Utility of flow cytometry for MRD monitoring in Myeloma: technical aspects and clinical impact

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Disease monitoring in multiple myeloma

• Different approaches to response assessment and disease monitoring
• Specific issues with enumerating neoplastic plasma cells by flow cytometry
• Results from the MRC Myeloma IX trial
Multiple Myeloma

- Proliferation of neoplastic plasma cells
  - >10% in the bone marrow
  - most cases secret a monoclonal immunoglobulin (M- or para-protein)

- Multi-system effects
  - Bone lesions and hypercalcemia
  - Renal failure
  - Anemia/Thrombocytopenia

- Treatment involves
  - combination chemotherapy induction e.g. CVAD, CDT
  - +/- high dose therapy (HDT) often with melphalan (HDM) with autologous stem cell rescue (ASCT)
  - Maintenance e.g. interferon, thalidomide (Thal)
Overall survival: general population data (HMRN)
Response Criteria

- **PR:** > 50% reduction of serum M-protein and reduction in 24 hours urinary M-protein by >90% or to < 200 mg/24 h
  - serum/urine M-protein unmeasurable: > 50% decrease in the difference between involved and uninvolved FLC levels
  - Serum/urine M-protein & sFLC are not measurable: > 50% reduction in plasma cells provided baseline plasma cell percentage was > 30%
  - In addition to the above listed criteria, if present at baseline, a > 50% reduction in the size of soft tissue plasmacytomas is also required
- **VGPR:** Serum and urine M-protein detectable by immunofixation but not on electrophoresis
  - or > 90% reduction in serum M-protein plus urine M-protein level < 100 mg/24 h
- **CR:** Negative immunofixation on the serum and urine and disappearance of any soft tissue plasmacytomas and < 5% plasma cells in bone marrow
- **sCR:** CR as defined below plus normal FLC ratio and absence of clonal cells in bone marrow by immunohistochemistry or 2-4 color flow cytometry

International Myeloma Working Group (IMWG)

Uniform Response Criteria for Multiple Myeloma
Immunofixation

- Gold standard for response assessment
- Paraprotein levels do not directly correlate with tumour burden
  - ~3% non secretory
  - 10 – 15% light chain only
Paraprotein can have a long half-life

112:814-9
Serum Free Light Chain

- Kappa:lambda ratio
- Much lower half-life than complete immunoglobulin
- MGUS: sFLC abnormal in 33% (379/1148)
Issues with disease monitoring

- Paraprotein quantitation and immunofixation (PP & IF):
  - Simple serum measurement, widely available, sensitive
  - Indirect measurement with variable applicability
  - Immunoglobulin may have a long half-life (IgG ~ 23 days)

- Serum Free Light Chain (sFLC):
  - Short half-life ➔ real-time measure of change in tumour burden
  - Indirect measurement
  - Relatively insensitive (normalises if neoplastic <= normal PC)

- Trephine Biopsy:
  - Direct measurement
  - Relatively insensitive (1-5%)

- Minimal Residual Disease (MRD) by PCR or Flow
Evidence for the use of MRD detection

• Allele-specific PCR in allogeneic transplantation:
  – ~60-70% of CR patients are MRD\textsuperscript{NEG}
  – 3/3 show significant improvement in PFS for MRD\textsuperscript{NEG} vs. MRD\textsuperscript{POS} CR.
  – Martinelli, JCO 2000; Corradini Blood 2003; Galimberti Leuk Res 2005

• Allele-specific PCR after high-dose melphalan
  – ~30% of CR patients are MRD\textsuperscript{NEG}
  – 6/7 show significant improvement in PFS for MRD\textsuperscript{NEG} vs. MRD\textsuperscript{POS} CR (1/7 trend)
  – Informative threshold ~ 0.01%
  – Swedin, BJH 1998; Cremer, BMT 2000; Fenk, Haematologica 2004; Bakkus, BJH 2004; Sarasquete, Haematologica 2005; Korthals Biol Blood Marrow Transplant 2012 (pre-Tx); Ladetto JCO 2012
Myeloma MRD: PCR

- Clonally related B-cells occur in the vast majority of patients
- There is no definitive evidence to indicate that the clonally related B-cells contribute to relapse
- Rottenburger et al, Heidelberg, BJH ’99

<table>
<thead>
<tr>
<th>Status</th>
<th>Fraction</th>
<th>ASO-PCR+ absolute</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td>CD20 +</td>
<td>1.9/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6/9 POS</td>
</tr>
<tr>
<td>CD20-</td>
<td></td>
<td>0.0/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4/9 POS</td>
</tr>
<tr>
<td>PD</td>
<td>CD20+</td>
<td>32/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5/5 POS</td>
</tr>
<tr>
<td>CD20-</td>
<td></td>
<td>334/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5/5 POS</td>
</tr>
</tbody>
</table>
Myeloma MRD: PCR

- MRD assessment more informative than IF
- $\text{MRD}^{\text{NEG}}$ = better PFS than $\text{MRD}^{\text{POS}}$
- Cut-off for predictive value of PCR: 0.01%
  - Within limit of sensitivity of MRD Flow
- Clonally related B-cells cause “false” positive PCR results?
- Assessable patients: ~ 75%
Next generation sequencing for MRD

http://www.nytimes.com/2011/07/21/science/21genome.html?_r=1&adxnnl=1&adxnnlx=1333714162-u0m1SB4dCQUaTYG12hmw1A

660 million wells on a chip

Approaches to improve PCR analysis:
- VH leader sequence
- Incomplete DJ rearrangements
- CD138 selection
Myeloma MRD: Flow

- Salamanca:
- 87 patients
- Panel:
  CD38/CD56/CD19/CD45
  CD138/CD28/CD33/CD38
  CD20/CD117/CD138/CD38
- Up to $2 \times 10^6$ cells acquired
- >30% of plasma cells neoplastic

Induction $\Rightarrow$ ASCT vs. Chemo
Myeloma MRD: Flow

- UK Myeloma VII
- 45 patients
- Panel:
  - CD45/CD138/CD38
  - CD45/CD3/CD38
  - CD45/CD19/CD38
  - CD45/CD56/CD38
- Up to $2 \times 10^5$ cells acquired
- $>10\%$ of plasma cells neoplastic

CVAMP Induction $\Rightarrow$ HDM ASCT
Flow cytometry is a better predictor of outcome than immunofixation

5-year PFS: 59% / 49% / 24% / 17%
P = 0.002


GEM2000: VBMCP/VBAD ➔ HDM ASCT
Flow cytometry is a better predictor of outcome than immunofixation.

"Achieving an immunophenotypic response translates into superior PFS and TTP compared with conventional CR or stringent CR."


GEM2005 >65y
Disease monitoring in multiple myeloma

• Different approaches to response assessment and disease monitoring
• Specific issues with enumerating neoplastic plasma cells by flow cytometry
• Results from the MRC Myeloma IX trial
Most flow cytometry issues already have a broad consensus resolution.

- Plasma cell percentage flow vs. morphology
- Gating strategy: need CD38, CD138, and CD45 in same tube
- Essential, recommended and suggested markers to discriminate Myeloma from normal plasma cells
Issues in obtaining informative results

• Sample quality
• Identifying plasma cells (gating)
• Defining neoplastic vs. normal
  – Which antibodies to use?
  – Clonality vs. phenotype
Sample quality

First aspirate ➔ morphology

Second aspirate ➔ laboratory
Discrepancy between morphology and flow cytometry

Flow cytometry vs. “first-pull” marrow film
~ 50% lower

Flow cytometry vs. “laboratory” film
~ 15% lower

Flow cytometry vs. paired cytoprep
~ equivalent
Assessing aspirate quality: the presence of B-progenitors

First Pull:
4.8% B-cells
67% progenitors

Second Pull:
0.6% B-cells
26% progenitors
Assessing aspirate quality:

Acceptable for reporting:
Normal PCs >0.05% (?)
B-progenitors >1% (?)

Issues in bortezomib / IMID era:
Depletion of both neoplastic and normal plasma cells in good quality aspirates

Aspirate quality in MyeIX poor in pre-treatment samples but usually good/OK post-treatment
Issues in obtaining informative results

• Sample quality
• Identifying plasma cells (gating)
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  – Which antibodies to use?
  – Clonality vs. phenotype
Gating strategy and antibody choice

Table 4. Identification of the optimal marker combination for gating plasma cells: overall performance of different combinations of plasma cell gating markers evaluated at the EMN workshop held in Leeds in May 2007.

<table>
<thead>
<tr>
<th>Gating markers</th>
<th>CD38</th>
<th>CD38 and CD45</th>
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<th>CD38, and CD45 and CD138</th>
</tr>
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<tbody>
<tr>
<td>Proportion of cases with detectable disease</td>
<td>42%</td>
<td>28%</td>
<td>42%</td>
<td>61%</td>
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<td>Median percentage of plasma cells in cases with detectable disease</td>
<td>8.1% (1.6-35%)</td>
<td>0.8% (0.2-26%)</td>
<td>7.6% (0.5-39%)</td>
<td>2.7% (0.07-33%)</td>
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<td>67%</td>
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*percentage of cases with concordant results between participants.
Where are plasma cells situated on a CD38 vs SSC plot

- Plasma cells
- B-progenitors
- Monocytes
Where are plasma cells situated on a CD38 vs SSC plot.
Where are plasma cells situated on a CD38 vs SSC plot

Gated on CD138/SSC
Where are plasma cells situated on a CD38 vs SSC plot

Gated on CD138/SSC
Where are plasma cells situated on a CD38 vs SSC plot

Gated on CD138/SSC
CD38 and CD45

• CD45 is very helpful to exclude the non-specific binding events
• CD45 with tandem conjugates: false positive binding to apoptotic or necrotic plasma cells
• CD45 is expressed by neoplastic plasma cells in some cases: caution discriminating from B-progenitors
Differences in CD138 antibodies

- B-B4 clone appears to give better signal:noise than other clones
- Anecdotal: less epitope decay with time
**Gating strategy and antibody choice**

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Issues in obtaining informative results

- Sample quality
- Identifying plasma cells (gating)
- Defining neoplastic vs. normal
  - Which antibodies to use?
  - Clonality vs. phenotype
Abnormal CD19 expression by neoplastic plasma cells
Abnormal CD19 expression by neoplastic plasma cells

<table>
<thead>
<tr>
<th>Cytoplasmic κ:λ Ratio</th>
<th>CD19+ PC</th>
<th>CD19- PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norm1</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Norm2</td>
<td>2.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Norm3</td>
<td>1.8</td>
<td>1.1</td>
</tr>
<tr>
<td>Norm4</td>
<td>2.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Norm5</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Norm6</td>
<td>1.9</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Myeloma patients in complete remission after high-dose therapy

Myeloma IX / XI experience with Thal/R containing induction: early recovery of CD19-CD56- and CD19-CD56+ plasma cells common
# European Myeloma Network Suggested Markers

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Normal expression profile (percentage expression on normal plasma cells)</th>
<th>Abnormal expression profile</th>
<th>Percentage of myeloma cases with abnormal expression</th>
<th>Requirement for diagnosis and monitoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19</td>
<td>Positive (&gt;70%)</td>
<td>Negative</td>
<td>95%</td>
<td>Essential</td>
</tr>
<tr>
<td>CD56</td>
<td>Negative (&lt;15%)</td>
<td>Strongly positive</td>
<td>75%</td>
<td>Essential</td>
</tr>
<tr>
<td>CD117</td>
<td>Negative (0%)</td>
<td>Positive</td>
<td>30%</td>
<td>Recommended</td>
</tr>
<tr>
<td>CD20</td>
<td>Negative (0%)</td>
<td>Positive</td>
<td>30%</td>
<td>Recommended</td>
</tr>
<tr>
<td>CD28</td>
<td>Negative/weak (&lt;15%)</td>
<td>Strongly positive</td>
<td>15-45%</td>
<td>Recommended</td>
</tr>
<tr>
<td>CD27</td>
<td>Strongly positive (100%)</td>
<td>Weak or negative</td>
<td>40-50%</td>
<td>Recommended</td>
</tr>
<tr>
<td>CD81</td>
<td>Positive (100%)</td>
<td>Weak or negative</td>
<td>Not published</td>
<td>Suggested</td>
</tr>
<tr>
<td>CD200</td>
<td>Weakly positive</td>
<td>Strongly positive</td>
<td>Not published</td>
<td>Suggested</td>
</tr>
</tbody>
</table>
GEIL consensus proposal:

Gating: CD38 / CD138 / CD45
(1) CD36 / CD117+ CD34
(2) Lambda / Kappa
(3) CD19 / CD56

Myeloma
IX

Frebet/Feuillard – Clinical Cytometry 2011, 80B; 176.
# 8-color EuroFlow panels for PC dyscrasias

| Tube   | PB V450 | V500 | FITC | PE  | PerCP-Cy5.5 | PE-Cy7 | APC   | APC-H7 APC-C750 |
|--------|---------|------|------|-----|-------------|--------|-------|----------------|-----------------|
| Baseline | CD45   | CD138 | CD38 | CD56 | β2micro     | CD19   | cylgκ | cylgλ         |                 |
| Baseline | CD45   | CD138 | CD38 | CD28 | CD27        | CD19   | CD117 | CD81         |                 |
| MRD     | CD138  | CD27  | CD38 | CD56 | CD45        | CD19   | CD117 | CD81*        |                 |
Differences between normal CD19- and neoplastic CD19- plasma cells

- Median Fluorescence Intensity

- CD27, CD38, CD39, CD40, CD56, CD63, CD81, CD117, CD138, CD200, B2m

- Normal CD19+
- Normal CD19-
- Myeloma
Case 1

Case 2

Case 3
Added value of CD81

Pavia/San Miguel, Leukemia
2012, 26: 1862-9
SLAM family

CD319 : SLAMF7 / CS1 / CRACC – elotuzumab in clinical use
Issues in obtaining informative results

• Sample quality
• Identifying plasma cells (gating)
• Defining neoplastic vs. normal
  – Which antibodies to use?
  – Clonality vs. phenotype
Clonality plus CD19 expression

- CD138, CD38 & CD45 for gating
- clgκ and clgλ
- κ:λ ratio on CD19+ and CD19- plasma cells
- Effective* in 71% (44/62)

* >0.01% CD19- PC with light chain restriction or <0.01% CD19- PC
The problem with clonality (1): not quantitative

<table>
<thead>
<tr>
<th>Neoplastic PC</th>
<th>Normal B cells</th>
<th>Normal PC</th>
<th>Consensus IgH-PCR</th>
<th>Plasma cell κ/λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% +</td>
<td>0.1%</td>
<td>0.05%</td>
<td>+</td>
<td>6.5 + 0.3 +</td>
</tr>
<tr>
<td>0.1% —</td>
<td>5%</td>
<td>0.5%</td>
<td>—</td>
<td>1.5 — 1.4 —</td>
</tr>
<tr>
<td>0.01% +</td>
<td>0%</td>
<td>0%</td>
<td>+</td>
<td>&gt;10 + &gt;10 +</td>
</tr>
<tr>
<td>0.01% ?</td>
<td>0.1%</td>
<td>0.05%</td>
<td>?</td>
<td>2.0 — 1.0 —</td>
</tr>
<tr>
<td>0.01% —</td>
<td>5%</td>
<td>0.5%</td>
<td>—</td>
<td>1.5 — 1.4 —</td>
</tr>
</tbody>
</table>
The problem with clonality (2): poor specificity

Oligoclonal bands & non-neoplastic paraprotein common after high dose therapy and confer good prognosis
- CD138, CD38 & CD45 for gating
- CD56 and CD27
- Aberrant phenotype on CD19- plasma cells
- Effective* in 94% (136/145)

* >0.01% CD19- PC with aberrant CD56 &/or CD27 or <0.01% CD19- PC
Methods of response assessment

- Serum/urine measurement of disease status have significant limitations
  - measure of change rather than direct assessment of tumour burden
  - CR may take several months after end of Rx, or patients enter CR prior to high-dose or maintenance treatment and the impact of these interventions can only be assessed by PFS/OS after many years
- ASO-PCR provides improved prediction of outcome over immunofixation but
  - Clonally related B-cells limit sensitivity, only 75% of cases amplify
- Flow cytometry
  - Sensitive and directly quantitative; complicated but single-tube 6-8 CLR assays now available
  - Issues on gating, preparation methods and immunophenotypic characterisation: consensus documents from EMN, GEIL, Euroflow
Disease monitoring in multiple myeloma

- Different approaches to response assessment and disease monitoring
- Specific issues with enumerating neoplastic plasma cells by flow cytometry
- Results from the MRC Myeloma IX trial
MRC Myeloma IX—Trial Design

N = 1,960

Intensive

Randomisation

Clodronate CVAD
Zoledronic acid CVAD
Clodronate CTD
Zoledronic acid CTD

MEL-200 ASCT

Randomisation

=Thal +Thal

Non-intensive

Randomisation

Clodronate MP
Zoledronic acid MP
Clodronate CTDa
Zoledronic acid CTDa

Maximal Response

Randomisation

=Thal +Thal
Key Results from Myeloma IX

- Improved outcome for CTD induction vs. CVAD
- Difference in progression-free but not overall survival with thalidomide maintenance
- Zoledronate offers clear PFS and OS benefit
  - Reduced mortality by 16% (P=0.0118)
    • Median 50.0 vs. 45.5 months
  - Improved PFS by 12% (P=0.0179)
    • Median 19.5 vs. 17.5 months
  - Similar AE rate except higher osteonecrosis of the jaw with zoledronate (4% vs. <1%)
Impact of achieving MRD-negativity (<0.01% neoplastic plasma cells)

- Intensive arm
  - Median PFS: 27 vs. 40 months  P<0.001
  - 5yr OS*: 61% vs 70%  P = 0.028

- Non-intensive arm
  - Median PFS: 12 vs. 16 months  P=0.03
  - Median OS: 32 vs. 48 months  P=0.048

* Median not reached
MRD negativity: Superiority of CTD/CTDa.

<table>
<thead>
<tr>
<th></th>
<th>CVAD</th>
<th>CTD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post induction (n=774)</td>
<td>8%</td>
<td>16%</td>
<td>0.0029</td>
</tr>
<tr>
<td>Day 100 (n=526)</td>
<td>38%</td>
<td>53%</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>M &amp; P</th>
<th>CTDa</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of treatment (n=510)</td>
<td>2%</td>
<td>13%</td>
<td>0.000007</td>
</tr>
</tbody>
</table>
Differences between immunofixation and flow cytometry

CR after induction usually MRD$^{\text{POS}}$ (~0.7%)
20% of PR at Day 100 are MRD$^{\text{NEG}}$
Outcome according to MRD status and conventional response.

The graph shows the proportion progression free over years from trial entry for different MRD status and conventional response categories. The log rank test indicates a statistically significant difference among the groups with a p-value of <0.001.
PFS according to end of induction and day 100 post-Tx MRD status.

![Graph showing PFS according to end of induction and day 100 post-Tx MRD status. The graph includes a log rank P-value of 0.0015.](image)
Results according to conventional assessment:
- No significant change in response status
- Modest improvement in PFS: meta-analysis to demonstrate impact of Thal maintenance
Impact of maintenance therapy according to MRD status

Proportion Progression Free

Years from trial entry

Log rank P <0.005
Changes in neoplastic plasma cell levels after HDM +/- Thalidomide

![Graph showing changes in neoplastic plasma cell levels after HDM +/- Thalidomide.](image)

- **D100**: Day 100
- **2YR**: 2 years
- **No Thalidomide**: N=78
- **Thal maintenance**: N=80

Neoplastic plasma cells (% of bone marrow leucocytes)
- Dark red: 10 - 100
- Red: 1 - 10
- Orange: 0.1 - 1.0
- Yellow: 0.01 - 0.1
- Green: <0.01

Myeloma IX

HMDS - Haematological Malignancy Diagnostic Service
Maintenance or consolidation?

Become MRD-negative:

- No Thal: 4/39
- Thal: 11/34

P = 0.04

Become MRD-positive:

- No Thal: 18/39
- Thal: 9/46

P = 0.01
Flow cytometry in Myeloma

- MRD status at 0.01% level
  - Reproducibly attainable by FC and PCR
  - Better prediction of PFS than immunofixation
  - More sensitive (reproducible) than “stringent” CR
  - Particularly suited to assessment of multicomponent treatment schedules and maintenance strategies

- Response assessment in clinical trials
  - sFLC for initial response/primary refractory
  - MRD after treatment: Induction, HDM, Consolidation (maintenance)
  - Paraprotein better to measure relapse

- Therapy stratification according to MRD status?
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