement showed genetic response or stability, whereas 4 others with improved or stable FC patterns, exhibited genetic progression.

Among the six patients who subsequently underwent allo-HSCT, four in complete remission showed disappearance of all abnormal erythroid clusters, although the immature compartment remained below normal levels. Two patients who relapsed displayed highly abnormal FC patterns.

Discussion of Studies III and IV

The ability of FlowSOM to detect rare or "hidden" populations renders it an attractive diagnostic tool for disorders characterized by phenotypical deviations of the normal. At the same time, this function evokes the concept of measurable residual disease (MRD), based on the identification of rare populations with leukemia-associated immunophenotypes (LAIPs). Both the diagnostic and monitoring potential of FlowSOM in MDS were explored in our studies. Study III was an exploratory and descriptive investigation based on a small number of pathological cases, both clonal and non-clonal, primarily aiming to assess the performance of FlowSOM in detecting aberrancies. The identification and description of these aberrancies therefore served mainly for hypothesis generation. Study IV, which included a larger cohort of MDS cases and integrated genetic data, confirmed and expanded the hypotheses generated in the previous study, identifying clearly aberrant patterns. Going one step further, Study IV also explored the potential of FlowSOM as a monitoring tool during treatment.

Why clustering matters.

In conventional MFC, three erythroid subpopulations are identified: the immature $CD117^{+}/CD105^{+}$ and $CD117^{-}/CD105^{+}$ compartments and the mature $CD117^{-}/CD105^{-}$ one. The latter, often comprising > 90% of the cell line, is visualized as one big clump in the bivariate plots that cannot be further subdivided. Indeed, this is one of the limitations of bidimensional visualization, in which maturation stages are presented as a continuum without distinct borders. Other parameters that could further distinguish the three erythroid compartments in smaller subpopulation, like CD45 or SSC are usually not considered; it is not impossible to do so, but it is labor-intensive.

Another aspect of this limited clustering in conventional MFC is that normal references for marker expression intensities are usually defined for the total erythropoiesis. Taking CD36 and CD71 for example, their expression is usually examined collectively for the total erythropoiesis, both the immature and mature compartments. However, the immature erythroid compartment (both $CD117^{+}/CD105^{+}$ and $CD117^{-}/CD105^{+}$) normally exhibits higher expression of CD36 and CD71 compared to the mature one. In these early cells, aberrantly decreased expression of these two markers would still fall

into the normal range as defined for the total erythropoiesis and remain therefore undetected.

Dividing erythropoiesis into multiple subpopulations allows for an in-depth study of each one of them. This does not necessarily mean that all these subpopulations are meaningful or that they can provide important diagnostic information. But it is worth exploring the possibility, and the high performance and speed of FlowSOM make this exploration feasible and far from laborious.

How did we exploit this clustering?

By segregating erythropoiesis, we identified aberrant populations that could not be discerned by traditional MFC and subsequently explored their diagnostic value. Some examples are the abnormal early and intermediate dividing clusters (designated EARLY DIV A and INT DIV A) as well as the mature dividing cluster (LATE DIV A), characterized by decreased CD36 and/or CD71 expression compared to their normal counterparts (Table 9). None of these clusters could be identified by a classical two-dimensional gating strategy (Figure 25). This is one of the greatest advantages of FlowSOM: it "materializes" the aberrant populations, extracting them from a "soup" of mixed normal and aberrant cells and presenting them as well-defined populations.

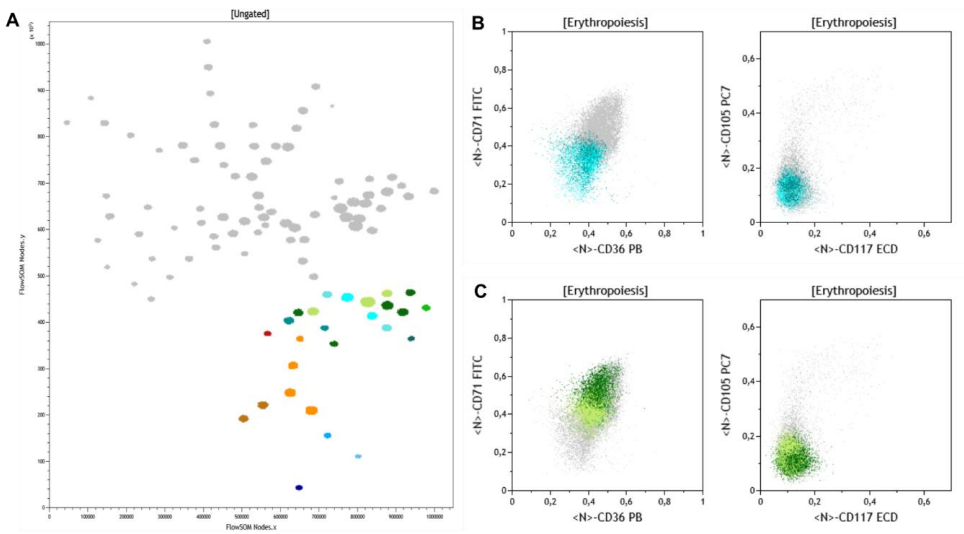

Figure 25. A. MST of an MDS case showing a mixture of normal and abnormal erythroid clusters. The populations detected by FlowSOM could not have been discerned in the traditional bivariate plots, as presented in B. showing abnormal mature erythroid clusters (suspected as such by their low CD71 and CD36 expression), and C. showing normal mature erythroid clusters.

The identified aberrant clusters could subsequently be scrutinized to determine the ones that significantly distinguished MDS from NBM. In keeping with the common practice of evaluating FC parameters collectively (as in scoring systems) instead of individually,[69,78,199] we identified five FC patterns. Interestingly, the increased complexity of these patterns was associated with the number of mutated genes. This finding is consistent with other studies, in which accumulating phenotypic changes in various cell compartments in BM have been associated with the number of mutations.[92,200]

Monitoring

The concept of an MRD-like analysis in MDS has started to gain increasing interest.[201,202] The introduction of the MDS/AML category by the ICC[7], including MDS cases with 10-19% blasts in BM or PB (roughly corresponding to the WHO category of MDS with increased blasts-2), has certainly contributed to this. In addition, in the revised 2023 IWG response criteria for MDS, MRD assessment by FC or NGS is included as a provisional response category.[46]

Few studies monitoring MRD in MDS are available; most of them apply the same principles as in acute leukemias, i.e. they focus on MDS with blast increase, interrogating only blasts based on their LAIP.[203–205] MDS, however, is more complex, since the clonal hematopoiesis expands not only in the aberrant blasts but also in the maturing compartments. The latter, with their various maturation stages and phenotypical variations per stage, render their monitoring extremely difficult. In addition, these aberrant populations do not form visually distinct groups in conventional bivariate plots. Subira et al have proposed a monitoring scheme including phenotypical aberrancies in other populations than blasts, in which they visually assess maturation patterns rather than individual marker expression.[93] However, this approach could be prone to subjectivity.

In this context, the clustering potential of FlowSOM could prove beneficial; by identifying and clearly visualizing the aberrant maturing subpopulations, it renders them accessible to monitoring. The unsupervised analysis can also enhance objectivity. The potential of FlowSOM in MRD assessment has been demonstrated by Vial et al, who applied the algorithm to AML monitoring achieving a level of detection (LOD) of 0.09%.[206] In our study, we demonstrated a tendency towards normalization during treatment with 5-aza. The phenotypically aberrant clusters diminished or completely disappeared, whereas changes in the size of normal populations – particularly the decrease of early dividing erythroid clusters – were more persistent. Quantitative changes, however, do not per se indicate clonal hematopoiesis if not associated with respective phenotypical changes. Besides, this persistent decrease of early diving precursors could be attributed to treatment effect; 5-aza, besides being a

hypomethylating agent, can act as an antiproliferative agent as well. In cases with available genetic data during treatment with 5-aza, FC improvement was to some extent parallel to the genetic response.

Technical considerations

Based on our experience with FlowSOM to date, and as Study IV is still in progress, several technical considerations have emerged that need to be addressed.

Comparability between Studies III and IV. As explained in the Methodology about FlowSOM, some approaches applied in the two studies differed. Different normal BM cohorts were used to define normal references, due to slightly altered settings of the ERY panel. The approach chosen to calculate normal references and to define aberrant subpopulations in Study IV was also refined, as our insights into the method increased. For this reason, the abnormal erythroid clusters identified in Study IV were fewer than in the Study III (nine instead of 18).

Side scatter. As explained in the section about the ERY panel in Methodology, one of the weaknesses of using non-lysed samples is that erythrocytes can create aggregates with nucleated cells; these aggregates, if present in large numbers, can interfere with the light scatter parameters (Figure 11), thereby rendering their interpretation less reliable. Although this artifact affects only a minority of cases, in Study IV we chose not to include SSC in the parameters defining abnormal subpopulations. Interestingly, Duetz et al performed a FlowSOM analysis in MDS using a lysis protocol and identified increased SSC of mature erythroid precursors as one of the most discriminative features between MDS and non-clonal cytopenias.[207] This finding is of course intriguing, although the effect of lysis in SSC, especially in the mature erythroid compartment, should be kept in mind.

Number of nodes. We observed that when we apply our FlowSOM protocol to the entire BM, the number of erythroid nodes generated roughly corresponds to the percentage of erythroid cells in the total sample. This means, if erythropoiesis comprises 20% of total, approximately 20 erythroid nodes will be created. Accordingly, in a BM with 5% erythropoiesis, 5 nodes will be generated. However, less erythropoiesis does not necessarily mean fewer subpopulations; "underclustering" in cases with reduced erythropoiesis could therefore result in missing meaningful subpopulations. One of the recognized issues of the method is to determine the optimal number of nodes, without cluttering or missing information. Creation of a large number of nodes – overclustering – could be balanced by the process of metaclustering. Underclustering on the other hand, would require to forcing the generation of additional nodes. One of the next steps in this ongoing study will therefore be to explore whether generating an identical

number of erythroid nodes for all cases, regardless of the size of erythropoiesis, can provide more information and increase comparability.

"Frozen" MSTs. In the FlowSOM script originally described by Van Gassen, there is the possibility of creating a template MST based on a reference sample that can be subsequently applied to other samples.[169,208] This allows us to detect quantitative and phenotypical deviations from the reference populations and facilitates comparability. This approach was also applied in the MRD study by Vial et al.[206] Of course, such a possibility would be meaningful in our monitoring study. However, the script available to us was only a limited version of FlowSOM, which together with my limited knowledge of R, did not allow us to explore this possibility in this phase of our study.

Looking ahead

As already stated, Study IV is an ongoing project with many issues still open. As a next step, correlation of our findings with clinical response and outcomes will be explored.

Among the technical considerations explained above, applying the same number of erythroid nodes in all cases and testing the "frozen" MSTs, i.e. the same layout for diagnostic and follow-up BM, are also included in our to-do-list.

Finally, examining a cohort of non-clonal cytopenias would contribute significantly to identifying the aberrancies that can more reliably distinguish MDS and to determining the method's sensitivity and specificity.

In all sincerity, could FlowSOM be used in diagnosis of MDS?

Yes. FlowSOM offers several features that may prove beneficial for diagnosis and disease monitoring in MDS. In addition, emerging areas in the understanding of MDS, such as the in-depth characterization of $CD34^+$ MyP and hematopoietic stem cells (HSC) – discussed in the section about future perspectives – provide a fertile ground for the application of clustering and population-recognition methods.

Yet for a method to become established, validation through standardized, multicenter studies is required. As artificial intelligence becomes increasingly integrated into research and clinical practice, new tools are continuously being developed: Principal Component Analysis (PCA), t-distributed stochastic neighbor embedding (t-SNE), visualizing data using t-SNE (viSNE), Spanning-tree Progression Analysis of Density-normalized Events (SPADE) are some of the currently available options. This continuously growing variety of available tools, however, may fragment the field, with many small, incomparable studies. Overclustering is not always beneficial!

Conclusions, future perspectives and Greek philosophy

Conclusions

Flow cytometry is already an indispensable part of the diagnostic work-up of MDS. Besides, it was never meant to act as a stand-alone diagnostic tool; and as a complement to morphology, it works excellently. In the clinical context of MDS, FC is routinely applied by clinical laboratories to:

- Accurately enumerate blast count, the cornerstone of MDS diagnosis and prognosis. This accuracy can be further enhanced by applying a non-lysis protocol.
- Provide adequate evidence to support an MDS suspicion or even diagnosis.
- Exclude other hematolymphoid disorders that comprise differential diagnoses, like acute leukemia and lymphoproliferative disorders.
- It has a central role in the diagnosis of PNH which can be associated with MDS or comprise a differential diagnosis.

Less established applications of FC in MDS are the prognostic and predictive information it can provide as well as the phenotype-genotype associations it may reveal.

And of course, the undisputed strength of FC lies in its ability to analyze vast numbers of cells within a very short turnaround time.

In this context, our studies supported the less explored contribution of erythropoiesis in the diagnosis of MDS. In Study I, we demonstrated the advantages of a non-lysis approach, mainly in the quantification of BM populations; the drastic effect of lysis in the percentage of the erythroid compartment as well as an impact on phenotype have been elucidated. Adding imaging in flow cytometry in Study II offered insights on the biology of dyserythropoiesis. Although the data about Imaging flow cytometry in MDS and hematology in general are limited, this method – after extensive validation studies – could be suggested as an objective alternative to the morphologic assessment of

dysplasia. Automated analysis was used in studies III and IV to reveal populations that conventional flow cytometry cannot discern and to follow them during treatment.

Regarding our suggested non-lysis approach, I need to admit that although the accuracy of such an approach has been acknowledged, the practical advantages of lysis, in particular ensuring common routines in sample handling, seem to prevail. Common routines are after all the cornerstone of standardisation.

Indeed, lack of standardisation and interlaboratory – technical – variation remain the main challenges in the application of FC in MDS. In contrast to the paradigm of leukemia monitoring and MRD assessment, where well-defined protocols – including mandatory antibody panels – have been established by international organisations, like the European Leukemia NET (ELN) and the Nordic Society of Paediatric Haematology and Oncology (NOPHO) or the Children's Oncology Group (COG) in the USA, a universal approach for the diagnosis of MDS has not been achieved to date. And that, despite the efforts of the ELN international Working Group for Flow Cytometry in MDS to provide concrete recommendations regarding both preanalytical and analytical issues.

I would dare say that the main reason for their efforts not to have been widely adopted yet is the generalization of NGS in the diagnosis of myeloid neoplasms. The most intensive attempts to establish FC as a diagnostic tool in MDS started in the mid-2000s and peaked in the mid – late 2010s. But, since the beginning of 2020s and in parallel with the introduction of the IPSS-M scoring system, molecular testing for patients with strong clinical suspicion of MDS has been more widely applied. The subsequent inclusion of MDS categories with defining molecular genetic abnormalities in the 5th edition of WHO and the ICC in 2022 has firmly cemented the indispensability of molecular testing in MDS. This is also reflected in our cohorts: In Study I, amongst 68 MDS cases retrieved between 2017 and 2018, 28 (41%) had available molecular genetic data. In Study IV, in which cases were referred between 2019 and 2022, molecular data were available for 30 of 34 MDS cases (88%).

Is this the end of FC in MDS? Absolutely not! As already stated in the beginning of this section, FC provides crucial information, which in many cases (like in PNH or in lymphoproliferative disorders) is not achievable by other methods. But its role in MDS could be redefined and updated; to this end, new applications and new modalities are already being explored. Let's get to know some.

Future perspectives

Minimalizing invasion

One of the ongoing projects of the ELN iMDSFlow WG is FC analysis of peripheral blood as a screening method to guide further diagnostic steps in patients with MDS suspicion. A pilot study has already been published by the group,[209] but similar attempts have appeared in literature over time.[210–213] Once this endeavor is achieved, its application in disease monitoring could be the next step. Importantly, in the panel suggested by the ELN group, the inclusion of MyP, although in low counts in PB, has proved among the most informative markers.

The MyP and beyond

The significance of $CD34^+$ MyP has already been outlined in the Introduction. In short, the FC aberrancies within this population have consistently shown the most robust diagnostic, prognostic, and predictive relevance. Going further back in the hematopoietic hierarchy, several studies focus on the hematopoietic stem and progenitor cells (HSPC) and their potential diagnostic and prognostic relevance in MDS. Aberrant immunophenotypes detected within this compartment, especially loss of phenotypical heterogeneity, have been shown to carry adverse prognostic implications.[214–217] The study of HSPC is also an ongoing project within the ELN iMDSFlow WG.

The role of immune regulation in MDS

Although much of the effort to understand MDS pathogenesis focuses on its molecular mechanisms, some dared to ask a different question: what is the role of inflammation in MDS? Over a decade, evidence has accumulated, suggesting that inflammation and immune (dys)regulation are indeed key components in the evolution and progression of MDS.[21,22,218–220] These insights resulted in bringing together a consortium of multidisciplinary experts, the International Integrative Innovative Immunology for MDS (i4MDS), acting under the umbrella of the European Hematology Association (EHA). In 2024 the group published their first recommendations, in which flow cytometry plays a central role in the immunological characterization of MDS.[221] The group's ultimate aim is to identify predictive markers and provide innovative immunotherapeutic perspectives. This is indeed an intriguing topic, and I am looking forward to developments in the field.

Monitoring and MRD

The principle of measurable residual disease in MDS, as known from acute leukemia, is applicable mainly in high-risk MDS with increased blasts. The introduction of the category MDS/AML by the ICC,[7] as well as the inclusion of both FC- and NGS-based MRD as a provisional response category in the recent IWG criteria,[46] could suggest that MRD assessment is beginning to find a place in MDS. However, certain challenges are involved in MRD monitoring of MDS, mainly because the clonal nature of the disorder is not restricted to the immature compartment – the blasts – but expands to all maturing populations as well. The approach we follow in Study IV, i.e. the FlowSOM-based clustering of the mature compartment to distinct, clearly visualized and easy-to-follow populations could be proposed as a way to overcome this specific challenge.

Artificial intelligence and automated analysis

Various applications of Artificial Intelligence (AI) in FC in general, and in the field of MDS in particular, have been briefly presented throughout this thesis. To fully explore the whole range of AI possibilities in FC would provide sufficient material not for one but for many doctoral theses. Undoubtedly, AI is here to stay, and the growing dimensionality of FC data is likely to benefit from it. Importantly, AI offers a means to address and reduce the interpretive limitations associated with human analysis. Still, some caution needs to be expressed, since AI remains vulnerable to pre-analytical variations highly dependent on the quality of training data. Carefully designed validation studies and appropriate regulatory frameworks are prerequisites for integrating AI into clinical practice.

Anecdote

This is a case that illustrates some of the strengths and weaknesses of flow cytometry.

On a late afternoon, we received a blood sample of a 61-year-old patient with B-lymphoblastic leukemia with *BCR*::*ABL1* fusion (p190) under treatment and in complete remission, because of blast increase in PB, which coincided with treatment with G-CSF. Hematologists wanted to exclude relapse.

An overview panel including a combination of myeloid and lymphatic markers was performed and detected 21% $CD34^{+}/CD33^{dim}$ myeloid blasts without expression of any B-cell markers. The hematopathologist responsible for the case, brilliant but less experienced, reported the finding as an effect of G-CSF. However, this being an uncommon case, my colleague left it on my desk for me to review the day after. By

reviewing the files, my attention was drawn to a weaker expression of CD38 in the myeloid blasts in question; this observation in combination with the AML-defining count of blasts made me feel uneasy. I wanted to know more about these blasts before confidently attributing them to G-CSF treatment. I discussed the case with the hematologists, who, although they could resonate with the explanation of G-CSF effect, they also felt concerned by this, admittedly uncommon, clinical manifestation. We agreed to perform more extended antibody panels. In these, no blasts with a leukemia associated immunophenotype were observed. The comprehensive myeloid panel however revealed stronger expression of CD13 and weaker expression of CD117 in these blasts. I did report the blasts as phenotypically abnormal.

After another discussion with the clinicians, we agreed to examine a BM sample; in this the percentage of $CD34^+$ myeloid blasts was 3.8% and the same aberrant phenotype was observed. MRD of the patient's B-ALL was <0.001%. Soon after, PCR for *BCR::ABL* came with a negative result, confirming that this finding was irrelevant to the patient's *BCR::ABL* fusion. At the same time, the blast count in the peripheral blood progressively decreased until normalization. However, due to the uncommon nature of the case (and to my strong belief that these blasts were abnormal), the hematologists ordered a myeloid NGS panel to the actual BM sample as well as to the one in which B-ALL diagnosis was made. A DNMT3A mutation was detected with 15% VAF in the follow-up BM sample and 1% in the diagnostic one.

So, what was this case? A CHIP that, after the pressure of G-CSF, had its 15 minutes of fame as a transient AML. Although there were no clinical consequences for the patient and no treatment modification was needed, the finding could justify an adjusted monitoring strategy, considering not only the patient's B-ALL but also the myeloid clone.

What are the issues highlighted by this example:

Flow cytometry can indeed reflect genotype. As already discussed in the introduction, many phenotypical aberrancies have been associated with specific cytogenetic aberrancies and mutations.

The robust diagnostic information yielded by the study of the $CD34^+$ myeloid progenitors is also underscored.

However, to be able to detect finer aberrancies, deep understanding of normal maturation patterns and phenotypes is required. Awareness of the phenotypical changes attributed to treatment is also crucial. This case illustrates that different levels of experience can lead to interobserver variability, a well-recognised weakness in the interpretation of FC data (and in the manual interpretation of every kind of data, for

that matter). An answer to this weakness could be automated, unsupervised methods and machine learning algorithms. Although these possibilities are still under exploration, their implementation in clinical practice is a matter of time.

And a question: In the case above, would the blasts have increased that much if they were not clonal?

Greek philosophy

As a person of Greek origin, I am probably expected to cite the wise words of ancient Greek philosophers. I will not let you down:

> Ἓν οἶδα ὅτι οὐδὲν οἶδα (*Plato, Apologia Socratis* 21d)

Populärvetenskaplig sammanfattning

Myelodysplastiska syndrom (MDS) är en form blodcancer, där de blodbildande stamcellerna i benmärgen inte utvecklas som de ska. Det kan leda till att blodvärdena sjunker och till ökad risk för utveckling av akut leukemi. Diagnosen ställs genom att påvisa låga blodvärden, avvikande celler i benmärgen och förändringar i arvsmassan. Flödescytometri, en metod som mäter olika egenskaper hos enskilda celler, har visat sig vara till hjälp både för diagnos och för att förutsäga sjukdomsförloppet. Den röda blodbildningen har studerats i mindre utsträckning med denna metod. Många tidigare studier har dessutom använt metoder som kan skada de omogna röda cellerna.

I Studie I utvecklade vi ett sätt att undersöka den röda blodbildingen utan att använda någon nedbrytning av röda blodkroppar (lysering). Med denna metod, ERY-panelen, fick vi resultat som stämde bättre överens med räkningen av celler med mikroskop. När proverna lyserades såg vi däremot tydlig påverkan avseende både cellmängder och uttrycket av olika cellproteiner. Genom att undersöka benmärgsprover från 68 MDS-patienter, 43 med godartade blodbrister och 29 friska, hittade vi MDS-typiska avvikelser i två markörer: CD36 och CD105.

I Studie II använde vi bildflödescytometri, en metod som kombinerar mikroskopibilder med flödescytometri, för att undersöka de typiska utseendeförändringarna vid MDS. Genom att jämföra 26 MDS-prover med 12 normala kunde vi visa att ökad komplexitet i cytoplasman, ökad cellstorlek och felaktigt formade cellkärnor är typiska för MDS.

I Studie III använde vi ett datorbaserat verktyg för gruppering av celler, FlowSOM, på benmärgsprover från 11 MDS-patienter och fem med godartade blodbrister och jämförde resultaten med en tidigare framtagen normalreferens. Vi hittade 18 avvikande cellgrupper, framför allt med abnorma nivåer av CD36 och CD71.

I Studie IV analyserade vi 34 MDS-prover och 11 normala prover med FlowSOM. Tre avvikelser var vanligare hos MDS patienter: minskning av de tidiga omogna röda cellerna och två grupper av omogna röda celler i senare stadier med avvikande CD36 och CD71. Kombinationen av dessa tre avvikelser gav fem olika mönster, där mer komplexa mönster hörde ihop med fler genmutationer. Hos patienter som behandlades med läkemedel mot MDS (azacitidin) såg vi att deras flödescytometriska värden förbättrades över tid.

Sammanfattningsvis visar studierna att undersökningen av avvikelser i de röda cellernas utveckling är viktiga för MDS-diagnos, att vår metod utan nedbrytning för röda blodkroppar ger stabila resultat, och att avancerade tekniker som bildflödescytometri och FlowSOM kan ge värdefull ytterligare information.

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A huge applause to the flow technicians, past and present, for their patience when we run in the lab at 3 pm and ask them to mix and match a little bit of this and a little bit of that panel to help us retrieve that rare population. And for their engagement and genuine devotion to the patient.

And finally, a deep bow to case 1, case 2, case 3, aka Inger, Mohammed, Johan and to all the patients behind the anonymized numbers, whose pain, hope, and generosity make the research world go round.

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