CD34+ CELL ENUMERATION ON FROZEN SAMPLES: VIABILITY ASSESSMENT

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CELL PROCESSING: AUTO-BMT

• The CPL’s (cell processing lab) primary role in supporting an autologous transplant program is

• 1) to preserve cellular therapy product viability during storage

• 2) to prevent the introduction of microbial contamination at all stages of processing
MINIMAL MANIPULATION OF THE GRAFT: which methods can be used?

METHODS USED SHOULD NOT MODIFY CELL FUNCTION AND STRUCTURE

Standards for Haemopoietic Progenitor Cell Collection, Processing & Transplantation- JOINT ACCREDITATION OF EBMT AND ISCT: JACIE
• MOBILIZATION/ PBSC COLLECTION
CD34 MEASUREMENT BEFORE AND AFTER CRYOPRESERVATION,
• HSC MATURITY (CD34 subsets: 38neg,CD133+)
• CLONOGENIC POTENCY
• CELLULAR COMPOSITION (PMNs, Plts etc.)
• Collection procedure (blood volume processed, collection efficiency)
GRAFT QUALITY: ISSUES TO BE DEALING WITH

- **SCT**: NEUTROPHIL AND PLATELET ENGRAFTMENT;
- HOSPITALIZATION TIME,
- No. OF INFECTION DISORDERS, DAYS ON ANTIBIOTICS, number of transfusions of blood component (plts and/or RBC),
- IMMUNOLOGICAL RECONSTITUTION,
- ORGAN TOXICITY, SAE, EARLY DEATH,
- QOL ASSESSMENT,
- DISEASE RELAPSE.
Hierarchical pathway of hemopoiesis by colony assay

- SCID Repop cells
- CFU-B
- CFU-T
- LTC-IC
- CFU-Blast
- HPP-CFC
- Pre-CFC
- CFU-GEWM
- CFU-GM
- BFU-Mk
- BFU-E

CD34 Neg

CD34
HLA-DR
CD38
CD45RA
CD45R0
CD90
CD117-c-Kit-R
CD33
Repopulating Potential of Hematopoietic Stem Cells - Animal Models

Donor

Bone Marrow

Recipient

Stem Cells

Isolation

Purification

Lethal Irradiation

Transplantation

Secondary Transplantation
PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

REFERENCE PARAMETERS:

u **CD34+ CELLS**

u > 20 uL (mobilised blood)

u > 2-5 x 10^6/kg (graft)

u **CFU-GM**

 (> 6-8 x 10^4/kg)

Hematology Unit- Cremona
CD34+ CELL ABSOLUTE COUNTING: Technical aspects

RBC LYSIS, WASHING, FIXATION METHOD
DETECTION OF VIABLE CELLS
USE OF NEGATIVE AND POSITIVE CONTROLS
MULTIPARAMETRIC ANALYSIS: 2-3-4 COLOR ANALYSIS (CD45, CD34+ SUBSETS, 7-AAD)
SINGLE OR DUAL PLATFORM (hematology analyzer for absolute leukocyte count)
TYPE OF SAMPLE ANALYZED (BM, CB, APHERESIS, fresh, thawed, age of the sample)
ASSESSMENT OF THE PERFORMANCE OF THE FLOW CYTOMETER
INTERNAL AND EXTERNAL QUALITY CONTROL
Flow Cytometric Methods for CD34 Enumeration

- Milan → ISHAGE
- Single parameter → Multiparameter
  - CD34, CD45, 7-AAD
  - CD34 subsets
- Dual platform → Single Platform
- Automated methods → Abs counts
Sequential gating For True viable CD34+ Cells (ISHAGE GUIDELINES)*

- R1 = selection of leukocytes (CD45^{+} from dim to bright)
- R2 = selection of CD34^{+} cells among the leukocytes
- R3 = selection of CD45^{dim}, SSC^{low} HPC
- R4 = selection of FSC^{low} to intermediate HPC
- R8 = selection of apoptotic/dead cells: gate out applied to all dot-plots

Optimal Transplant Cell Dose (CD34+/kg)

- Probability of platelet recovery correlated with the number of CD34+ cells transplanted


- In a retrospective study, lack of full platelet recovery (>150 x 10^9/L) was associated with lower CD34+ cell doses
Patients (%) achieving ≥ 6 million CD34+ cells/kg by apheresis day (ITT population)

HR = 2.54, p < 0.0001

Kaplan-Meier estimate of proportion of patients reaching ≥ 6 × 10^6 CD34+ cells/kg

G-CSF + plerixafor (n = 148)

G-CSF + placebo (n = 154)

Individual Quality Assessment of Autografting by Probability Estimation for Clinical Endpoints: A Prospective Validation Study from the European Group for Blood and Marrow Transplantation

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Quality Assessment of Hematopoietic Stem Cell Graft Committee

Chairman: Lanza F (Cremona-Irly)
Secretary: Johnsen H. (Alborg)

Quality Assessment of Autografting: A prospective registration study

http://www.ebmt.org/7Directory/committees/qahscg.htm
Multivariate analysis

1. Pediatric patients resulted to have less toxicity (p=0.0001)
2. 1 or 2 apheresis (p=0.001) predicted a good outcome
3. Toxicity increased with higher CD34+ volume reinfused (>500ml) (p=0.002)
4. PBSC COLLECTION: CD34+ cells collected > 4 x 10^6/kg in one apheresis (AL excluded)
5. CD34+ cells infused > 5 x 10^6/kg
6. Patients who experienced toxicity had a poor quality transplant (p=0.0001)
Engraftment and blood cell recovery are not the only clinical end points in autografting.

### 8.1. Proposed graded clinical end points in quality assessment

<table>
<thead>
<tr>
<th>Objective</th>
<th>End point</th>
<th>Grading</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary: Efficacy</strong></td>
<td>Days on antibiotics, transfusion of blood components, days in hospital</td>
<td><strong>Favourable:</strong> = 7 days on antibiotics and no transfusions</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Intermediate:</strong> = 7 days on antibiotics and transfusions OR &gt; 7 days on antibiotics and no transfusions</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Unfavourable:</strong> &gt; 7 days on antibiotics and transfusions</td>
</tr>
<tr>
<td><strong>Secondary: Toxicity</strong></td>
<td>Days to ANC &gt;0.5 x 10^6/L and Platelets &gt;20 x 10^6/L</td>
<td><strong>Favourable:</strong> ANC and platelets recovery before 14 days</td>
</tr>
<tr>
<td></td>
<td>Other organ toxicity if appropriate</td>
<td><strong>Unfavourable:</strong> ANC or platelets recovery after 14 days</td>
</tr>
<tr>
<td><strong>Tertiary: Safety</strong></td>
<td>Death or disease recurrence</td>
<td><strong>Favourable:</strong> Alive and without disease progression after 12 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Unfavourable:</strong> Death or disease progression before 12 months</td>
</tr>
</tbody>
</table>
Engraftment and blood cell recovery are not the only clinical end points in autografting

- Primary end points should evaluate **efficacy**, i.e. health economic considerations, including **antibiotic administration**, **transfusion of blood components** and **time in hospital**

- Secondary end points should evaluate **toxicity**, in accordance with f.x. Common Toxicity Criteria (CTC), including **mucositis, enteritis** and **haematological toxicity**

- Tertiary end points should evaluate **safety**, i.e. the **risk of regimen related death** or **disease progression** within the first 3 months following graft reinfusion
EWGCCA-EUROGRAFT CD34 subsetting trial: 4 sendouts (37 centres)

Intra-site variation < 5% (CD90: 43-46%; CD133: 46-57%); Intersite variation (CD90: 37%- 28% CV; CD133: 39%- 31% CV)

<table>
<thead>
<tr>
<th>Staining</th>
<th>MEDIAN</th>
<th>10th percentile</th>
<th>90th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstained</td>
<td>2.1</td>
<td>0.61</td>
<td>7.15</td>
</tr>
<tr>
<td>CD90</td>
<td>42.1</td>
<td>20.6</td>
<td>53</td>
</tr>
<tr>
<td>CD133</td>
<td>61.2</td>
<td>22.8</td>
<td>75.5</td>
</tr>
</tbody>
</table>
CD34+ CELL ENUMERATION IN CRYOPRESERVED BAGS IMPLICATION FOR TRANSPLANTATION

0.5 x 10^6 WBC

20 min (RT)

SYTO-13 (FL1)
CD45-FITC (FL1)
CD34-PE (FL2)
7-AAD (FL3)

500 µL

CD34 (8G12 clone)
CD45 (IMMU 19.2 clone)
SYTO13 (50 nM final concentration)
7-AAD (2µg/mL)

MACSQuant (Miltenyi)
Advantages of the new methodology

- Staining of intact live cells
- Immunophenotyping of whole blood after thawing
- Erythrocyte discrimination by SYTO-13 + CD45-FITC staining
- Avoids lysis, washing and centrifugation steps
- No cell depletion
- Manufacturer-independent technology
Significant numbers of CD34+AnnV+ events were found within the 7AAD-gated population.

AnnV assessed CD34 dose predicts CFUs, especially after thawing.

The method complements the standard enumeration and returns a more qualitative evaluation of the units.
CD34 CELL COUNTING IN CORD BLOOD UNITS

- In UCBT, CD34+ cells count has not been as successful as for PBSC transplant in predicting the engraftment as a single parameter.
- CD34 + cells counting is well standardized on fresh and its inter-laboratory reproducibility was tested also on CBU.
- However flow-cytometry analysis of thawed CB samples requires an adaptation of both the acquisition setting and the gating strategy, with reference to the standard technique (Flores et Al. 2009; Scaradavou et Al. 2010).
- There is room for an improvement and standardization of CD34+ cells counting of thawed CBU samples.
The definition of “High Quality Units” is generally referred to large size units, containing a high number of haematopoietic progenitors to ensure faster engraftment.

However the concept of Quality in CB Banking is more in general referred to the consistency of the CBU data reported by banks to the registries.

Discrepancies between CBU data as reported by the bank and at transplant center have been reported, possibly affecting the selection of the most suitable CBU and therefore clinical outcomes.
Neutrophil recovery after single UCBT for patients with malignant disorders after myeloablative conditioning regimen

HLA 6/6 (n=150) 90%
HLA 5/6 (n=686) 88%
HLA 4/6 (n=730) 86%
HLA 3/6 (n=87) 74%

CD34 infused < 1.5 x10⁵/kg (n=557) 86%
CD34 infused > 1.5 x10⁵/kg (n=607) 90%
P<0.0001
Assessment of cord blood unit characteristics on the day of transplant: comparison with data issued by cord blood banks

Eric Wagner, Michel Duval, Jean-Hugues Dalle, Hugo Morin, Sonia Bizier, Josette Champagne, and Martin A. Champagne

In some cases, CB measures known to be predictive of engraftment were found much lower than reported by CBBs. Important differences may have accounted for the lack of neutrophil engraftment.
ASSESSMENT OF CD34+ CELL COUNT IN THAWED CORD BLOOD UNITS

Steering Committee:

- Riccardo Saccardi, Eurocord, Paris, Careggi Hospital, Florence
- Vanderson Rocha, Eurocord, Paris
- Annalisa Ruggeri, Eurocord, Paris
- Francesco Lanza, ISCT, Cremona
- Sergio Querol, Barcelona, London
- Gesine Koegler, Dusseldorf
- Jerome Larghero, Paris
- Etienne Baudoux, Netcord, Liege
- Eliane Gluckman, Eurocord, WMDA, Paris
CD34+ counting on thawed CBU: objectives

- Main objective:
  - generate a consensus on processing and Flow Cytometry assessment of CD34+ cells in thawed CBU samples

- Secondary objectives:
  - to validate the protocol through a multiple-laboratory exercise
  - To assess the impact of the protocol in determining the feasibility of the attached segment
OBJECTIVES

- The main objective of this study is to determine whether there is a “Cord Blood Banking Effect” by evaluating the impact of variables related to Cord Blood Banking procedures on the clinical outcomes.
- 100 days non relapse mortality is the primary endpoint.
- Other secondary outcomes are: myeloid engraftment (PMN and Plt recovery), acute GVHD, chronic GVHD and overall survival.
- Data concerning the CBUs used for the transplants will be collected from the Banks through a specific questionnaire.
### CD34+ counting on thawed CBU

<table>
<thead>
<tr>
<th>City</th>
<th>Flow Cytometer</th>
<th>Beads</th>
<th>MoAbs</th>
<th>Solution</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barcelona</td>
<td>EPICS XL</td>
<td>FLOWCOUNT</td>
<td>CD34(PE); CD45(FITC); 7AAD annexin tube: CD34(PE), CD45(ECD)</td>
<td>NYCBB</td>
<td>1:1 /1:3 /1:10</td>
</tr>
<tr>
<td>Cremona</td>
<td>FACS CANTO</td>
<td>TruCOUNT</td>
<td>CD34(PE); CD45(FITC); 7AAD</td>
<td>NYCBB</td>
<td>1:3</td>
</tr>
<tr>
<td>Dusseldorf (old)</td>
<td>EPICS XL</td>
<td>FLOWCOUNT</td>
<td>CD34(PE) and CD45(FITC) + 7AAD</td>
<td>NYCBB</td>
<td>1:1</td>
</tr>
<tr>
<td>Dusseldorf (new)</td>
<td>FACS CANTO</td>
<td>TruCOUNT</td>
<td>CD34(PE) and CD45(FITC) + 7AAD</td>
<td>NYCBB</td>
<td>1:1 /1:3 /1:10</td>
</tr>
<tr>
<td>Firenze</td>
<td>FACS CALIBUR</td>
<td>TruCOUNT</td>
<td>CD34(PE); CD45(FITC); 7AAD</td>
<td>NYCBB</td>
<td>1:3</td>
</tr>
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<td>Liege</td>
<td>FACS CALIBUR</td>
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<td>CD34(PE); CD45(FITC); 7AAD annexin tube: CD34(PE), CD45(ECD)</td>
<td>NYCBB</td>
<td>1:1 /1:3 /1:10</td>
</tr>
<tr>
<td>Nottingham</td>
<td>FACS CALIBUR</td>
<td>TruCOUNT</td>
<td>CD34(PE); CD45(FITC); 7AAD</td>
<td>Dext/ABS/PBS</td>
<td>1:1</td>
</tr>
<tr>
<td>Paris</td>
<td>FC500</td>
<td>FLOWCOUNT</td>
<td>Stem-kit reagents</td>
<td>Albumin 5%</td>
<td>1:5</td>
</tr>
</tbody>
</table>

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**Becton Dickinson**  **Beckman Coulter**

**Eurocord - International Registry on Cord Blood Transplantation**
Cord blood: 4 European centres

Post-thaw CD34+ Recovery

- ISHAGE: 75.8%
- Modified ISHAGE: 84.3%
ASSESSMENT OF CRYOPRESERVED AND THAWED PBSC

• A prospective, observational trial is being performed in 4 European Centers to assess the reproducibility and feasibility of a fully automated method for washing thawed cellular products.

• The pre-clinical phase has been finalized

• The clinical phase (10 consecutive auto-HSCT/Center) has been done and data analysed
Fig 3. In the pre-clinical study 20/40 samples showed an after thawing recovery of viable CD34+ cells lower than 70%. In all of them the washing procedure resulted in an improvement of the recovery ranging between 5 and 61%.
FULLY AUTOMATED WASHING OF CRYOPRESERVED PBSC IN A MULTICENTER STUDY

Pre-clinical Trial: Viable CD34+ recovery

Time after washing (hours)
CD34 + cells counting is well standardized on fresh specimens and its inter-laboratory reproducibility has been tested in various samples (PB, PBSC, CB, BM).

CD34, CD45 and 7-AAD allow an easy identification of lived progenitor cells, but either necrotic and apoptic cells are not recognizable using this method in most of the thawed cells.

The flow-cytometry analysis of thawed PBSC and CB samples requires an adaptation of both the acquisition setting and the gating strategy, with reference to the standard technique. This hypothesis has been tested and validated in a large number of specimens from various sources.
CD34+ ASSESSMENT IN THAWED CBU: SUMMARY

- Standardization of sample processing, acquisition and gating strategy resulted in a satisfactory level of reproducibility across different European labs in thawed CBU.

- In thawed samples, FC standard ISHAGE method lead to an overestimation of viability of thawed CD34+ cells. A new FC methodology is therefore proposed.

- If validated, this methodology might improve the reliability of quality controls on stored CBU and reduce discrepancies between CD34+ counting carried out at Banks and Transplant Units, respectively.
THANKS TO

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- Di Stefano R

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