CD34 numeration through the French external quality control: Impact in the implementation of new technical chapters at the European Pharmacopoeia

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26/04/14
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Summary

- General missions of ANSM
- External quality control of cell therapy products
- Method Validation
- CD34+ numeration results
  - Through the External Quality Control campaign
  - Through a Cord Blood Multicentric Study
- Monograph proposal at the European Pharmacopoeia
  - « Cell Therapy Products » Working group
  - 2.7.23 technical chapter for CD34+ cell numeration
- Conclusion
General Presentation of ANSM

- Public establishment **on the authority** of the Ministry of health
- Resumption of the missions, responsibilities, and competences exercised by the AFSSAPS
- Introduction of new functions → **consolidation of the health system**

**December 29th 2011 law**
Related to the reinforcement of the drug and health product safety

**ANSM Creation**

**Implementation 2012, the 1st of May**

**New organization, 2012, the 1st of October**
ANSM Missions

- Participation in the development of "... laws and regulations in the manufacture, preparation, conservation or utilization (...)". health products for human. "(Articles L.5311 - 1 and L.5311 - 2 of the Code of Public Health)

- Health Safety Mission
  - Assessment
  - Inspection
  - Information
  - Control

Laboratories Control Division (CTROL)

French « Official Medicines Control Laboratories » (OMCL)
Cells and CTP

Subsequent product regulatory

First grafts of bone marrow in the 60’s

1st law of bioethic (1994)

July 1998 Law
Afssaps creation = competent authority
Organs, tissues, cells = health products

October 2001 decree and February 2003 order

September 2008 decree and October 2011 order

Etablissement Authorisation

Products Authorisation
after preparation process and indication evaluation
(quality, efficacy and safety)

29 December 2011 law ANSM Creation
Controls by ANSM Laboratories

- Within national program: External quality control (until 2013) and dedicated studies

- Emergencies following notification of an incident or accident related to a product:
  - An alert
  - A biovigilance notification
  - A request from assessment department: scope of evaluation file
External Quality Control

- 2 to 3 checks per year until 2012,

- 33 sites of production in 2014 for Hematopoietic Stem Cells (HSC)
  - Participation rate: 75 to 95% according to the round
  - Sending of more than 1600 samples of HSC since October 1999
  - Analyses carried out in parallel by the producers and sending of the results to the ANSM for comparison
Nature of Controlled Products

Preponderance of the haematopoietic products and their therapeutic use (most frequently for haematopoietic reconstitution in the case of malignant diseases like lymphoma, myeloma or leukemia):

- Peripheral Blood Stem Cells (PBSC) after mobilization in an autologous or allogeneic context
- Bone marrow
- Umbilical cord blood cells
- Donor lymphocytes

For all these products, the contents in cells of interest varies according to various parameters (origin, pathology, collection etc...)
CD34+ Cell Numeration

- **Principle**
  Labelling of CD34 and CD45 membrane antigens by direct immunofluorescence and analysis of this labelling by flow cytometry.

This analysis is based on the following criteria: «true CD34 + cells express the CD34 antigen, weakly express the CD45 antigen and have a low SS (Side Scatter) with a low to intermediate FS (Forward-Scatter) characteristic of progenitor cells». From «The ISHAGE guidelines for CD34+ cell determination by flow cytometry». Sutherland et al (1996), J. Hematotherapy, 5:213-226

These characteristics allow to define a region containing true CD34 + cells to count.


See also the 2.7.23 chapter of European Pharmacopoeia
CD34 enumeration – Validation and Quality Assurance

◆ Study of repeatability, reproducibility, linearity, robustness, for fresh and thawed products in the presence of 7-AAD.

◆ Autostandardization System before analysis
  ● PMT adjustment (FlowSet fluorospheres)
  ● Fluorescence compensation
  ● Analysis system checked with StemTrol (a CD34+ calibrated cell line)

**Mean = 1228/µl +/- 5% (n=38)**

**StemTrol expected value = 1250/µl +/- 15%**
CD34 enumeration – Validation Stage

Linearity of StemKit in the presence of 7-AAD for a fresh leukapheresis product at 120,000 cells/µl and 0.83% CD34+

\[ y = 1.13x - 5.39 \]

\[ R^2 = 0.989 \]
EXAMPLE OF A CD34 ANALYSIS OF A LEUKAPHERESIS

CD34+ number/µl =
(mean CD34 events in D / number of beads events) x beads concentration (nb/µl)

Autologous PBSC with 708 viable CD34+ /µl (0.48%), total viability 96.9% with 7-AAD.

CD34.10⁶/kg = 708/µl x bag volume (µl)/ patient weight
Follow-up of differences for the CD34 numeration between Producers and ANSM from 2000 to 2013

Significant difference between the mean gap observed in 2000 and those observed in 2012-2013 (p<0.001)
Follow-up of external quality control centre by centre

Distribution of the differences between the producer and ANSM for each controlled PBSC. Variation ≤ to 20% is considered to be acceptable.

Distribution of the average differences obtained by each centre and for each analysis. For the CD34 analysis, the average of these differences is 13.7%. The variations are most important for the CFU – GM functional assay.

Useful data to the process/product files evaluated by the Commission of Cellular Therapy.

Follow-up of the quality in inter-laboratory conditions as a technical validation tool.
## Differences according to the method used by the producer - Superiority of the single-platform method

<table>
<thead>
<tr>
<th>Products</th>
<th>CD34 Mean Difference between Producers and ANSM (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Double-Platform</td>
<td>Single-Platform</td>
</tr>
<tr>
<td>PBSC</td>
<td>16.4 ± 13.1 (n=393)</td>
<td>12.9 ± 9.7 (n=386)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>19.6 ± 16.6 (n=63)</td>
<td>13.3 ± 10.6 (n=54)</td>
</tr>
<tr>
<td>Thawed PBSC (Viable CD34+/Prod)</td>
<td>27.5 ± 21.7 (n=87)</td>
<td>22.3 ± 16.5 (n=55)</td>
</tr>
<tr>
<td>(Total CD34+/Prod)</td>
<td>21.2 ± 15 (n=69)</td>
<td>14.7 ± 10.9 (n=36)</td>
</tr>
<tr>
<td>Producer Recovery for CD34+ cells (%)</td>
<td>90.8 ± 28.6 (n=24)</td>
<td>60.5 ± 23.1 (n=36)</td>
</tr>
</tbody>
</table>
EXAMPLE OF A CD34 ANALYSIS OF A BONE MARROW

A) Gating of total CD34

B) Gating tightened on the bright CD34+ cells

RESULTS: With A) 88 viable CD34+/µl, with B) 60 viable CD34+/µl leading to a 32% difference.
Practices investigation of the 34 sites concerning the gating – september 2012

Producer answers for 5 bone marrow examples

- A: Total CD34+ cell gate
- B: Bright CD34+ cell gate

- A; 63%
- B; 18%
- A et B; 14%
- ND; 5%
EXAMPLE OF A CD34 ANALYSIS OF A THAWED PLACENTAL BLOOD

With 7-AAD

12 /µl viable CD34+ cells

Without 7-AAD

15 /µl total CD34+ cells

CD34+ cell viability = 80% whereas Total CD45+ cell viability = 42.5%
7-AAD Toxicity? Or damaged products?

- For 132 thawed PBSC, difference analysis of viability between assay and negative control:
  - Mean Difference: 2.8 ± 3.4 (-1.3 to 16.4)
  - 16.7% with a difference higher than 5 pts, mean in this group: 8.55 ± 3.6

- These PBSC are related to a mean difference with the producer of 44.4% ± 22 for viable CD34+ cells

- And related to a weaker CFU-GM cloning efficacy (6.5% ± 3.6) for 57% of these or for 33% to no growth in culture or a NS result.
Cord Blood Multicentric Study

- More and more cell banks have to thaw placental blood, but few benchmarks as to the reliability for the CD34+ cell number: normal low value or technical problem?

- Use of downgraded Cord Blood Units
  - 14 participants: Thawing by the cell bank and sent to the ANSM
  - CD34 result comparison and recovery analysis according to the flow cytometry method.

Panterne et al, Transfusion Clinique et Biologique 17 (2010) 41-46
CD34 Analysis in thawed umbilical cord blood

<table>
<thead>
<tr>
<th></th>
<th>All methods (n=48)</th>
<th>Double-platform method (n=15)</th>
<th>Single-platform method (n=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34 gap</td>
<td>29 ± 23</td>
<td>47 ± 25</td>
<td>21 ± 16</td>
</tr>
<tr>
<td>Cell banks/ANSM (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34 Recovery on the thawing site (%)</td>
<td>82 ± 60</td>
<td>126 ± 90</td>
<td>62 ± 20</td>
</tr>
</tbody>
</table>

Significant Difference between SP and DP methods, p<0.05

Low dispersion for the SP method, increased dispersion with the DP one
Weak correlation, decision on the TNC number can lead to a poor cord blood unit for its CD34+ content.
CD34+ Correlation before cryopreservation and after thawing

A single-platform method was used before cryopreservation and after thawing for CD34+ cell determination, $R^2=0.75$

Single or dual-platform method was used by participant sites at thawing for the CD34+ cell determination, $R^2=0.46$
Practice Evolution of the 33 French cell banks for the CD34+ cell numeration

% of Single-Platform Users

% 100
90
80
70
60
50
40
30
20
10
0

Year

0 10 20 30 40 50 60 70 80 90 100
EUROPEAN CTP WORKING GROUP

- Standardization work undertaken by the European Pharmacopoeia on the initiative of France and on the basis of several years of external control for CTP.

- 6 monograph projects of which 4 have been prepared by ANSM were adopted by the European Pharmacopoeia

  - **With implementation to the 1/01/07**
    - Human haematopoietic stem cells (23.23)
    - **Numeration of CD34+/CD45+ cells in haematopoietic products (2.7.23)**
    - Flow cytometry (2.7.24)
    - Microbiological control of cellular products (2.6.27)

  - **With implementation to the 1/01/08**
    - Colony-forming cell assay for human haematopoietic progenitor cells (2.7.28)
    - Nucleated cell count and viability (2.7.29)
Monograph Proposals: Who is doing the demand?

ANSM
- French Pharmacopoeia Committees
- French Manufacturers
- National Pharmacopoeia authorities at NORSTA Department

EDQM
- Others: Individuals, Administrations, Associations...
- EDQM Working groups ie CTP group
- Non European Manufacturers
- Others: Administration, Associations...

European Commission of Pharmacopoeia

National Authorities

European Manufacturers
Monograph development process at the French and European Pharmacopoeia

5 French Pharmacopoeia Committees
- Draft proposals

- ANSM Laboratories
- ANSM Internal expertise

Public Survey (NTPP): publication of texts in the Official Journal
- Information of the EDQM and the other EU States

ANSM
- Text Adoption

Publication in the French Pharmacopoeia

European Commission of Pharmacopoeia
- Text Adoption

Publication in the European Pharmacopoeia

Working groups through European Pharmacopoeia
- Draft proposals

- ANSM Laboratories
- ANSM Internal expertise

Public inquiry: publication of the texts in Pharmeuropa

ANSM Laboratories
- ANSM Internal expertise

ANSM Laboratories
- ANSM Internal expertise
2.7.23. NUMERATION OF CD34/CD45+ CELLS IN HAEMATOPOIETIC PRODUCTS

This chapter describes immunolabelling and analysis by flow cytometry (2.7.24) to determine the number of CD34/CD45+ cells contained in haematopoietic products. The determination is carried out by a single platform method using calibrated fluorospheres, after lysis of the sample red blood cells if necessary.

This method applies to all types of preparations and whole blood. However, its level of precision makes it particularly suitable for preparations containing very low percentages of CD34/CD45+ cells.

Graft quality assessment by CD34/CD45+ cell enumeration

A variety of studies have established that the 1-3 per cent of cells in the bone marrow that express the CD34 cell surface antigen are capable of reconstituting long-term, multilineage haematopoiesis after myeloablative therapy. CD34/CD45+ cells are also found in the peripheral circulation of normal individuals but are extremely rare (0.01-0.1 per cent). However, CD34/CD45+ cells may also be mobilised from marrow to the peripheral circulation in greater numbers by haematopoietic cytokines such as granulocyte colony-stimulating factor and/or chemotherapy.

The technique used for enumeration of CD34/CD45+ cells must meet the following requirements:

- high sensitivity, since haematopoietic stem cells are rare events;
- accuracy, to provide clinically relevant results;
- reproducibility, to provide clinically reliable results;
- speed, to provide real-time analysis.
E. P. 2.7.23 main recommendations

- Use of a single-platform method (with calibrated fluorospheres) to determine the absolute count of CD34+/CD45+ cells

- Use of a viability marker at least for old specimens (more than 24H) and for thawed products

- Choice of antibody i.e. class III for CD34 labelling, conjugated to the brightest fluorochrome for rare events.

- Gating strategy:
  - commercial kit, apply the gating recommended by the manufacturer (dedicated software available)
  - In-house assay, apply a currently recommended strategy

- Autostandardization for settings of PMT and compensation, system checking by a control preparation (i.e. a positive calibrated cell line)
CD34+ Cell Numeration – Conclusion

- To make reliable CD34 value for the graft is also to allow the follow-up of the cellular outputs of the production systems

Follow-up of the variations centre by centre with the form of control charts

Use of these charts by the producers in the files of requests for authorization of process/product

- Robust technical tools with single-platform methods
- Low CD34 recovery allows alert: loss of quality
- Interest of viable CD34+ cell determination at thawing

Reinforcement of the data robustness
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