Flow Cytometry
Immunophenotyping For
Myelodysplastic Syndromes

Sa A. Wang, MD
Dept. of Hematopathology
UT MD Anderson Cancer Center
Houston, TX
Myelodysplastic Syndromes

Definition:
A group of heterogeneous clonal hematopoietic stem cell diseases
1) Ineffective hematopoiesis with peripheral cytopenia(s),
2) Morphological dysplasia
3) Propensity for development of acute myeloid leukemia (AML)

Etiology
- Primary (de novo)
- Therapy-related
Clinical Scenario for MDS Bone Marrow Work-up

Patients Presented with Cytopenia(s), Cytopenia(s) often:

- Unremitting ≥ 6 months
  - Hb < 10
  - Absolute Neutrophil count (ANC) 1.8
  - Plt: 100

But, cytopenia can be less severe, and presents in a shorter duration

Hematologists and primary physicians often conduct extensive clinical/laboratory work-up, or even give empirical treatment (iron, B12, folate, epo), however, when/if there is a need to rule out:

- A bone marrow process
As a Pathologist/hematopathologist, what we expect to find in BM that possibly explain cytopenia(s):

1. **Intrinsic Bone Marrow Stem Cell Neoplasm**
   1. MDS or MDS/MPN or MPN (CIMF)
   2. Acute leukemia (AML, ALL)

2. **Bone marrow infiltrative processes**
   1. B cell lymphoma, especially low grade
   2. T-cell neoplasm: Large granular lymphocytic leukemia
   3. Plasma cell Neoplasm
   4. Metastatic carcinoma

3. **Bone marrow Failures** (Congenital, infection, immune-mediated, PNH, Drug/toxin, et al)
   1. Aplastic Anemia
   2. Single lineage aplasia/hypoplasia
      - Red cell/Myeloid/Mega
Normal Bone Marrow

BM Biopsy

Normal fat/cellular distribution, megakaryocytes are normal in number and morphology

BM Aspirate

Trilineage Hematopoiesis, orderly maturation, and normal morphology
MDS Bone Marrow (Biopsy)

Altered bone marrow topography
Adipocyte clustering, hematopoietic cell clustering, immature cells away from the bone paratrabeculae, increased histiocytes, stromal cells, vasculature, and dysplastic megakaryocytes.
Morphological Dysplasia

Erythroid Dysplasia
Morphological Dysplasia
dysmegalakaryopoiesis
Myeloid Dysplasia
Blasts in MDS

1. Count 500 cells on BM aspirate smears, and 200 cells on peripheral blood smears
2. Myeloblasts: granular and agranular type
3. The presence of Auer rods would qualify a case as RAEB-2
4. MDS progress to acute leukemia, almost always AML, cases of lymphoblastic leukemia transformation are rarely reported
## 2008 World Health Organization Classification of Myelodysplastic Syndromes

<table>
<thead>
<tr>
<th><strong>MDS subcategories</strong></th>
<th><strong>Diagnostic features</strong></th>
</tr>
</thead>
</table>
| **Refractory cytopenias with unilineage dysplasia (RCUD)**  
  • Refractory anaemia (RA);  
  • Refractory neutropenia (RN);  
  • Refractory thrombocytopenia (RT) | One or two cytopenia(s) with unilineage dysplasia  
  <1% blasts in blood  
  <5% blasts in bone marrow |
| **Refractory anaemia with ring sideroblasts (RARS)** | Dyserythropoiesis with ≥15% ring sideroblasts  
  <1% blasts in blood  
  <5% blasts in bone marrow |
| **Refractory cytopenia with multilineage dysplasia (RCMD) Cytopenia(s) with or without Ringed-sideroblasts (RCMD-RS)** | Dysplasia in ≥ two myeloid lineages  
  <1% blasts in blood  
  <5% blasts in bone marrow |
| **Refractory anaemia with excess blasts-1 (RAEB-1)**  
  Unilineage or multilineage dysplasia  
  5-9% blasts in bone marrow  
  <5% blasts in blood | |
| **Refractory anaemia with excess blasts-2 (RAEB-2) Cytopenia(s)** | 1. Unilineage or multilineage dysplasia  
  2. 10-19% blasts in bone marrow  
  3. 5-19% blasts in blood  
  4. If Auer rod present |
| **Myelodysplastic syndrome - unclassified (MDS-U)** | 1. RCUD or RCMD with 1% blasts in blood  
  2. <10% dysplasia, with cytogenetic abnormality as presumptive evidence of MDS  
  3. RCUD with pancytopenia |
| **MDS associated with isolated del(5q)** | 1. Isolated del(5q) cytogenetic abnormality  
  2. Normal to increased megakaryocytes with hypolobated nuclei  
  3. <5% blasts, no Auer rods  
  4. <1% blasts in blood |
Morphological Dysplasia

The MDS categories in the red boxes

- BM blasts are <5%,
- more than 50% of these cases have a normal karyotype.
- To diagnose a case of MDS in those categories, all depend on the presence of morphological dysplasia.
Morphological Dysplasia

Morphological dysplasia can be seen in a number of non-MDS conditions

1. Nutritional deficiency (B12, folate, copper)
2. Drug/Toxin (arsenic and alcohol, grow factor treatment, chemotherapy)
3. Metabolic disorders, chronic diseases
4. Infection: HIV
5. Collagen Vascular Diseases
6. Hemolysis
7. Aplastic anemia, treated
8. Pediatric congenital disorders
9. ...

Worst of all, MDS can coexist or evolve from these conditions
Non-MDS Mimics

HIV Bone marrow

Systemic Lupus
Dysplasia in the Therapy-Related Setting

Morphological dysplasia is extremely problematic in post-therapy related setting:

- We retrospectively reviewed in the past 10 years of therapy-related myeloid neoplasm with a normal Karyotype at MDACC:
  - 196 cases showed significant dysplasia that pathologists raised a diagnosis of t-MDS at least in the comments
  - Only 65 were real t-MDS after long-term follow-up (follow-up biopies, clinical/Lab data and outcome)
Suboptimal BM Specimens

- Assessment of morphological dysplasia is further confounded by Suboptimal Specimens
  - No good spicules for evaluation
  - “Dried Smears”
  - Poorly stained smears: over- or under-stained smears
  - No smears
The Utility of Flow Cytometry Immunophenotyping in MDS

- Normal Hematopoiesis is characterized by highly reproducible patterns of antigen expression during myeloid maturation.

- MDS is a stem cell neoplasm, neoplastic stem cells may show immunophenotypic aberrancies.

- It would be an objective method, the historic reliance on subjective morphologic criteria is likely to be lessened, especially when the material and morphology are suboptimal.
MDS Flow Cytometry, Literature

- The first paper actually used flow cytometry in clinical patients was published by Stetler-Stevenson M et al (Blood. 2001 Aug 15;98(4):979-87)

- The CD11b/CD16 and CD13/CD16 are still widely used combinations to assess myeloid cells.

- Many publications since then, where different panels, different markers, different scoring systems have been used

- Difficult for people to follow
Diagnostic Utility of FCI in Myelodysplastic Syndromes

Finally, a Consensus of Flow Cytometry in Diagnosing MDS is made by the European group

Standardization of flow cytometry in myelodysplastic syndromes: report from the first European LeukemiaNet working conference on flow cytometry in myelodysplastic syndromes.

Arjan A van de Loosdrecht, Canan Alhan, Marie Christine Béné, Matteo G Della, Porta, Angelika M Dräger, Jean Feuillard, Patricia Font, Ulrich Germing, Detlef, Haase, Christa H Homburg, Robin Ireland, Joop H Jansen, Wolfgang Kern, Luca, Malcovati, Jeroen G te Marvelde, Gulham J Mufti, Kiyoyuki Ogata, Alberto,Orfao, Gert J Ossenkoppele, Anna Porwit, Frank W Preijers, Stephen J Richards, Gerrit Jan Schuurhuis1, Dolores Subirá, Peter Valent, Vincent HJ van der Velden, Paresh Vyas, August H Westra, Theo M de Witte, Denise A Wells, Michael R Loken, Theresia M Westers

Haematologica. 2009 Aug;94(8):1124-34.
In this article, there listed

Recommendations for Standardization

- Acknowledging the utility of FCI in diagnosing MDS and stratifying MDS risks
- Recommendation for markers and panels
- Recommendation for scoring
- Recommendation for interpretation: descriptive in nature, with a statement that findings could be consistent with MDS
Flow cytometry immunophenotype of MDS

- After 10 years efforts, where is the position of FCI in MDS?
  - The diagnostic utility is acknowledged by 2008 WHO, and
  - Acknowledged by the MDS international working group
Immunophenotyping
Published studies on immunophenotyping by flow cytometry in MDS have focused on several strategies, including determining the size and immunophenotype of the blast population and assessing the maturation pattern of the myeloid cell population. More specifically, these studies included immunophenotyping of CD34+ cells, application of scoring systems, and pattern recognition strategies using multicolor analysis and comparison with normal/reactive PB and BM.

There is generally good correlation between the percentage of blasts as determined by morphologic examination of routine smear or imprint or immunohistologic preparations and percentage of CD34+ cells determined by flow cytometry (FC). However, in some cases there may be significant discordance due to significant myelofibrosis and haemodilute samples. As a result, FC percentages of CD34+ cells cannot replace differential counts on smears. However, FC may be informative if abnormal phenotypes of CD34+ cells are detected; this could be additional evidence of dysplasia. In addition, an emerging pathological population of CD34 or CD117 cells in low-grade MDS could suggest evolution of the disease [1622].

Maturation patterns of erythroid, granulocytic, and monocytic differentiation in the normal/reactive BM, as well as the immunophenotype of the mature cells in PB, have been thoroughly described using four-color FC. Erythroid abnormalities as determined by the pattern of expression of H-ferritin, CD71 and CD105 in glycophorin A (GPA) positive nucleated cells reportedly can predict morphological erythroid dysplasia with 98% sensitivity [550]. Aberrant maturation patterns in granulopoiesis could predict morphological dysplasia and abnormal cytogenetics in approximately 90% of cases [1212]. Thus, flow cytometry results correlate well with morphology and cytogenetics in MDS.

However, in cases with borderline dysplasia by morphology and no cytogenetic abnormalities, FC results are highly suggestive for MDS only if there are three or more aberrant features in erythropoietic, granulocytic or monocytic maturation; single aberrant features by FC are not significant. Cases with inconclusive morphologic and cytogenetic findings and three or more aberrant features by flow cytometry should be reevaluated over several months for definitive morphologic or cytogenetic evidence of MDS.
### Minimal Diagnostic Criteria in MDS

<table>
<thead>
<tr>
<th><strong>(A) Prerequisite criteria</strong></th>
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<tr>
<td>Constant cytopenia: Hb &lt;11 g dL; ANC &lt; 1500 μL or platelets &lt;100K</td>
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<tr>
<td>Exclusion of all other hematopoietic or non-hematopoietic disorders as primary reason for cytopenia/dysplasia</td>
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<tr>
<th><strong>(B) MDS-related (decisive) criteria</strong></th>
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<tbody>
<tr>
<td>Dysplasia in at least 10% of all cells in a respective lineage or &gt;15% ringed sideroblasts (iron stain)</td>
</tr>
<tr>
<td>5-19% Blasts in bone marrow smears</td>
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<tr>
<td>Typical chromosomal abnormality (by conventional karyotyping or FISH)</td>
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<tr>
<th><strong>(C) Co-criteria (for patients fulfilling ‘A’ but not ‘B’, and otherwise show typical clinical features, e.g. macrocytic transfusion-dependent anemia)</strong></th>
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</thead>
<tbody>
<tr>
<td>Abnormal phenotype of bone marrow cells clearly indicative of a monoclonal population of erythroid or/and myeloid cells, determined by flow cytometry</td>
</tr>
<tr>
<td>Clear molecular signs of a monoclonal cell population in HUMARA assay, gene chip profiling, or point mutation analysis (e.g. RAS mutations)</td>
</tr>
<tr>
<td>Markedly and persistently reduced colony-formation (±cluster formation) of bone marrow or/and circulating progenitor cells (CFU-assay)</td>
</tr>
</tbody>
</table>

Leuk Res. 2007 Jun;31(6):727-36
Flow Cytometry Panel For Cytopenia Work-up

- Include B-cell clonality
- Include basic T-cell markers, especially the markers detect large gradular lymphocytes (CD8, CD56, CD57)
- If possible, take a look at plasma cells (normal plasma cells CD38+++; CD19+, CD56-; neoplastic plasma cells CD38+++; CD19+, CD56-)
- MDS work-up
FCI in MDS: The Analytical Approaches

- Bone Marrow cell Populations
  - Precursors
  - Myeloid cells
  - Monocytic cells
  - Erythroid
  - Megakaryocytes

(nearly impossible to assess by flow cytometry)
Erythroid Lineage (not very popular)

Problems with Erythroid Lineage assessed by Flow cytometry

1. **Limited markers** are commercially available (CD71, CD235a/glycophorin, CD36)
2. **Red cell lysis** (lyse late stage hemoglobinized nucleated red blood cells, not the entire spectrum of nucleated RBC available for analysis)
3. **Non-specific**
   1. Reactive erythroid hyperplasia, left-shifted maturation, maturation arrest of erythroid can produce similar flow cytometry findings as seen in MDS
   2. Increased Sideroblasts can alter the maturation pattern, but often not MDS
4. Easy to assess ring sideroblasts by iron stain

Della Porta’s group showed some utility in assessing erythroid cells by flow cytometry:

- **Markers can be used:** CD71, CD105, cytosolic H-ferritin, cytosolic L-ferritin and mitochondrial ferritin (MtF)
- **Changes observed:**
  - Decreased CD71 and increased HF in MDS
  - Increased proerythroblasts, left-shifted
  - MtF correlate with the presence of ringed sideroblasts

Mature vs Immature myeloid cells: they have different immunophenotypes

- **Immature** (promyelocytes, myelocytes and early metamyelocytes)
  - CD10−, CD64+, CD33bright+, CD15low+, CD13heterogenous+, CD16heterogenous+, and CD11b heterogenous+

- **Mature** (late metamyelocytes, bands and segmented neutrophils)
  - CD10+, CD64 dim/−, CD33dim+, CD15bright+, CD13bright+, CD16bright+, and CD11b bright+) populations.
Four Patterns of Myeloid Maturation

The solid circles: immature myeloid; and the dash-circles: Mature myeloid

Diagnostic pitfalls and caveats

- Separate the myeloid cells into mature and immature can help to recognize a left-shifted myeloid maturation; hemodilute specimen, increased eosinophils... which could be misinterpreted as MDS

- Aged specimen can show alterations mimicking MDS
- Aged Specimen
- Increased Eosinophils
- Hemodilute Specimen
- Left-shifted myeloid Maturation
Maturing Myeloid Cells Assessed by FCI, our experience

- **Hypogranulation: Useful**
  - However, growth factor treatment, regenerating BM can produce hypogranulation

- Significant alterations in CD11b/CD16 and CD13/CD16: Useful
  - be aware increased eosinophils, PNH cells, aged specimens

- **CD56 (if high percent, and high MFI), useful**

- **Decreased CD13, CD33, not specific**
  - Genetic polymorphism

- **Synchronous left-shifted Maturation (left-shifted, not dysplasia)**

- **Increased CD64, CD14 neutrophils**, often activation markers, not MDS

- **CD10 decreased alone: not specific**, 
  - Can be seen autoimmune neutropenia, aplastic anemia, drug induced neutropenia
Monocytes
Monocyté Changes in MDS
Monocyte Changes in MDS, Our experience

- Decreased CD45/SSC: useful

- Decreased CD64, CD14, CD11b, CD13, CD33, CD38, HLADR, CD184 (or increased), not specific

- Increased CD15, CD65, not specific

- Increased CD56 (high percent and high MFI), useful

- Aberrant CD2 expression, useful but very uncommon
A Prospective Study on Clinical Cytopenia Patients by using myelomonocytic maturation approach by our group

- We included 102 patients who presented with cytopenia

- Their marrows showed either no morphological dysplasia or only mild changes insufficient to diagnose MDS.

- All patients had a normal karyotype.
Follow-up with repeated Bone Marrow biopsy, lab work-up, hematologists’ assessment

<table>
<thead>
<tr>
<th></th>
<th>Group1- Myelodysplastic syndrome</th>
<th>Group2- Cytopenia due to various secondary causes</th>
<th>Group3- Cytopenia of unknown causes</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>9</td>
<td>4</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>52</td>
<td>15</td>
<td>69</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>61</td>
<td>29</td>
<td>102</td>
</tr>
</tbody>
</table>

A positive FCM result has a positive predictive value of 69% and a negative FCM result has a negative predictive value of 95%.

Truong F et al Leukemia Research, 2009
Myelomonocytic Maturation Pattern Approach

- Overall: Sensitive, but not very specific

- Require Expertise in Interpretation
  - Be aware of pitfalls and caveats

- Useful in patients who have not been treated previously, and who are not acutely ill (most clinic patients)

- Become less reliable in patients with other underlying medical conditions, or who are undergoing various treatment for the medical conditions
CD34+ Precursor Based FCI Approach

- Blasts in MDS have an immunophenotype of committed myeloid precursors CD34(+)CD38(+)HLA-DR(+)CD13(+)CD33(+), regardless of the disease subtype

- CD34-based assay is useful in low grade MDS
  - A high FCI score was detected in 16/27 low grade MDS regardless of karyotype and none of the 90 controls (sensitivity 59%, specificity of 100%)
Our Panels

We implemented CD34-based assay at MDACC, where most of the patients have received or undergone various treatment

- Panels
  - FITC PE PerCP PE-Cy7 APC V450 V500
  - Tube 1: CD16 CD11b CD34 CD33 CD13 CD15 CD45
  - Tube 2: CD65 CD64 CD34 CD10 CD2 CD14 CD45
  - Tube 3: DR CD123 CD34 CD10 CD184 CD56 CD45
  - Tube 4: CD7 CD5 CD34 CD117 CD38 CD19 CD45
  - Tube 5: Kappa Lambda CD19 CD20 CD4 CD5 CD45
  - Tube 6: CD57 CD94 CD4 CD3 CD8 CD56 CD45

- First 4 tubes:
  - analyze three populations: CD34 blasts; myeloid and monocytes
  - CD10 can separate myeloblasts and hematogones; mature and immature myeloid elements

- Tube 5: B-cell tube, and Tube 6: T-cell tube
CD34 Positive Precursors in Normal Bone Marrow versus in MDS

Normal

MDS: discrete population; decreased CD45
CD34 Positive Precursors: Loss of Diverse Differentiation in MDS or aberrant expression

**Normal**

**MDS**
Loss diverse differentiation

**MDS**
Aberrantly increased expression of CD64, CD65 and CD15
CD34+ precursors: Alterations of Levels of Expression in MDS

Normal

MDS: Increased CD117 MFI, increase CD184, decreased CD38, increased CD13

MDS
CD34+ Precursors: **Aberrant Antigenic Expression**
A Very Hemodilute Specimen

The morphology of this case is inadequate; FCI shows that the CD34 cells: no hematogones; CD38dec, CD5+, CD13inc, CD56+, findings are consistent with MDS
Rule out Lymphoproliferative Processes

Always check B cells and T cells: upper panel: a case of chronic lymphocytic leukemia; the lower panel: a case of LGL leukemia detected by MDS panel
Diagnostic Criteria
(Our FCI panel)

- **Positive**
  - If one aberrant lymphoid antigen expression in Blasts
  - If two significant alterations of level of expression
  - If no blast abnormality identified (very few cases)
    - Very significant CD13/CD16; CD11b/CD16 pattern in myeloid cells, and/or significant lymphoid antigen (CD56) expression, and significant hypogranulation.

- **Indeterminate** (we have very few cases in this category)
  - If only one significant alteration of levels of expression of CD34 cells

- **Negative**
  - If no blasts abnormality, only mild abnormalities in myelomonocytic cells
MDACC Experience

- First MDS or rule out MDS diagnosis at MDACC
- We tested 259 patients in one year period (5/2009 to 4/2010) with follow-up information
  - **147 MDS** (62 normal karyotype, 76 abnormal, 9 not available)
    - 6 RARS
    - 59 RCMD
    - 3 MDS-U
    - 25 RAEB
    - 5 5q-
    - 18 MDS/MPN
    - 35 t-MDS
  - **112 non-MDS cytopenia**
    - 44 patients s/p chemotherapy
    - 17 status post stem cell transplant
    - 6 Aplastic anemia, treated
    - 45 other medical cytopenia (ITP, hemolytic anemia-CLL, liver, kidney problems, LGL, viral, low grade B cell lymphoma...)
Sensitivity and Specificity of Antigenic Alterations

<table>
<thead>
<tr>
<th>Stage 1 Hematogone (≤10%)</th>
<th>Control (n=112)</th>
<th>MDS (n=147)</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>Accuracy %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>124</td>
<td>84</td>
<td>73</td>
<td>80</td>
</tr>
<tr>
<td>Plasmcytoid dendritic precursors (&lt;5%)</td>
<td>35</td>
<td>104</td>
<td>71</td>
<td>69</td>
<td>70</td>
</tr>
<tr>
<td>Abn CD13/CD33</td>
<td>19</td>
<td>89</td>
<td>61</td>
<td>83</td>
<td>70</td>
</tr>
<tr>
<td>Inc CD117</td>
<td>10</td>
<td>75</td>
<td>51</td>
<td>91</td>
<td>68</td>
</tr>
<tr>
<td>Inc CD123</td>
<td>11</td>
<td>73</td>
<td>50</td>
<td>90</td>
<td>67</td>
</tr>
<tr>
<td>Abn CD45/SS</td>
<td>8</td>
<td>55</td>
<td>37</td>
<td>93</td>
<td>63</td>
</tr>
<tr>
<td>Inc CD34</td>
<td>2</td>
<td>45</td>
<td>31</td>
<td>98</td>
<td>60</td>
</tr>
<tr>
<td>Dec CD38</td>
<td>1</td>
<td>45</td>
<td>31</td>
<td>99</td>
<td>60</td>
</tr>
<tr>
<td>Inc CD184</td>
<td>6</td>
<td>39</td>
<td>27</td>
<td>95</td>
<td>56</td>
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<tr>
<td>CD34 (≥3%)</td>
<td>6</td>
<td>42</td>
<td>29</td>
<td>95</td>
<td>57</td>
</tr>
<tr>
<td>Lymphoid antigen</td>
<td>8</td>
<td>54</td>
<td>37</td>
<td>93</td>
<td>61</td>
</tr>
<tr>
<td>Mature myelomonocytic antigen</td>
<td>8</td>
<td>27</td>
<td>18</td>
<td>93</td>
<td>50</td>
</tr>
</tbody>
</table>

The FCI has a sensitivity of 90.5%; specificity of 88%; PPV of 91%; NPV of 88%, and an accuracy of 90%.
Summary

- FCI Assays for clinical cytopenia without an established diagnosis should be able to:
  - Detect B cell clonality
  - Identify aberrant T cells
  - May be plasma cell neoplasm (CD38/CD19/CD56, gate on bright CD38 cells)
  - Stem cell neoplasm (MDS or related neoplasm)
Summary

MDS assays

Myelomonocytic based assay
- Sensitive, can be applied to the community setting, but less specific
- Needs experience in interpretation
  - Recognize reactive conditions, specimen quality, and mimics

CD34+ Blast based assay
- Specific, and sensitive. Sensitivity can be improved by utilizing more markers
- Especially useful in patients who have been treated for hematological or non-hematological disorders
Summary

Flow Cytometry Immunophenotyping is very useful in Diagnosis of MDS

- FCI is particularly useful in low grade MDS with a normal karyotype and borderline dysplasia.
  - Either it is mild dysplasia or dysplasia is difficult to assess because of sample quality
  - It is very important to rule out a case not of MDS

- FCI positive cases, at least require close follow-up
  - Some older patients may receive empirical treatment for MDS
Acknowledgement

- Jeffrey L. Jorgensen, MD PhD
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