**DIAGNÓSTICO Y CLASIFICACIÓN DE LEUCEMIAS AGUDAS CON LOS PANELES EUROFLOW**

**CANCER RESEARCH CENTER, UNIVERSITY & UNIVERSITY HOSPITAL of SALAMANCA (SPAIN)**

Curso Avanzado de Actualización en Oncohematología por Citometría de Flujo, Buenos Aires, 31 de mayo de 2011

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**DIAGNOSTICS IN HEMATO-ONCOLOGY**

1. **Making the diagnosis**
   - Normal vs reactive/regenerating vs malignant
   - Annually ∼ 300,000 new patients with a hematological malignancy in developed countries

2. **Classification of hematopoietic malignancies**
   - Relation with prognosis
   - Relevance of risk-group definition in treatment protocols
   - Based on differentiation characteristics and particularly on chromosome aberrations, resulting in fusion gene transcripts or aberrantly (over)expressed genes

3. **Evaluation of treatment effectiveness**
   - Detection of minimal residual disease (MRD):
     - MRD-based risk-group stratification (treatment reduction or treatment intensification)
     - Annually ∼ 400,000 follow-up samples in leukemia patients (ALL, AML, CML)

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**REQUISITED DEVELOPMENTS IN FLOW CYTOMETRY**

(challenges in 2005)

- **Immunobeads**
  - Introduce combined cellular/immunobead assays
  - Special immunobeads for leukemias

- **Novel antibodies**
  - Test new (academic) antibodies for application in intracellular stainings
  - Development of new antibodies against oncproteins and aberrant signalling pathways

- **Multicolor flow cytometry**: all color comprehensive panels
  - Inclusion of solid state violet laser
  - Selection of appropriate fluorochromes
  - Compare conjugated antibodies (multiple companies)

- Development of novel software for complex pattern recognition
  - Combining multiple tubes: calculate data & multivariate analyses
  - Mapping of diagnosis and follow-up leukemia samples against templates of reference "normal/control" samples

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**THE EUROFLOW APPROACH TO LEUKEMIA/LYMPHOMA IMMUNOPHENOTYPING**

- Clinical question
- Diagnostic screening test
- "Diagnostic classification" panel
- MRD monitoring
- Majority of diseases?
- Majority of cases?
- New disease entities?

**Clinical request/need: CONVENTIONAL PANEL DESIGN**

- Clinical request/need
- Knowledge
- Experience
- Evaluation
- Reference profiles

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**SET UP OF A FCM LABORATORY FOR LEUKEMIA/LYMPHOMA TYPING**

- Purchase a flow cytometer
- Design of MAb panels (Disease category vs cell lineage oriented)
- Training
- Immunophenotypic diagnostic activity started

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STANDARDIZATION EFFORTS FOR IMMUNOPHENOTYPIC STUDIES

- CLSI (Clinical Laboratory Standards Institute):
- CCS (Clinical Cytometry Society):
- ESCCA (European Society for Clinical Cell Analysis: www.escca.eu)
- European Leukemia Net (www.leukemia-net.org)
- Consenso Latinoamericano (Clin Cytometry, 1998 y 2006)

CONSTRUCTION OF EUFLOW LEUKEMIA/LYMPHOMA IMMUNOPHENOTYPING ANTIBODY PANEL

- Clinical request/need
- Medical indication
- Design of MAb panels (medical indication-oriented) & immunophenotyping strategy
- Panel optimization (re-design)
- Panel evaluation vs conventional in-use panels

LEUKEMIA /LYMPHOMA IMMUNOPHENOTYPING: EVALUATION OF ANTIBODY PANELS

- Single center panel
- Multicenter panel
- Single center experience/evaluation
- Multicenter evaluation possible

- Experience-based (subjective)
- Long time required
- Limited by new instruments/techniques/markers
- Consensus recommendations
- Shared experience
- Prospective evaluation
- Experimentally supported

CONSTRUCTION OF EUFLOW PANELS: MEDICAL INDICATION ORIENTATION/SCREENING & CLASSIFICATION PANELS

- Panel evaluation vs conventional in-use panels
- Panel optimization (re-design)

- 2-8 cycles

- ALOT: 1 tube
- BCP-ALL: 4 tubes
- T-ALL: 4 tubes
- AML/MDS: 4 to 7 tubes
CONSTRUCTION OF EUROFLOW ANTIBODY PANELS

Step 0: Design strategy
Step 1: Selection of fluorochromes
Step 2: Selection of markers
Step 3: Selection of antibody reagents
Step 4: Selection of antibody combinations
Step 5: Panel constructed

ALOT (Acute Leukemia Orientation Tube)

- Designed for assessment of the nature of immature blast cell populations in acute leukemia samples
- Designed to choose appropriate immunophenotypic panel(s)

ALOT: B-cell precursor ALL

BM stained with ALOT 8-color tube

CyCD3
CD7
CD19
CyCD79a
CyMPO
CD34
CD19
CD7
sCD3
CD19
CyCD79a
CD45
CD34

ALOT: T-cell acute lymphocytic leukemia (T-ALL)

BCP-ALL

AML
Single « virtual » merged tube/data file

Responsible scientist: Ludovic Lhermitte

ALOT (Acute Leukemia Orientation Tube)
ALOT: IMMUNOPHENOTYPIC CLASSIFICATION OF BLASTS

Development of immunostainings protocols – 8-color combinations

BCP-ALL panel:

T-ALL panel:

Development of immunostainings protocols – 8-color combinations

BCP-ALL panel:

T-ALL panel:

Responsibility scientist: Ludovic Lhermitte
### T-ALL panel

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### Positive Diagnosis

- **Differential Diagnosis**
- **Ambiguous lineage acute leukemia**

### Classical classification

- **Maturation stage**

### Alternative T-ALL classification

- **Maturation stage**
- **Well-defined molecular abberations**
**BCP-ALL panel**

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**Positive Diagnosis**

**Differential Diagnosis**

&

**Ambiguous lineage acute leukemia**

**Maturation stage (EGIL)**

**Alternative classification**

**Immunophenotypic features associated with well-defined molecular aberrations**

**Prognosis markers**

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BCP-ALL panel

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  - CD19
  - CD15
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- **Pac Orange**
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  - CD21
  - CD9
  - TdT
  - CD13
  - CD22
  - CD24
  - CD21

- **FITC**
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  - SMCD3

- **PE**
  - CD3
  - CD45
  - CD79a

- **PerCP-Cy5.5**
  - SMIgM
  - SMIgK

- **PE-Cy7**
  - APC
  - APC-H7

LAP markers

BCP-ALL panel

**Parameters**

- **PE-Cy5.5**
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  - SMCD3

- **PE**
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- **FITC**
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- **Pac Blue**
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  - CD34
  - CD19

- **Pac Orange**
  - CD58
  - CD66c

- **APC-H7**
  - APC

NORMAL BM B-CELL MATURATION vs REGENERATION

B-CELL MATURATION IN NORMAL BM

31 parameters
Fluorescence intensity

- Definition of which subpopulations to analyse
  - Usually blast cells
  - Less frequently: maturing neutrophils, monocytes, erythroid cells
- Define which markers/combinations of markers
- Define the myeloid lineages involved
- Define the phenotypic alterations to be considered:
  - Numerical changes
  - Fluorescence intensity
  - Asynchronous expression of maturation-associated markers

WHO CLASSIFICATION OF AML*

*SM-A9N2NID
**IMMUNOPHENOTYPIC CHARACTERIZATION OF MDS**

**HOW SIMILAR ARE NEOPLASTIC CELLS TO NORMAL CELLS?**
- Reflect cell lineage and maturation stage.

**IN WHAT DO NEOPLASTIC CELLS DIFFER FROM NORMAL CELLS?**
- Reflect derangement of protein expression (underlying genetic abnormalities and/or changes in the BM microenvironment?)

**WHO CLASSIFICATION OF AML**
AML with recurrent genetic abnormalities
AML with myelodysplasia-related changes

**AML NOS:**
- AML with minimal differentiation
- AML without maturation
- AML with maturation
- Acute myelomonocytic leukemia
- Acute myeloblastic leukemia
- Acute monoblastic leukemia
- Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis

Therapy-related myeloid neoplasms
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

**HEMATOPOIESIS**

Bone marrow
- Hematopoietic stem cell
- Lymphoid precursor
- Platelet
- Neutrophil
- Monocyte/Macrophage/Dendritic
- Eosinophil
- Mast cell
- Basophil
- Erythrocyte

Blood - tissues
- Dendritic cells
- Neutrophil
- Monocyte/Macrophage/Dendritic
- Eosinophil
- Mast cell
- Basophil
- Erythrocyte
- Platelets

**IMMUNOPHENOTYPIC IDENTIFICATION OF LINEAGE COMMITMENT OF CD34+ BM CELLS**

**Multi-tube EuroFlow classification panel for AML/MDS**

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**Multi-tube EuroFlow classification panel for AML/MDS (Part 2)**

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<td>CD34</td>
<td>CD117</td>
<td>CD36</td>
<td>CD15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Normal Maturation of CD34+ Neutrophil precursor cells in the BM

<table>
<thead>
<tr>
<th>STAGE I</th>
<th>STAGE II</th>
<th>STAGE III</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+</td>
<td>CD34+</td>
<td>CD34-</td>
</tr>
<tr>
<td>CD117+</td>
<td>CD117+</td>
<td>CD117-</td>
</tr>
<tr>
<td>HLADR+</td>
<td>HLADR-</td>
<td>HLADR+</td>
</tr>
<tr>
<td>CD105-</td>
<td>CD105+</td>
<td>CD105/-</td>
</tr>
<tr>
<td>CD71m</td>
<td>CD71a</td>
<td>CD71/lo</td>
</tr>
<tr>
<td>CD45m</td>
<td>CD45a</td>
<td>CD45-</td>
</tr>
<tr>
<td>MPO++</td>
<td>MPO++</td>
<td>MPO++</td>
</tr>
<tr>
<td>CD15/65</td>
<td>CD15/65-</td>
<td>CD15/65+</td>
</tr>
<tr>
<td>CD64-</td>
<td>CD64+</td>
<td>CD64-</td>
</tr>
</tbody>
</table>

HEMATOPOIETIC MATURATION IN NORMAL BM

<table>
<thead>
<tr>
<th>Normal BM</th>
<th>Gated CD34+ BM cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-SSC</td>
<td>CD45-PerCP</td>
</tr>
<tr>
<td>HLADR FITC</td>
<td>Erythroid DC</td>
</tr>
</tbody>
</table>

Normal Maturation of Erythroid Cells in the BM

<table>
<thead>
<tr>
<th>STAGE I</th>
<th>STAGE II</th>
<th>STAGE III</th>
<th>STAGE IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34-</td>
<td>CD34-</td>
<td>CD34+</td>
<td>CD34-</td>
</tr>
<tr>
<td>CD117+</td>
<td>CD117+</td>
<td>CD117-</td>
<td>CD117-</td>
</tr>
<tr>
<td>HLADR+</td>
<td>HLADR-</td>
<td>HLADR+</td>
<td>HLADR-</td>
</tr>
<tr>
<td>CD36lo</td>
<td>CD36+</td>
<td>CD36-</td>
<td>CD36-</td>
</tr>
<tr>
<td>CD105-</td>
<td>CD105+</td>
<td>CD105/-</td>
<td>CD105/-</td>
</tr>
<tr>
<td>CD71m</td>
<td>CD71a</td>
<td>CD71/lo</td>
<td>CD71/lo</td>
</tr>
<tr>
<td>CD45m</td>
<td>CD45a</td>
<td>CD45-</td>
<td>CD45-</td>
</tr>
<tr>
<td>MPO-</td>
<td>MPO-</td>
<td>MPO+</td>
<td>MPO+</td>
</tr>
<tr>
<td>CD15/65</td>
<td>CD15/65-</td>
<td>CD15/65+</td>
<td>CD15/65+</td>
</tr>
<tr>
<td>CD64-</td>
<td>CD64+</td>
<td>CD64-</td>
<td>CD64-</td>
</tr>
</tbody>
</table>

IMMUNOPHENOTYPIC IDENTIFICATION OF LINEAGE COMMITMENT OF CD34+ BM CELLS

<table>
<thead>
<tr>
<th>Monoctytic</th>
<th>pDC</th>
<th>Mast cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45 FITC</td>
<td>CD45 PE</td>
<td>HLA DR FITC</td>
</tr>
<tr>
<td>Erythroid</td>
<td>Eosinophil</td>
<td>Neutrophil</td>
</tr>
<tr>
<td>CD45 FITC</td>
<td>CD45 PE</td>
<td>CD117 FITC</td>
</tr>
</tbody>
</table>

IMMUNOPHENOTYPE OF CD34+ MYELOID-COMMITTED HPC

<table>
<thead>
<tr>
<th>CELL LINEAGE</th>
<th>SSC</th>
<th>IMMUNOPHENOTYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythroid</td>
<td>stable</td>
<td>CD36-, CD64-, CD45lo, CD105+</td>
</tr>
<tr>
<td>Megakaryocytic</td>
<td>high</td>
<td>CD61+, CD45lo</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>high</td>
<td>CyMPO+, CD13hi</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>high</td>
<td>CyMPO-, CD15/65+, CyEPO+</td>
</tr>
<tr>
<td>Basophil</td>
<td>low</td>
<td>CD123hi, HLADRlo, CD117lo, CD45hi, CD203c+</td>
</tr>
<tr>
<td>Monocytic</td>
<td>stable</td>
<td>CyMPO-, CD64+, DR+, CD117lo</td>
</tr>
<tr>
<td>Mast cell</td>
<td>low</td>
<td>CD117hi, HLADRlo, CD45hi</td>
</tr>
<tr>
<td>pDC</td>
<td>stable</td>
<td>CD123hi, HLADRlo, CD36+</td>
</tr>
</tbody>
</table>
Myeloid proliferations related to Down syndrome (DS):
- Transient abnormal myeloipoiesis
- Myeloid leukemia associated with DS
Blastic plasmacytoid dendritic cell neoplasm

*SM-AHRMD*
Monocytic maturation in normal BM: definition of maturation stages based on principal component analysis (PCA) of data on 10 parameters.

Antigen expression patterns during monocytic maturation in normal BM.
Erythroid maturation in normal BM: definition of maturation stages based on principal component analysis (PCA) of data on 10 parameters.

"DE NOVO" AML: ABERRANT PHENOTYPES & CLONAL HEMATOPOIESIS (n=68)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Polyclonal AML#</th>
<th>Clonal AML*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal phenotype</td>
<td>N=12</td>
<td>N=49</td>
</tr>
<tr>
<td>&lt;2 altered lineages</td>
<td>58%</td>
<td>2%</td>
</tr>
<tr>
<td>N. of altered lineages</td>
<td>0.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Total 12/61 (20%) 49/61 (80%)

- Two ISM-AML cases with KIT mutation restricted to mast cells
- Two cases shared coexistence of t(8;21) & D816V KIT mutation in all cellular compartments

WHO CLASSIFICATION OF AML

- AML with recurrent genetic abnormalities
  - AML with t(15;17)(q22;q22); RUNX1-RUNX1T1
  - AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
  - APL with t(15;17)(q22;q22); AML-SAR
  - AML with t(9;11)(p22;q23); MLT3-MKL
  - AML with t(9;22)(q21;q22); BCR-ABL
  - AML with t(9;21)(q22;q22); MUL-MUL
  - AML with mutated NPM1
  - AML with mutated CBFB

AL: GENOTYPIC-PHENOTYPIC ASSOCIATIONS

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Genetic lesion</th>
<th>Aberrant immunophenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCP-ALL</td>
<td>t(9;22)*</td>
<td>CD34+,CD10+,CD38+,CD13+</td>
</tr>
<tr>
<td></td>
<td>t(12;21)</td>
<td>CD34+,CD10+,CD20-,CD13+</td>
</tr>
<tr>
<td></td>
<td>11q23</td>
<td>CD34+,CD10+,7.1+,CD15+</td>
</tr>
<tr>
<td>AML</td>
<td>t(15;17)</td>
<td>CD34-,CD15-,CD2-,CD13+</td>
</tr>
<tr>
<td></td>
<td>inv(16)</td>
<td>MPO+,CD2-fl</td>
</tr>
<tr>
<td></td>
<td>t(8;21)</td>
<td>CD19-,CD56-</td>
</tr>
<tr>
<td></td>
<td>11q23</td>
<td>CD56-,7.1-,CD19-,CD2-</td>
</tr>
</tbody>
</table>

Bead-based flow cytometric assay for detection of fusion proteins

- FITC-conjugated anti-PML antibody
- Anti-RARA antibody

Preservation kit

beads coated with anti-RARA antibody

Cell lysate

FITC-conjugated anti-PML antibody

Beads coated with anti-RARA antibody

Kit for detection of fusion proteins
Results of PML–RARA fusion protein detection using the immunobead assay

At this moment the technical developments for 7 well-defined fusion proteins have (virtually) been completed:

- **CML:**
  - BCR-ABL: completed
  - BCR-ABL: RUO kit launched and published
- **Precursor-B-ALL:**
  - BCR-ABL: completed
  - TEL-AML: completed
  - E2A-PBX1: completed
  - MLL-AF4: completed
- **AML:**
  - AML1-ETO: completed
  - CBFB-MYH11: completed
  - PML-RARA: completed

Prototype testing completed

**Several examples:**

- **Core-factor tube**
- **Multiplex tube**
- **Precursor-B-ALL tube**

**MUCHAS GRACIAS**