Immunophenotyping of Hematologic Malignancy

Introduction of Euroflow System

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BRIEF INTRODUCTION OF FLOW CYTOMETRY
Basics of immunophenotyping: antigens

• Each cell has a unique proteins on its cell membrane and cytoplasm
• These proteins can be recognized by (monoclonal) antibodies
## Structure of Antibody

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Human and Mouse</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Light Chain</td>
<td>Subtype</td>
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<tr>
<td>IgA</td>
<td>κ or λ</td>
<td>IgA₁</td>
</tr>
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<tr>
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### Human

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<td>IgG₁</td>
<td>γ₁</td>
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<tr>
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<td>κ or λ</td>
<td>IgG₂</td>
<td>γ₂</td>
</tr>
<tr>
<td></td>
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<td>γ₃</td>
</tr>
<tr>
<td></td>
<td>κ or λ</td>
<td>IgG₄</td>
<td>γ₄</td>
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### Mouse

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<tr>
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</tr>
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<td>κ or λ</td>
<td>IgG₂</td>
<td>γ₂</td>
</tr>
<tr>
<td></td>
<td>κ or λ</td>
<td>IgG₂a</td>
<td>γ₂a</td>
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<td>κ or λ</td>
<td>IgG₃</td>
<td>γ₃</td>
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<tr>
<td></td>
<td>κ or λ</td>
<td>IgG₃</td>
<td>γ₃</td>
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</tbody>
</table>
Flow Cytometric Monoclonal Antibodies

- CD = cluster of differentiation
Light Scatter

**Forward scatter (FSC)**
- Dependent on cell size

**Side scatter (SSC)**
- Measure for internal structure (surface, nucleus, granule, etc)
Peripheral Blood
EuroFlow Consortium Participants

- 12 countries
- 17 labs
- Prof. dr. J. J.M. van Dongen, chair
- Prof. dr. A. Orfao, co-chair
Structure of EuroFlow Consortium

General Assembly
(one representative per participating organisation)

Coordination Committee (WP1)
J.J.M. van Dongen, coordinator
A. Orfao, vice-coordinator
M. Kneba
E.A. Macintyre

Advisory Committee
- M. Kok
- C. Romeo Casabona
- A.J.M. de Wild-Chardonnens
- J.F. San Miguel
- H.J.H.M. Claassen

Intellectual Property Committee
- E. van Oosterom, lawyer
- L.A.C.M. van Wezenbeek, patent attorney
- J.J.M. van Dongen, coordinator

Management team
- A. Steenbergen (project manager)
- W. Borsje (financial officer)
- B. van Bodegom (secretariat)

WP2 DYNAMICS
WP3 CYTOGNOS

WP4
A. Orfao

WP5
M. Kneba

WP6
E.A. Macintyre

EuroFlow
Standardization for Immunophenotyping

- CLSI (Clinical Laboratory Standards Institute)
  - Clinical flow cytometric analysis of neoplastic hematolymphoid cells
- CCS (Clinical Cytometry Society)
  - 2006 Bethesda International Consensus recommendations on the flow cytometric immunophenotypic analysis of hematolymphoid neoplasia
- ESCCA (European Society for Clinical Cell Analysis)
  - www.escca.eu
- European Leukemia Net
  - www.leukemia-net.org
- Latin American Consensus
2 Reasons for Consensus not Useful

• **Focus on lists of markers without** specific recommendations about
  – Reagent clones
  – Fluorochrome conjugates
  – Optimally designed antibody combinations
2 Reasons for Consensus not Useful

• Fail to provide robust protocols for the selection of the most appropriate
  – Combinations of fluorochromes and fluorochrome-conjugated reagents in a panel,
  – Sample preparation techniques
  – Standard operating procedures (SOPs) to establish instrument settings prior to the measurements
  – The most adequate strategies for data analysis
Achievement of the EuroFlow Consortium

- Full technical standardization of multicolor flow cytometry (≥8 colors)
  - Standardization of instrument settings and laboratory protocols
  - Selection of fluorochromes and selection of antibody clones per marker
  - EuroFlow protocols work on all tested ≥8 colors flow cytometers:
    - DAKO Cyan, LSR-II, FACS Canto-II;
    - "late arrivals" (Navios and Gallios) still to be tested (new Workpackage)
- Implementation and further development of novel software: Infinicyt
  - Fast and easy data handling with automated pattern recognition
  - Combining multiple tubes: calculation and APS (principle component analysis)
  - Mapping of diagnosis and follow-up leukemia samples against templates of “normal/control” samples
- Development of 8-color antibody protocols for diagnosis, classification and monitoring of hematological malignancies
  - 8-color panels are based on recognition of normal cells & differentiation pathways
  - Diagnosis and classification tubes are ready; MRD tubes in development
  - Flexibility within panels: deletion and inclusion of markers and tubes is possible
- Large EuroFlow data base linked to Infinicyt software
EuroFlow Antibody Protocols

• Screening tubes (include recognition of normal leukocyte subsets)
  – Acute leukemia orientation tube (ALOT): 1 tube (L Lhermitte)
  – Lymphoid screening tube (LST): 1 tube (J Flores Montero)
  – Small sample screening tube (SST): 1 tube (AW Langerak)
  – Plasma cell screening tubes (PCST): 1 tube (J Flores Montero)

• Multi-tube panels for characterization per disease category
  – B-cell precursor ALL (BCP-ALL) protocol: 4 tubes (L Lhermitte)
  – T-cell ALL (T-ALL) protocol: 4 tubes (V Asnafi)
  – AML/MDS protocol: 7 tubes (VHJ van der Velden)
  – B chronic lymphoproliferative diseases (B-CLPD): 5 tubes (S Böttcher)
  – T chronic lymphoproliferative diseases (T-CLPD): 6 tubes (J Almeida)
  – NK chronic lymphoproliferative diseases (NK-CLPD): 3 tubes (J Almeida)
ALOT
(ACUTE LEUKEMIA ORIENTATION TUBE)
ALOT (Acute Leukemia Orientation Tube)

Design for

- Initial assessment of the nature of immature populations of hematopoietic cells in acute leukemia samples
- Allow appropriate orientation towards the complementary BCP-ALL, T-ALL and AML/MDS antibody panels

<table>
<thead>
<tr>
<th>Pac Blue</th>
<th>Pac Orange</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP Cy5.5</th>
<th>PE Cy7</th>
<th>APC</th>
<th>APC H7</th>
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<tbody>
<tr>
<td>cyCD3</td>
<td>CD45</td>
<td>cyMPO</td>
<td>cyCD79a</td>
<td>CD34</td>
<td>CD19</td>
<td>CD7</td>
<td>smCD3</td>
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</table>

<table>
<thead>
<tr>
<th>Target Antigen</th>
<th>Fluorochrome conjugate</th>
<th>Gating markers (first level)</th>
<th>Gating Markers (second level)</th>
<th>Immaturity markers</th>
<th>Lineage markers</th>
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<td>X</td>
<td>X</td>
<td></td>
<td>My B, T</td>
</tr>
<tr>
<td>cyCD79a</td>
<td>PE</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34</td>
<td>PerCP Cy5.5</td>
<td>X</td>
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<td>PE CY7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD7</td>
<td>APC</td>
<td></td>
<td></td>
<td></td>
<td>B, My T, My</td>
</tr>
<tr>
<td>smCD3</td>
<td>APC H7</td>
<td></td>
<td></td>
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<tr>
<td>cyCD3</td>
<td>Pacific Blue</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CD45</td>
<td>PO</td>
<td>X</td>
<td></td>
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</tbody>
</table>
Assessment of Blast Lineage

AML vs BCP-ALL
- CD19: 32%
- CyMPO: 27%
- CyCD79a: 20%
- CD45: 8%
- CyCD3: 6%

AML vs T-ALL
- CyMPO: 26%
- CD7: 26%
- CyCD3: 26%
- CD34: 15%
- SmCD3: 6%

BCP-ALL vs T-ALL
- CD19: 23%
- CyCD3: 22%
- CD7: 22%
- CyCD79a: 14%
- CD34: 9%
Performance of ALOT

• 98.3% efficacy for non-ambiguous lineage cases
  – 483 newly diagnosed acute leukemia cases, tested prospectively at different centers.
LST
(LYMPHOCYTE SCREENING TUBE)
LST (Lymphocyte Screening Tube)

Design for

- Phenotypically aberrant and assessment clonal mature lymphocytes
- Identification of the abnormal lymphocytes and their discrimination from normal and reactive cells

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</thead>
<tbody>
<tr>
<td>CD20-CD4</td>
<td>CD45</td>
<td>CD8-Smlgλ</td>
<td>CD56-Smlgκ</td>
<td>CD5</td>
<td>CD19-TCRγδ</td>
<td>SmCD3</td>
<td>CD38</td>
</tr>
</tbody>
</table>
## LST (Lymphocyte Screening Tube)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Main normal population identified</th>
<th>Positive diagnosis</th>
<th>Population subsetting</th>
<th>Diagnostic subclassification</th>
<th>Potential minimal disease value</th>
<th>Prognostic relevance</th>
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<tbody>
<tr>
<td>CD45</td>
<td>Mature lymphocytes and B-cell precursors</td>
<td>X</td>
<td>X</td>
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<tr>
<td>CD19</td>
<td>B-cells, T- and NK-cells by exclusion</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>CD20</td>
<td>B-cells, T- and NK-cells by exclusion</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>SmIgκ and λ</td>
<td>SmIg+ B-cells</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>CD38</td>
<td>Plasma cells and B-cell precursors</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>SmCD3</td>
<td>T-cells, B- and NK-cells by exclusion</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>CD4+ T-cells</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>CD8</td>
<td>CD8hi T-cells and CD8lo NK-cells</td>
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<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>CD56</td>
<td>NK-cells</td>
<td>X</td>
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<tr>
<td>TCRγδ</td>
<td>TCRγδ+ T-cells</td>
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<tr>
<td>CD5</td>
<td>T-cells</td>
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<td>CD20-CD4</td>
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<td>CD8-SmIgλ</td>
<td>CD5</td>
<td>CD5</td>
<td>CD19-TCRγδ</td>
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</table>

**Identify**

- Non-Hematopoietic cells (CD45, CD56, CD38, FSC, SSC)
<table>
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<tr>
<th>Pac Blue</th>
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<td>CD19-TCRγδ</td>
<td>SmCD3</td>
<td>CD38</td>
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**Identify**
- Non-Hematopoietic cells (CD45, CD38, CD56)
- **Hematopoietic cells**
  - B-cell (CD19, CD20, CD45)
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<th>Pac Blue</th>
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<td>CD19-TCRγδ</td>
<td>SmCD3</td>
<td>CD38</td>
<td></td>
</tr>
</tbody>
</table>

**Identify**

- Non-Hematopoietic cells (CD45, CD38, CD56)
- Hematopoietic cells
  - B-cell (CD19, CD20, CD45)
    - Kappa (CD19, CD20, CD45, SmIgκ)
    - Lambda (CD19, CD20, CD45, SmIgλ)
Identify

- Non-Hematopoietic cells (CD45, CD38, CD56)
- Hematopoietic cells
  - B-cell (CD19, CD20)
    - Kappa (CD19, CD20, CD45, SmIgκ)
    - Lambda (CD19, CD20, CD45, SmIgλ)
  - T-cell (SmCD3, CD45)
Identify

- Non-Hematopoietic cells (CD45, CD38, CD56)
- Hematopoietic cells
  - B-cell (CD19, CD20)
    - Kappa (CD19, CD20, CD45, Smlgκ)
    - Lambda (CD19, CD20, CD45, Smlgλ)
  - T-cell (SmCD3, CD45)
    - Helper T (SmCD3, CD45, CD4)
    - Cytotoxic T (SmCD3, CD45, CD8)
    - γδ+ (SmCD3, CD45, TCRγδ)
Identify

- **Non-Hematopoietic cells** (CD45, CD38, CD56)
- **Hematopoietic cells**
  - **B-cell** (CD19, CD20)
    - Kappa (CD19, CD20, CD45, SmIgκ)
    - Lambda (CD19, CD20, CD45, SmIgλ)
  - **T-cell** (SmCD3, CD45)
    - Helper T (SmCD3, CD45, CD4)
    - Cytotoxic T (SmCD3, CD45, CD8)
    - γδ+ (SmCD3, CD45, TCRγδ)
  - **NK-cell** (SmCD3, CD56, CD45, CD38)
Identify

- Non-Hematopoietic cells (CD45, CD38, CD56)
- Hematopoietic cells
  - B-cell (CD19, CD20)
    - Kappa (CD19, CD20, CD45, SmIgκ)
    - Lambda (CD19, CD20, CD45, SmIgλ)
  - T-cell (SmCD3, CD45)
    - Helper T (SmCD3, CD45, CD4)
    - Cytotoxic T (SmCD3, CD45, CD8)
    - γδ+ (SmCD3, CD45, TCRγδ)
  - NK-cell (SmCD3, CD56, CD45, CD38)
  - Plasma cells (CD19, CD38, CD45, CD56)
Identify

- Non-Hematopoietic cells (CD45, CD38, CD56)
- Hematopoietic cells
  - B-cell (CD19, CD20)
    - Kappa (CD19, CD20, CD45, SmIgκ)
    - Lambda (CD19, CD20, CD45, SmIgλ)
  - T-cell (SmCD3, CD45)
    - Helper T (SmCD3, CD45, CD4)
    - Cytotoxic T (SmCD3, CD45, CD8)
    - γδ+ (SmCD3, CD45, TCRγδ)
  - NK-cell (SmCD3, CD56, CD45, CD38)
  - Plasma cells (CD19, CD38)
  - B-CLPD backbone markers (CD19, CD20, CD45)
Performance of LST

- **B-CLPD**
  - Aberrant B-cell detection rate: 99.4% (149/150)
    - Only 1 neoplastic B-cells overlapped with normal B-cells

- **T-CLPD**
  - Aberrant T-cell detection rate: 94% (61/65)

- **NK-CLPD**
  - Aberrant NK-cell detection rate: 94.4% (17/18)
PCST (PLASMA CELL SCREENING TUBE)
PCD (PLASMA CELL DYSCRASIA)
CONSTRUCTION OF EuroFlow MRD PANELS: MM

Identify PC

Select PC

Principal component analysis
(n=12 markers)

Merge PC
(n-cases)

MOST INFORMATIVE MARKERS

CD19  19.83
CD56  19.02
CD81  12.29
CD45  11.47
CD27  9.95
CD117 9.34
CD38  5.18
PCST (Plasma Cell Screening Tube)

<table>
<thead>
<tr>
<th>Pac Blue</th>
<th>Pac Orange</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP Cy5.5</th>
<th>PE Cy7</th>
<th>APC</th>
<th>APC H7</th>
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<tbody>
<tr>
<td>CD45</td>
<td>CD138</td>
<td>CD38</td>
<td>CD56</td>
<td>CD27</td>
<td>CD19</td>
<td>CyIgκ</td>
<td>CyIgλ</td>
</tr>
</tbody>
</table>
## PCD (Plasma Cell Dyscrasia)

<table>
<thead>
<tr>
<th>Tube</th>
<th>Target Antigen</th>
<th>Identification of plasma cells</th>
<th>Aberrant markers</th>
<th>2\textsuperscript{nd} diagnostic level marker</th>
<th>Assessment of plasma cell clonality</th>
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</thead>
<tbody>
<tr>
<td>BB markers</td>
<td>CD38</td>
<td>X</td>
<td>C</td>
<td></td>
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<tr>
<td></td>
<td>CD138</td>
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<tr>
<td></td>
<td>CD19</td>
<td>X</td>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube 1</td>
<td>CyIgκ</td>
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<td></td>
<td></td>
<td>X</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>CD56</td>
<td>C</td>
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<td></td>
<td>β2 Micro</td>
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<td></td>
<td>X</td>
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<tr>
<td>Tube 2</td>
<td>CD27</td>
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<td>X</td>
<td></td>
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<tr>
<td></td>
<td>CD28</td>
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<td>X</td>
<td></td>
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<tr>
<td></td>
<td>CD117</td>
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<tr>
<td></td>
<td>CD81</td>
<td></td>
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</tbody>
</table>
Performance of PCD Panel

- 100 samples
  - 38-MM
  - 23-MGUS
  - 2-PCL
  - 6-Suspect MM
    - 2-MGUS, 2-MM, 1-plasmacytoma, 1-IgM paraproteinemia
  - 10-Non-MM sample
  - 13-Regeneration marrow from Non-PCD
  - 8-Normal healthy

- Aberrant plasma cell detection: 63/63
- Normal/Reactive plasma cell: 88/88
- Co-existing aberrant & normal PC: 49/49
SST
(SMALL SCREENING TUBE)
Distribution of Cell Populations in Normal/Reactive CSF Samples

Adapted from University hospital of Salamanca
## Normal/Reactive Leukocyte Population in CSF (n=120) and Vitreous biopsy (n=21)

<table>
<thead>
<tr>
<th>Group of samples</th>
<th>% B-cells</th>
<th>SmIgκ/SmIgλ ratio</th>
<th>% T-cells</th>
<th>CD4/CD8 ratio</th>
<th>% Monocytes</th>
<th>% Neutrophils</th>
<th>% Other undefined events</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF (n=120)</td>
<td>2.0 (0-32)</td>
<td>1.4 (1.0-3.0)</td>
<td>50.8 (0-100)</td>
<td>1.8 (0.1-10.9)</td>
<td>2.1 (0-17)</td>
<td>15 (0-99)</td>
<td>31.7 (0-100)</td>
</tr>
<tr>
<td>CSF (MS cohort)</td>
<td>1.1 (0-4)</td>
<td>1.3 (1.0-2.5)</td>
<td>48.2 (0-89)</td>
<td>2.8 (1.7-7)</td>
<td>3.6 (0-14)</td>
<td>23 (0-99)</td>
<td>21.9 (0-100)</td>
</tr>
<tr>
<td>CSF (other HM suspicion) (n=9)</td>
<td>0.2 (0-1)</td>
<td>NA</td>
<td>40.8 (0-100)</td>
<td>1.2 (0.2-3.5)</td>
<td>0</td>
<td>9 (0-14)</td>
<td>49.8 (0-100)</td>
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<tr>
<td>CSF (lymphoma suspicion) (n=96)</td>
<td>4.8 (0-32)</td>
<td>1.4 (1.0-3.0)</td>
<td>63.4 (0-100)</td>
<td>1.7 (0.1-10.9)</td>
<td>2.6 (0-17)</td>
<td>13 (9-59)</td>
<td>23.4 (0-100)</td>
</tr>
<tr>
<td>Vitreous fluid (n=21)</td>
<td>0.5 (0-4)</td>
<td>NA</td>
<td>51.7 (3-100)</td>
<td>2.1 (0.3-49)</td>
<td>2.6 (0-14)</td>
<td>18 (0-53)</td>
<td>14.1 (0-100)</td>
</tr>
</tbody>
</table>

CSF: cerebrospinal fluid; HM: hematological malignancy; MS: multiple sclerosis; NA: not applicable due to lack of B-cells in most samples; Sm: surface marker

Leukemia. 2012 Sep;26(9):1908-75
SST (Small Screening Tube)

**Design for**

- Pauci-cellular or ‘small’ with obtainable maximal information from minimal numbers of cells

<table>
<thead>
<tr>
<th>Screen</th>
<th>PB</th>
<th>PO</th>
<th>FITC</th>
<th>PE</th>
<th>PerCPCy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
<th>APC-H7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD20</td>
<td>CD45</td>
<td>CD8-Smlg(\lambda)</td>
<td>CD56-Smlg(\kappa)</td>
<td>CD4</td>
<td>CD19</td>
<td>CD3-CD14</td>
<td>CD38</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>PO</td>
<td>FITC</td>
<td>PE</td>
<td>PerCPCy5.5</td>
<td>PE-Cy7</td>
<td>APC</td>
<td>APC-H7</td>
</tr>
<tr>
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<tr>
<td>Screen</td>
<td>CD20</td>
<td><strong>CD45</strong></td>
<td>CD8-SmIgλ</td>
<td><strong>CD56-SmIgκ</strong></td>
<td>CD4</td>
<td>CD19</td>
<td>CD3-CD14</td>
<td><strong>CD38</strong></td>
</tr>
</tbody>
</table>

**Identify**

- **Non-Hematopoietic cells (CD45, CD38, CD56, SSC, FSC)**
<table>
<thead>
<tr>
<th>PB</th>
<th>PO</th>
<th>FITC</th>
<th>PE</th>
<th>PerCPCy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
<th>APC-H7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screen</td>
<td>CD20</td>
<td>CD45</td>
<td>CD8-Smlg(\lambda)</td>
<td>CD45-Smlg(\kappa)</td>
<td>CD4</td>
<td>CD19</td>
<td>CD3-CD14</td>
</tr>
</tbody>
</table>

**Identify**
- Non-Hematopoietic cells (CD45, CD38, CD56, SSC, FSC)
- **Hematopoietic cells**
  - **B-cell** (CD19, CD20, CD45)
Identify

- Non-Hematopoietic cells (CD45, CD38, CD56)
- Hematopoietic cells
  - B-cell (CD19, CD20, CD45)
    - Kappa (CD19, CD20, CD45, SmIgκ)
    - Lambda (CD19, CD20, CD45, SmIgλ)
Identify

- Non-Hematopoietic cells (CD45, CD38, CD56)
- Hematopoietic cells
  - B-cell (CD19, CD20)
    - Kappa (CD19, CD20, CD45, SmIgκ)
    - Lambda (CD19, CD20, CD45, SmIgλ)
  - T-cell (SmCD3, CD45)
<table>
<thead>
<tr>
<th></th>
<th>PB</th>
<th>PO</th>
<th>FITC</th>
<th>PE</th>
<th>PerCPCy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
<th>APC-H7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screen</td>
<td>CD20</td>
<td><strong>CD45</strong></td>
<td>CD8-SmIgλ</td>
<td>CD56-SmIgκ</td>
<td>CD4</td>
<td>CD19</td>
<td><strong>CD3-CD14</strong></td>
<td>CD38</td>
</tr>
</tbody>
</table>

**Identify**

- Non-Hematopoietic cells (CD45, CD38, CD56)
- Hematopoietic cells
  - B-cell (CD19, CD20)
    - Kappa (CD19, CD20, CD45, SmIgκ)
    - Lambda (CD19, CD20, CD45, SmIgλ)
  - T-cell (SmCD3, CD45)
    - Helper T (SmCD3, CD45, CD4)
    - Cytotoxic T (SmCD3, CD45, CD8)
<table>
<thead>
<tr>
<th>Screen</th>
<th>PB</th>
<th>PO</th>
<th>FITC</th>
<th>PE</th>
<th>PerCPCy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
<th>APC-H7</th>
</tr>
</thead>
<tbody>
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<td>CD45</td>
<td>CD8-</td>
<td>CD56-</td>
<td></td>
<td></td>
<td>CD19</td>
<td>CD3-CD14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SmIgλ</td>
<td>SmIgκ</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tbody>
</table>

### Identify
- Non-Hematopoietic cells (CD45, CD38, CD56)
- Hematopoietic cells
  - B-cell (CD19, CD20)
    - Kappa (CD19, CD20, CD45, SmIgκ)
    - Lambda (CD19, CD20, CD45, SmIgλ)
  - T-cell (SmCD3, CD45)
    - Helper T (SmCD3, CD45, CD4)
    - Cytotoxic T (SmCD3, CD45, CD8)
  - NK-cell (SmCD3, CD45, CD56, CD38)
### Identify

- **Non-Hematopoietic cells** (CD45, CD38, CD56)
- **Hematopoietic cells**
  - B-cell (CD19, CD20)
    - Kappa (CD19, CD20, CD45, SmIgκ)
    - Lambda (CD19, CD20, CD45, SmIgλ)
  - T-cell (SmCD3, CD45)
    - Helper T (SmCD3, CD45, CD4)
    - Cytotoxic T (SmCD3, CD45, CD8)
  - NK-cell (SmCD3, CD45, CD56, CD38)
  - **Monocyte** (CD45, CD14)
Identify

- **Non-Hematopoietic cells** (CD45, CD38, CD56)
- **Hematopoietic cells**
  - B-cell (CD19, CD20)
    - Kappa (CD19, CD20, CD45, SmIgκ)
    - Lambda (CD19, CD20, CD45, SmIgλ)
  - T-cell (SmCD3, CD45)
    - Helper T (SmCD3, CD45, CD4)
    - Cytotoxic T (SmCD3, CD45, CD8)
  - NK-cell (SmCD3, CD45, CD56, CD38)
  - Monocyte (CD45, CD14)
  - **Plasma cell** (CD19, CD38, CD45)
Analysis of SST

SST analysis of CSF sample

SST analysis of vitreous biopsy
## Performance of SST

<table>
<thead>
<tr>
<th>Category</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Suspected lymphoma localized in CSF (n = 115)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aberrant/clonal B-cell populations&lt;sup&gt;a&lt;/sup&gt; (1 CLL, 1 BL, 1 FL, 1 MCL, 2 DLBCL, 1 B-cell lymphoma, 2 unknown)</td>
<td>9/115</td>
<td>7.8%</td>
</tr>
<tr>
<td>Aberrant T-cell populations (T-cell lymphoma, PTLD)</td>
<td>2/115</td>
<td>1.7%</td>
</tr>
<tr>
<td><strong>Suspicious of other hematological malignancies in CSF (n = 11)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aberrant plasma cell populations</td>
<td>2/11</td>
<td>18.1%</td>
</tr>
<tr>
<td><strong>Vitreous biopsy (n = 23)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aberrant/clonal B-cell populations</td>
<td>2/23</td>
<td>8.7%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>15/149</td>
<td>10.0%</td>
</tr>
</tbody>
</table>

Abbreviations: BL, Burkitt lymphoma; CNS, central nervous system; CLL, chronic lymphocytic leukemia; CSF, cerebrospinal fluid; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; FNA, fine needle aspirate; IOL, intraocular lymphoma; PTLD, post-transplant lymphoproliferative disease; SST, small sample tube.<sup>a</sup>In one case a parallel FNA brain biopsy was analyzed next to the CSF sample, showing the same aberrant B-cell population;<sup>b</sup>All positive cases had a final diagnosis of CNS lymphoma or IOL based on histopathological analysis, imaging techniques and/or the clinical behavior of the disease, while none of the negative cases by the SST labeling was diagnosed as having CNS or IOL.
### FCM vs CC for Detection of CSF Involvement in Aggressive B-NHL (n=123)

<table>
<thead>
<tr>
<th>Conventional Cytology</th>
<th>Flow Cytometry</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Negative</td>
<td>95/123</td>
<td>77</td>
<td>17/123</td>
</tr>
<tr>
<td>Positive</td>
<td>1*/123</td>
<td>1</td>
<td>7/123</td>
</tr>
<tr>
<td>Suspicious</td>
<td>--</td>
<td>--</td>
<td>3/123</td>
</tr>
</tbody>
</table>

*The presence of neoplastic cells in this patient was ruled out by further immunocytochemical analyses (one cytospin slide was fixed in acetone and stained with CD20 monoclonal antibody L-26 [Dako, Glostrup, Denmark] using the ABC method).

<table>
<thead>
<tr>
<th></th>
<th>FCM</th>
<th>Cytology</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF (+)</td>
<td>22% (27/123)</td>
<td>6% (6/123)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

New Analysis Software
Where did Infinicyt come from?

Industry + Academia → Infinicyt®

Based on University of Salamanca patents (Spain)

Licensed and developed by Cytognos, SL (Spain)

Contribution and validation by EuroFlow
What is the need for Infinicyt?

New Technology

More Laser

More Colors

More Files

More Diagrams

Difficult Interpretation
Functions of Infinicyt
Manual Analysis
Manual Analysis

Traditional Analysis

Multiparametric Diagrams
Automatic Population Separator

2D Dot Plot

APS Diagrams

Separation using TWO parameters
- FSC
- SSC

Separation using ALL parameters
- FSC
- SSC
- FL1
- FL2
- FL3
- FL4
- FL5
- FL6
- FL7
- FL8
Automatic Population Separator

2D Dot Plot

APS Diagrams
Automatic Population Separator
File Merge

- 1 unique file
- 500,000 events
- All marker’s information

CyMPO
CD79
CD7
CyCD3
CD3
CD58
CD66c
CD10
CD38
CD20
CyIgM
CD33
IgM+CD117
IgL
IgK
TdT
CD13
CD22
CD24
CD9
CD15
NG2
CD123
CD81
CD21

Common parameters
- FSC
- SSC
- CD45
- CD34
- CD19

File Merge

EuroFlow
File Merge

5 Single files → 1 Merged File
File Merge: Comparative Study

PHENOTYPE OF TONSIL vs. PERIPHERAL BLOOD vs. BONE MARROW PLASMA CELLS

Tonsil | Peripheral Blood | Bone marrow

Perez-Andres M et al, Clin Cytom 2010
SIMULTANEOUS ANALYSIS OF PLASMA CELLS FROM TONSIL, PB AND BM

Information from Perez-Andres M et al, Clin Cytom 2010
Calculate Data

Tube 1

Expression of CD27

Tube 2

Expression of CD200
Calculate Data: Validation

Real data (generated with antibodies conjugated with distinct fluorochrome)

Real

CD11c-APC
CD5-PE

Calculated data (originally impossible 2-dimensional dot-plot representations)

Calculated

CD11c-APC
CD5-APC

Real data (generated with antibodies conjugated with distinct fluorochrome)

Real

CD23-PE
sIgK-APC

Calculated data (originally impossible 2-dimensional dot-plot representations)

Calculated

CD23-PE
sIgK-PE
# Immunophenotypic Patterns of Different Types of B-CLPD


<table>
<thead>
<tr>
<th></th>
<th>sIg</th>
<th>CD5</th>
<th>CD10</th>
<th>CD20</th>
<th>CD11c</th>
<th>CD23</th>
<th>CD24</th>
<th>CD25</th>
<th>CD38</th>
<th>CD43</th>
<th>CD79b</th>
<th>CD103</th>
<th>FMC7</th>
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</thead>
<tbody>
<tr>
<td>B-CLL</td>
<td>d</td>
<td>+</td>
<td>-</td>
<td>d</td>
<td>-/+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-/+</td>
<td>+</td>
<td>d</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PLL</td>
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<td>-/+</td>
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<td>+</td>
<td>-</td>
<td>++</td>
<td>-/+</td>
<td>-/+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**Legend:**
- d: Diminutive
- +/-: Variable expression
- : Absent
- +: Present
- +: Strongly present
- +: Weakly present
- +: Moderate present
- : Not tested
Reference Image

Case Study
Compass Tool

Reference Cases from Group 1

Reference Cases from Group 2

Case Study

APS 1
Compass Tool

Two by two comparative

Single comparative
Compass Tool

Aids in diagnosis, not a diagnosis tool
Maturation Tool

- Identify cell population
- Draw maturation direction
Maturation Tool

Save Normal References and create Databases

Normal Case 1

Normal Case 2

Normal Case 3

Normalised Database for the Reference Cases
Maturation Tool

Compare a case with the Maturation Database

Case Study

Normalised Database

CD11b

Overexpression of CD11b

Under expression of CD11b

Neutrophils (Stages)
Thanks for your attention