Outline

- Response evaluation in hematologic malignancies
- Technical aspects of MRD measurement
  - Flow cytometry
  - Quantitative PCR
  - Next generation sequencing
- Incorporation of MRD results into patient management
  - Patient monitoring
  - Risk stratification and treatment decisions
  - Trial endpoints
- Summary
Response Assessments: The Basics

"With the enormous increase in the clinical use of drugs to control the growth of cancer, it becomes essential to develop an acceptable and meaningful terminology to describe the indications and therapeutic effectiveness of each agent or procedure. The orderly presentation of information would serve as a guide to the practicing physician and it would provide a common language to facilitate the collection and analysis of clinical experiences."

- Response Assessments:
  - Provide prognostic information
  - Simplify the evaluation of new agents

David A. Karnofsky

Clinical Pharmacology and Therapeutics

volume 2 number 6 November-December 1961
<table>
<thead>
<tr>
<th></th>
<th>Microscopy/Morphology</th>
<th>PET/CT or Radiology</th>
<th>Flow Cytometry</th>
<th>Serology</th>
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<td>±</td>
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<td>✓</td>
<td>±</td>
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<tr>
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<td>✓</td>
<td>✓</td>
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</table>

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; IHC, immunohistochemistry; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; PET-CT, positron emission tomography-computed tomography.

Timeline of Selected Response Criteria in Hematologic Malignancies – Multiple Myeloma

Key discoveries
- 1956: Light chains discovered in MM
- 1964: Use of immunofixation in MM
- 1939: M protein spike discovered in MM and use of serum protein electrophoresis in MM
- 1968: First response criteria for MM established
- 1986: First use of CR in MM

Response criteria/guidelines released
- 2004: Development of the serum FLC assay in MM
- 2006: IMWG response criteria guidelines published for MM

Techniques incorporated into guidelines
- 2011: Immunophenotypic and molecular CR introduced by IMWG
- 2016: IMWG MRD criteria introduced

FLC, free light chain; IMWG, International Myeloma Working Group.
Timeline of Selected Response Criteria in Hematologic Malignancies – Lymphoma

1939 M protein spike discovered in MM and use of serum protein electrophoresis in MM

1946 Light chains discovered in MM

1956 Use of immunofixation in MM

1964 First response criteria for HL assessment

1968 Development of CT scanning

1971 The first PET-CT scanner becomes available

1989 First response criteria for NHL released

1989 CT is recommended for HL assessment

1991 Immunophenotypic and molecular CR introduced by IMWG

1999 PET-CT is added to NHL assessment guidelines

2001 Development of the serum FLC assay in MM

2001 IMWG response criteria guidelines published for MM

2006 IMWG MRD criteria introduced

2011 PET-CT is added to NHL assessment guidelines

2016 IMWG MRD criteria introduced

References:
Complete Response

- Complete response or remission is often accepted as a predictor of clinical benefit

- Limitations of complete response
  - Presence of sanctuary sites
  - Sampling errors
  - Insensitive assays
Minimal/Measurable Residual Disease (MRD)

- MRD is the presence of malignant cells below the detection limit of conventional methods\(^1,2\)
- Routine MRD assessments have become a key element in the management of patients with hematologic malignancies\(^2,3\)

Graphical definitions of MRD.
Adapted from Bruggemann M, et al.\(^2\)

Measuring MRD
Selected Timeline of MRD Testing Development

1975: Development of monoclonal antibodies
1980: Use of fluorescence microscopy to identify leukemic cells
1982: Development of cloning of oncogenic gene rearrangements
1983: Invention of PCR
1987: Use of PCR to detect translocation of bcl-2 with the Ig heavy chain locus in FL
1988: Use of flow cytometry to detect MRD in B-cell ALL
1989: Use of PCR to amplify VDJ region in B ALL
1990: Use of multidimensional flow cytometry to detect MRD in AML
2015 - 2016: Development of 8-10 color flow cytometry for MRD detection

*2 light scattering channels and 3 fluorescence markers.
Selected Timeline of MRD Testing Development

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2015 - 2016
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1980
- Use of fluorescence microscopy to identify leukemic cells

1982
- Development of clonal analysis of oncogenic gene rearrangements

1988
- Use of PCR to amplify BCR-ABL1 mRNA in CML and ALL

1989
- Use of PCR to amplify TCR gene rearrangements in ALL

2012
- Use of NGS in ALL

*2 light scattering channels and 3 fluorescence markers.
NGS, next generation sequencing.
Techniques for Measuring MRD

- There are currently 2 established methods in use for determining MRD\(^1\)
  - Flow Cytometry: sensitivity of $10^{-3} - 10^{-4}$
  - PCR: sensitivity of $10^{-4} - 10^{-5}$

- Paired analysis shows high levels of concordance between the two methods\(^2,3\)

**Approximate Equipment Sizes**

- **PCR\(^4\)**: 33 cm x 20 cm
- **Next-generation sequencing\(^5\)**: 46 cm x 52 cm
- **Flow cytometry\(^6\)**: 67 cm x 91 cm

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Flow Cytometry Overview

- Flow cytometry measures properties of individual particles, typically cells, passing in single file between a laser and a detector\(^1\)
  - Light scatter conveys particle size and internal complexity\(^1\)
  - Light emission from fluorescently labelled antibodies or dyes\(^1\)
  - Capability to sort cells based on phenotype\(^2\)

- Sensitivity and specificity improves with more lasers and detectors\(^3\)
Flow Cytometry for MRD Detection

- Use of specific molecular or immunophenotypic markers and size to distinguish between malignant and normal hematopoietic cells

- Establishment of standardized vs patient-specific antibody combinations

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A) MRD+ patient, pre-chemotherapy
B) MRD+ patient, post-chemotherapy
C) MRD- patient

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Recommended 6-color flow cytometry panel for MM:

<table>
<thead>
<tr>
<th>Tube 1</th>
<th>Tube 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD27</td>
<td>CD56</td>
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<tr>
<td>CD81</td>
<td>CD117</td>
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<tr>
<td>CD19</td>
<td>CD19</td>
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<tr>
<td>CD38</td>
<td>CD38</td>
</tr>
<tr>
<td>CD138</td>
<td>CD138</td>
</tr>
<tr>
<td>CD45</td>
<td>CD45</td>
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</tbody>
</table>

Examples of Markers Used in B-cell ALL:

- CD10
- CD19
- CD34
- CD38
- CD45
- CD58
- CD123

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8-color flow cytometry also requires 2 tubes.

Sources of Error in Flow Cytometric Analysis

**Sample-based**
- Sample quality
- Event number
- Hemodilution

**Fluorescence-based**
- Spectral overlap
- Autofluorescence

**Operator-based**
- Gating

**Disease-based**
- Immunophenotypic shift

Quantitative Real-Time PCR (qPCR)

- Standard (qualitative) PCR determines presence or absence of mutated gene of interest\(^1,2\)
- qPCR allows for estimation of tumor burden (frequency)\(^2\)
  - Relies on a hybridization probe labeled with 2 different fluorescent dyes\(^3\)
    - Reporter dye/Quencher dye
  - Comparison to a standard curve allows quantitation

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Alternative Approaches to Disease Quantitation: Droplet Digital PCR (ddPCR)

- Sample DNA is partitioned into ~20,000 droplets, allowing multiple parallel reactions\(^1\)
- Provides quantitation without the need for standard curves\(^2\)
- Similar sensitivity and accuracy to qPCR\(^2,3\)
  - 88% sample concordance in ALL\(^2\) and strong concordance in pooled MM, MCL, and FL samples\(^3\) with correlation of coefficient of 0.94

Partitioning → Amplification → Detection

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qPCR in MRD Detection

- qPCR has the capability to detect:

  - Gene fusions\(^1,2\)
  - Point mutations/Insertions/Deletions\(^3,4\)
  - Gene rearrangements\(^1\)
Emerging Platforms: Next-Generation Sequencing (NGS)

- Sensitivity\(^1\): \(10^{-5} - 10^{-6}\)
  - High concordance with both flow cytometry and qPCR\(^1,2\)

Methods\(^3\)
- Sequencing by synthesis
- Pyrosequencing
- Sequencing by ligation
- Nanopore

Terms\(^4,5\)
- Library = a collection of DNA or cDNA for sequencing
- Adapters = synthetic DNA added during library generation
- Barcodes = unique DNA sequences that can be added to individual libraries for multiplexing
- Read = base pair information generated by sequencing
- Read length = number of bases sequenced
- Depth of Coverage = the number of distinct reads of a given DNA sequence

NGS Methods: Sequencing by Synthesis

- Nucleotides are passed over immobilized sample DNA fragments
- DNA synthesis occurs one base at a time
- Incorporation of nucleotide generates a signal
- Examples:
  - Fluorescently tagged nucleotides
  - Ion Torrent: nucleotide binding triggers a pH change

References:
NGS Methods: Nanopore

- Voltage current is applied across a nanopore
- DNA is drawn through the nanopore
  - Each nucleotide provides a distinct signal as it passes through
Amplicon vs Array-Based Sequencing

- During NGS, hundreds of millions of DNA segments are sequenced in parallel\(^1\)
- Targeted sequencing focuses only on genes of interest\(^2\)
  - Hybridization-capture: gene specific hybridization probes\(^2\)
  - Amplicon-based: gene specific primer sets\(^3\)
- Multi-array panels identify a broad scope of the genetic mutations present\(^4\)

**Hybridization-Capture Sequencing**

Sources of Error in NGS Based Analysis

Sample-based
- Sample degradation
- Low DNA input
- Sample contamination

PCR-based
- PCR amplification errors
- Library generation
- Sequencing reaction

Instrument/Computational-based
- Base calling
- Alignment
- Variant calling
# MRD Detection Methods: Important Considerations

<table>
<thead>
<tr>
<th>Method</th>
<th>Important Considerations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Cytometry¹</td>
<td>- Rapid&lt;br&gt;- Applicable in most patients</td>
</tr>
<tr>
<td>qPCR¹,²</td>
<td>- High sensitivity: $10^{-4} - 10^{-5}$&lt;br&gt;- High standardization and automation</td>
</tr>
<tr>
<td>NGS²-⁴</td>
<td>- High sensitivity: $10^{-5} - 10^{-6}$&lt;br&gt;- Limited standardization across laboratories&lt;br&gt;- Complex bioinformatics</td>
</tr>
</tbody>
</table>

MRD Testing: Bone Marrow (BM) vs. Peripheral Blood (PB)

- While BM samples are frequently used, PB as a less invasive sample option has been proposed\(^1\)
- Results vary by disease

<table>
<thead>
<tr>
<th>Similar Detection Levels</th>
<th>BM More Sensitive than PB</th>
</tr>
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<tbody>
<tr>
<td>T-cell ALL(^1)</td>
<td>B-cell ALL(^1,,2)</td>
</tr>
<tr>
<td>AML(^3)</td>
<td>MM(^4)</td>
</tr>
<tr>
<td>CML(^5)</td>
<td></td>
</tr>
<tr>
<td>FL(^6,a)</td>
<td></td>
</tr>
<tr>
<td>MCL(^6)</td>
<td></td>
</tr>
<tr>
<td>CLL(^7,b)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) A previous study using less sensitive testing methods reported BM having superior detection limits; \(^b\) In patients not undergoing treatment or chemotherapy. Advantages of PB or BM may vary by treatment.

![qPCR in T-ALL](n=149 paired samples)
![qPCR in BCP-ALL](n=532 paired samples)

Developing Detection Methods: Cell-Free Circulating Tumor DNA

- Shedding of apoptotic or necrotic cell DNA fragments into the bloodstream is physiologic\(^1\)
- In patients with cancer, a large fraction of cell-free DNA is tumor derived and termed circulating tumor (ct) DNA\(^{1,2}\)
- ctDNA has use in a variety of applications:\(^1\)
  - Detecting specific mutations
  - Assessing tumor burden
  - Monitoring therapy responses
  - MRD surveillance

\(^{bp}\), base pairs.

Established and Exploratory Uses of MRD in Disease Management
MRD in Patient Monitoring

- Once a patient achieves a complete remission/response, regular monitoring for relapse is important.
- Patient monitoring schedules provide specific time points at which MRD can be used to assess a patient’s response to treatment and predict disease progression.
- Example: The European LeukemiaNet guidelines for CML:
  - Monitoring can be performed using a molecular or cytogenetic test, or both.

![Diagram showing monitoring schedule]

MMR: major molecular response.
### Use of MRD in Heme Malignancies

MRD has been used for risk stratification and in guiding treatment decisions

<table>
<thead>
<tr>
<th>Disease</th>
<th>Patient Population</th>
<th>MRD Detection Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL¹</td>
<td>Adults, post-induction</td>
<td>qPCR</td>
</tr>
<tr>
<td>ALL²</td>
<td>Children, newly diagnosed</td>
<td>Flow cytometry and/or qPCR</td>
</tr>
<tr>
<td>AML³</td>
<td>NPM1-mutated AML</td>
<td>qPCR</td>
</tr>
<tr>
<td>AML⁴</td>
<td>Children with mainly de novo AML</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>MM⁶</td>
<td>Post-ASCT</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>CLL⁷</td>
<td>Post-chemotherapy</td>
<td>Flow cytometry</td>
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</table>
MRD in Choosing Post-Induction Treatment Regimens

- Treatment decisions based on MRD may allow for the personalization of therapy regimens$^1,2$

SCT, stem cell transplantation.
MRD in Choosing Post-Induction Treatment Regimens

- Treatment decisions based on MRD may allow for the personalization of therapy regimens\(^1,2\)

![Flowchart diagram showing the relationship between MRD, remission induction, consolidation/maintenance, and SCT.]

SCT, stem cell transplantation.  
MRD Can Identify Patients with ALL for SCT

- MRD status after induction determined treatment
  - MRD negative: maintenance therapy
  - MRD positive: hyper-fractionated cycle treatment (H/C) and SCT

Phase A: Induction/Consolidation MRD Study
- SCT (n = 34)
- toxicity (n = 16)
- relapse (n = 44)
  - No sampling (n = 7)
  - no probe (n = 23)

Completed: n = 142/236 (60.2%)
  - n = 112/142 (78.8%)

Phase B: MRD/Risk-Oriented Therapy

- MRD<sup>neg</sup> (n = 58)
- MRD<sup>SR</sup> (n = 10)

- MRD<sup>pos</sup> (n = 54)
- HR/VHR (n = 20)
  - (no donor)

Maintenance (non-VHR)

Allogenic SCT

Key:
- ◊ = Bone marrow examination
- ■ = Cranial irradiation (18 Gy)
- H / R = Autologous blood stem cell harvest / reinfusion
- TP = Timepoint

HR, high risk; SR, standard risk; VHR, very high risk.
MRD as a Surrogate Clinical Endpoint

- Traditional clinical endpoints such as OS are the “gold standard” primary endpoint\(^1\)
- Surrogate endpoints can predict clinical benefit without directly measuring clinical benefit itself\(^2\)
  - Potential to expedite drug development
- Since MRD status may correlate with patient outcomes and can be measured at earlier time points, it has potential for use as a surrogate endpoint\(^2\)

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MRD as a Surrogate Clinical Endpoint (cont.)

- **Challenges:**
  - Standardization of assays
  - Determination of proper MRD threshold
  - Optimizing measurement time points

- **CML:** accelerated US FDA approval for tyrosine kinase inhibitor was based on BCR-ABL transcript levels detected by qPCR

- Workshops have been held by the US FDA to discuss potential adoption of MRD as a surrogate endpoint for ALL, CLL, AML, and MM

- MRD is currently approved as a surrogate endpoint in CLL by the EMA
MRD Summary

- MRD is the presence of malignant cells under the detection limit of conventional methods\(^1\)
- Strong prognostic indicator of patient outcomes\(^2\)
- Potential for use in guiding treatment decisions\(^3\)
  - Intensity of therapy
  - Appropriateness of SCT
- Currently measured using flow cytometry or qPCR\(^2\)
  - Newer testing methods are emerging
- MRD is currently being evaluated as a potential surrogate endpoint, and may have the potential to replace or augment morphological CR as a response criteria in certain hematologic malignancies\(^4\)

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