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Ten Years of External Quality Control for Cellular Therapy Products in France

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1. Introduction

The French Health Products Agency (Afssaps) was created in 1998 within a national context of strengthened health monitoring and control. The Afssaps evaluates the safety, the efficacy and the quality of health products. The law 96-452 of May, 28th 1996 and the law 98-535 of July 1st 1998, published in the "Public Health Regulation" have established rules for the use of cell therapy products. The Laboratories and Controls Department (DLC) is in charge of external quality control of cell therapy products since 1999. In this French regular context and because haematopoietic cells are the main cellular products used in routine in Europe (Gratwohl et al., 2006) as they are in the US (Read & Sullivan, 2004), control was first developed for these. Haematopoietic stem cells are populations of primitive multipotent cells capable of self-renewal as well as differentiation and maturation into all haematopoietic lineages. They are found in small numbers in the bone marrow, in the mononuclear cell fraction of circulating blood (peripheral blood stem cells or PBSC) and in umbilical cord blood (umbilical or placental stem cells). For the twenty-five last years, important progresses have been obtained in the treatment of onco-hematology diseases, especially with the advent of therapeutic intensification following by a graft of haematopoietic stem cells (Copelan, 2006). The infused haematopoietic progenitor cell populations can originate from the recipient (autologous) or from another individual (allogeneic) but until now, most of the grafts are made in an autologous context. Also, 85% of the PBSC samples received for the control at Afssaps had an autologous status. Considering peripheral blood stem cells, the major criteria retained for clinical decision is the positive cell number for the CD34 antigen (Bender et al., 1993; Allan et al., 2002; Weaver et al., 1995). However, as there is no target value for CD34+ cells according to the nature of the product, there was a strong need for efficient methods to guarantee the CD34 value and thus make reliable the clinical decision. Numeration by flow cytometry method is currently

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the most sensitive and rapid one, but variations due to the different techniques yet persist. For the clinical studies and the therapeutic use of these cell products, a standardization of the methods was undertaken with the assistance of learned societies. Recommendations for the CD34+ cell numeration were published in 1996 (Sutherland et al., 1996) followed by their adaptation to the single- platform (SP) methods (Keeney et al., 1998). Then, different teams using this methodology provided data showing the strong reliability of CD34+ evaluation (Barnett et al., 2000 ; Gratama et al., 2003 ; Chang et al., 2004). However, double-platform method is still used to evaluate CD34+ cell quantity in haematopoietic graft products (Laroche et al., 2005; Timeus et al., 2006; Scaradavou et al., 2010).

Till now, one of the main criteria of survival after a cord blood allograft is the number of nucleated cells able to ensure the haematopoietic reconstitution (Gluckman et al., 1997). Also, the activity and the development of the "Réseau Français du Sang Placentaire" (RFSP) were done with strict criteria of quality where the number of total nucleated cells contained in a graft must be higher than $2.5 \times 10^7/\text{kg}$ (Rubinstein et al., 1998) even $3.5 \cdot 10^7/\text{kg}$ at that time (Grewal et al., 2003). This criterion and its corollary (the initial volume of the graft must be higher than 80 ml) result in a therapeutic use of only 30 to 40% of collected umbilical cord blood. However, the amount of CD34+ seems increasingly important in the choice of the Cord Blood Unit (CBU). Since 2000, it is shown that the contents in progenitors (CFC) are a better indicator than the number of nucleated cells (Migliaccio et al., 2000). It was then established that an amount of CD34+ lower than $1.7 \cdot 10^5/\text{kg}$ was associated with a lower rate of engraftment and that the negative effect of a HLA mismatch (to the maximum 2) can, at least partially, be compensated by a CD34+ amount higher than this threshold (Wagner et al., 2002). Currently, if CD34 numeration of PBSC is validated by an international multicentric study (Gratama et al., 2003), and the number of viable CD34+ after thawing is associated with engraftment (Allan et al., 2002; Lee et al., 2008), recent inter-laboratory studies show that there exists still a great variability of results for placental bloods (Moroff et al., 2006; Brand et al., 2008). Other parameters like cell viability are able to inform about thawing yield and more to predict if this operation would be optimal. In so doing, we particularly focused on product composition as the granulated cell fraction containing polynuclear neutrophils. An analysis of this parameter measured in the product after leukapheresis showed an unexpected influence on cell viability. For several years, it has been shown that PBSC could be preserved for a 24H before freezing (Beaujean et al., 1996) as for CBU (Campos et al., 1995), and confirmed by Calmels et al (Calmels et al., 2007). However, Moroff and his collaborators have shown that this time preservation could be extended to 3 days before freezing (Moroff et al., 2004). Nowadays, a 24H storage before cryopreservation is a well established process. However, cell quality at thawing is not still homogeneous according to the product and, other parameters in addition to storage conditions should be studied to try to prevent loss of quality.

Another important tool is the standardization of bacteriological control under the impulse of the work carried out by Afssaps. The investigation on practices in French cell banks, which has taken place in 1998 to implement the external control of cell therapy products, contrary to the measurement of CD34, showed a very large heterogeneity of the practices for the microbiological control. There was sometimes the use of not adapted techniques probably leading to an under-detection of contaminated products by bacteria or fungi. We initially had a transposition of the methods used for infectious samples to the products of cellular therapy. The first ones do not have a limitation of volume, of germ concentration and a positive result is generally obtained within 24 to 48H. This is completely different for a cell therapy product where volume available is very small, and the concentration of germs, if

there are, very low. At the time of implementation, based growth methods with rich medium as those for blood culture were used for the control of labile blood products (Brecher et al., 2001; McDonald et al., 2001; Dunne et al., 2005) as for bone marrow control (Schwella et al., 1998). Taking together, it was decided to evaluate an automated blood culture system for the microbiological control of cell therapy products. The work led at Afssaps with the active participation of French cell banks allowed us to develop specific recommendations for this control which has given the basis for the European Pharmacopoeia chapter 2.6.27 applied since 2007.

As a consequence of the French regulation for cell therapy products, an external quality control of haematopoietic stem cells (HSC) was implemented since 1999, based on an investigation for practices, a pilot and a feasibility study with all the French cellular therapy (CT) facilities and on collaborative studies for the bacteriological control. All the French CT facilities have participated to this control and it is now a robust tool used in order to check product specifications. Nowadays, external quality control takes place 2 to 3 times a year as a survey market where each French CT facility sends samples from graft product towards Afssaps. This chapter describes the main results obtained during the past 10 years since the implementation of the external quality control in 2000. These results are going to focus on the assessment site by site leading to a personalised follow-up, on the standardization of the CD34+ cell numeration, on the identification of parameters which could influence cell therapy product quality and on the standardization of the microbiological control for haematopoietic products.

2. Materials and methods

2.1 Materials

To organize external quality control for cell therapy products by the competent authority in France, facilities and technical assays have been implemented in 1998 in the Blood Products and Cell Therapy Unit at the Laboratories and Control Department (DLC), Afssaps in quality system management using the ISO17025 norma.

2.1.1 Cell samples for external quality control

Currently, 31 sites in France prepare haematopoietic products for grafts indicated in haematological diseases. After a pilot study to validate transportation conditions and technical procedures, these sites send samples of haematopoietic products according to the scheme designed by the Blood and Cell Therapy Products Control Unit at DLC, Afssaps. A procedure for the logistic and transportation has been defined with a transportation temperature from 4 to 12°C and a transfer from sites inferior to 24H (mean time is 16 hours). Each sample received is immediately analyzed for nucleated and CD34+ cell count, viability, CFU-GM progenitor and a microbiological control is performed using an automated blood culture system. In parallel, producers make the usual controls and send their results to Afssaps for comparison. In this context, 92% of the products sent to Afssaps were peripheral blood stem cells (PBSC); others were from bone marrow or from umbilical cord blood.

2.1.2 Cord blood units (CBU) for a multicentric study

Forty-two displaced units obtained from the AICT (Activity of Cellular and Tissue Engineering) at the French Blood Establishment in Besancon were sent by transport in nitrogen vapours to the 14 cellular therapy facilities participating to this study (gathered

according to their localization). Each site received a non reduced in volume CBU and 2 miniaturized CBU. They have then to defrost these CBU the day chosen in dialogue with Afssaps. The thawing was carried out according to the usual processes on the sites. After taking away for control, the remainder of the defrosted unit was sent towards Afssaps in the transfer bag at 4-12°C transportation according to the procedure used within the framework of external control. At the receipt, the temperature, the conditioning and the aspect of the CBU were controlled and usual analyses as described below were performed.

2.1.3 Collaborative studies for microbiological control

Contaminated cell samples were prepared at the DLC. Cellular materials were obtained from the French Blood Establishment according to a contract, they generally consisted in residual buffy-coats or thawed mononuclear cells. Bacterial strains were prepared by standard practices and sowed in cell suspension at a determined concentration. A panel of samples was sent to each participant who performed the analysis according to its own procedure. Each kind of sample is also controlled by Afssaps after different times of preservation at 5±3°C to cover the delay of transportation and the beginning of the analysis for the different participants. Results are collected and analyzed by Afssaps. Participants are the usual laboratories who perform the microbiological control of cellular products for the French cell therapy producers. One study takes place per year with at least 32 participants.

2.2 Techniques used at Afssaps

2.2.1 Nucleated cell numeration

Nucleated cell counts were measured with an automated cell counter (MaxM, Beckman Coulter, Miami, FL) validated for leukapheresis products. In particular, it is necessary to check the linearity of counts because of the necessary dilution of leukapheresis products for most cases. Here, the nucleated cell count was used to define the working dilution for the StemKit procedure by flow cytometry.

2.2.2 Viability

Viability was determined by flow cytometry using a nucleic acid intercalating agent, 7-AAD (7-aminoactinomycine D). The percentage of 7-AAD positive cells was based on the use of the single-platform ISHAGE gating strategy developed by Keeney and Sutherland (Keeney et al., 1998) and determined during the CD34/CD45 labeling using StemKit methodology (Beckman Coulter, Miami, FL). The use of 7-AAD permits to check viable CD34+ cells especially for thawed products (Brocklebank & Sparrow, 2001).

2.2.3 CD34+ cell numeration

CD34/CD45 labelling using StemKit in the presence of StemCount fluorospheres to determine directly the CD34+ absolute count by flow cytometry and according to the ISHAGE guidelines (Sutherland et al., 1996). Quickly, 20µl of a mix of CD45-FITC and CD34-PE antibodies and 20µl of 7-AAD (final concentration: 1µg/mL) were added to 100 µl of a cell suspension containing 15,000 to 30,000 cells/µl and incubated for 20 minutes at room temperature, in the dark. Lysis of red blood cells was then performed using chloride ammonium 1x (10x solution provided with StemKit) during an incubation of ten minutes at room temperature and in the dark. Then, 100 µl of FlowCount calibrated fluorospheres (Beckman Coulter) were added to allow the determination of the absolute CD34+ cell value.

Acquisition of sample tubes was performed according to the protocol designed by Keeney and Sutherland (Keeney et al., 1998).

2.2.4 CFU-GM evaluation

A clonogenic assay was performed using ready-to-use semi-solid media from StemCell Technologies, Vancouver, Canada and from Stem alpha, St-Genis l'Argentière, France. Several references of methylcellulose medium were used due to the external quality control context. Each sample of PBSC received at Afssaps (in mean 16h after the collection) was analyzed with the reference of medium used by the producer as much as possible to allow a better comparison of results. Samples were plated in duplicate in 35-mm culture dishes at a concentration targeted to 200 to 300 viable CD34+ cells/ml. The dishes were incubated at 37°C in a humidified 5% CO₂ incubator for 14 days. CFU-GM colonies were scored at the day 14 using an inverted microscope. The CFU-GM clonogenicity (number of CFU-GM/100 CD34+ cells) was determined for each sample.

2.2.5 Estimation of the granulated fraction

We have analyzed 271 fresh PBSC samples to study the influence of granulated cell fraction on the cell viability. The percentage of granulated cells was defined in a SSC/CD45 region during the CD45/CD34 procedure. This determination can be supplemented by a CD45/CD14/CD15 labelling (antibodies from Beckman Coulter) when this population is dense and in continuity with the monocytic population for more precision. The linear regression analysis between this CD15+ fraction and the granulated cell region obtained on a SSC/CD45 graph gave a correlation coefficient R² equal to 0.97 and this correlation was significant (p<0.01) between these 2 groups.

2.2.6 Bacteriological and fungal control

Automated growth-based method during 10 days on aerobic and anaerobic media (BactAlert system, BioMérieux). In case of positive controls, germs are identified on a Vitek 2 Compact automated system (BioMérieux) and an antibiogram is realized by the same apparatus with dedicated cards.

2.3 Techniques used by French producers

Techniques	Producers (at the time of the last survey in 2009)
Nucleated cell numeration	Automatic counting by an haematology automate
Viability	7-AAD for 25 sites, Trypan Blue for 3 sites and no viability determination before preservation for 2 sites
CD34+ cell numeration	Single platform method for 22 sites (StemKit : 16 sites, Procount or SCE : 5 sites, CD34 Count Kit : 1 site) and double-platform for 8 sites.
CFU-GM clonogenic assay	23 sites use a medium from StemCell Technologies and 6 from Stem alpha. One site does not perform this assay at this step For 12 of them (41%), they use a medium without Epo.

Bacteriological and fungal control	90% of the French sites implied in the cell therapy product preparation use an automated growth-based system (Bactec or BactAlert) with an incubation ranging from 7 to 10 days.
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2.4 Statistical analysis

Means and standard deviations have been determined for each sample group with Excel 2003 software. The sample groups were compared using the comparison of the 2 observed means with $\alpha=0.05$ with Statgraphics software. Concerning the analysis of the results of numerations, being paired series, the existence of a significant correlation between the participating sites and Afssaps was analyzed using a variance analysis of linear regression using the Statgraphics software with a 5% risk. The differences (expressed in %) between the results of the site and those of Afssaps for each pair were determined and gathered according to various criteria (technique, thawing, cell nature...).

3. Results

3.1 External quality control of haematopoietic products

3.1.1 Results site by site from 10 years of control

The French Health Products Agency, Afssaps, is an evaluation and assessment agency for the whole of these products. For ten years, the Blood Products and Cell Therapy Unit is in charge of external quality control of cell therapy products, essentially peripheral blood stem cells after mobilization. This control allowed us to define quality points adapted to these products, following the measure variation between the producers and Afssaps. Upon those ten years, this work supplies the thirty French preparation sites of cell therapy products with a global statement of the control operation and a follow-up site by site. This allowed us to identify the strong points of the control with operations to carry on and/or to develop and to define controls to check the quality of cell products

To perform these controls, validation for viable CD34+ cell enumeration using a single-platform method was made. It was available for leukapheresis, bone marrow, cord blood cells and thawed products. After a ten year activity and for this site by site study, we have analysed 775 fresh peripheral blood stem cells (PBSC) samples from 30 French preparation facilities. The mean number of sent products per site was equal to 26 fresh PBSC with a range from 11 to 73 directly linked to the site activity. On these 775 received products, 31 (4%) were degraded at reception in Afssaps, generally because of not observed transport conditions and were excluded from this assessment. For each site, we have followed the variation between the laboratory and Afssaps, established control cards for cell numerations and CFU-GM assay. Correlations between CD34 and CFU-GM doses have been studied. For the nucleated cell numeration, 67% of the laboratories obtained a deviation lower than 10% with those made in Afssaps. For CD34 cell numeration, a mean deviation lower than 20% with the Afssaps value is obtained for each site and for 60% of them, it is lower than 15%. Finally, the mean deviation in 2009 was only $11\% \pm 7$ between producers and Afssaps. These results show the good evolution for this parameter and constitute a very satisfactory result on the national plan. CFU-GM results show higher differences between the producer and Afssaps, however 79% of facilities give a mean deviation with Afssaps lower than 35%. About the correlation between the numerations of CD34+ cells and CFU-GM, 31% of the

sites obtain a R^2 higher than 0.7. The results of comparisons between Afssaps and each site obtained for the ten years of external control activity are detailed in the following tables and graphics.

3.1.2 Mean deviation between French sites and Afssaps

After ten years of external quality control, deviations for pairs of results between the production site and Afssaps have been studied site by site for the different analysis and for all the fresh PBSC sent by each of them. Main results are presented on table 1 showing the range of the mean deviations between each site and Afssaps in comparison with the mean deviation for all the products received in 2009 between the producers and Afssaps for the corresponding analysis. Thus, each site can check if its result approaches the up-date mean observed in 2009.

Fresh PBSC analysis	Mean deviation for the all the 30 sites (%) (black bar on figure 1) and range [min-max]	Mean deviation in 2009 between Afssaps and sites for 62 fresh PBSC (%)
Nucleated cell numeration	8.9 ± 2.5 [3.9 - 13.8]	10.4 ± 9
CD34+ cell numeration	13.7 ± 2.8 [7.9 - 18.4]	11.1 ± 6.8
CFU-GM assay	30.3 ± 6.8 [17.7 - 46.9]	32.4 ± 21.9

Table 1. Mean deviations \pm SD between French cell therapy sites and Afssaps. After ten years of external quality control, deviations for pairs of results between the production site and Afssaps have been studied site by site for the different analysis and for all the fresh PBSC sent by each of them.

The mean deviation between Afssaps and the site for each of the 30 ones are represented on the graphic 1 below. Each of them has received a personalised map with its own result appeared in a marked point.

The results show quite small variations (ranging between 4 and 14%) for the numeration of the total nucleated cells (TNC) because of the method used which is a simple and robust technique with a mean deviation equal to $9\% \pm 2.5$. Good results were also obtained for CD34+ cell numeration with variations ranging between 8 and 18.4% and with a mean deviation for all sites equal to $13.7\% \pm 2.8$. The most important variations are observed for the numeration of the CFU-GM whose experimental conditions make this test more difficult to standardise, the average deviation of 30.5% is however acceptable (regarding results obtained in Proficiency Testing Studies organised by StemCell Technologies with 150 to 200 participants, coefficient variation for the CFU-GM number are ranged from 30 to 50%). As the variations observed are close to 10% for the CD34 and TNC numerations and close to 25% for the CFU-GM assay and for some sites, the objective is to maintain or reach these values for all the producers.

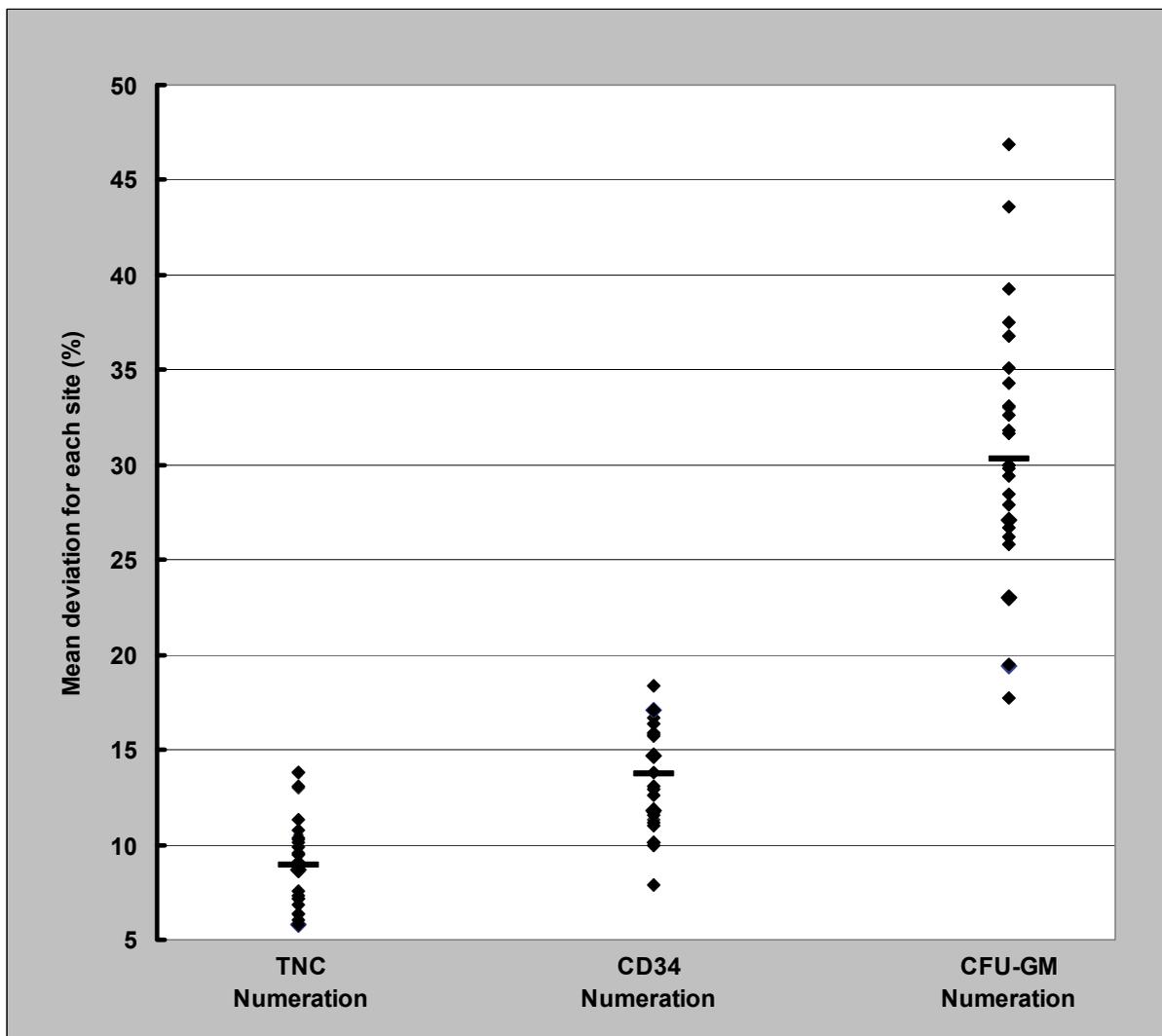
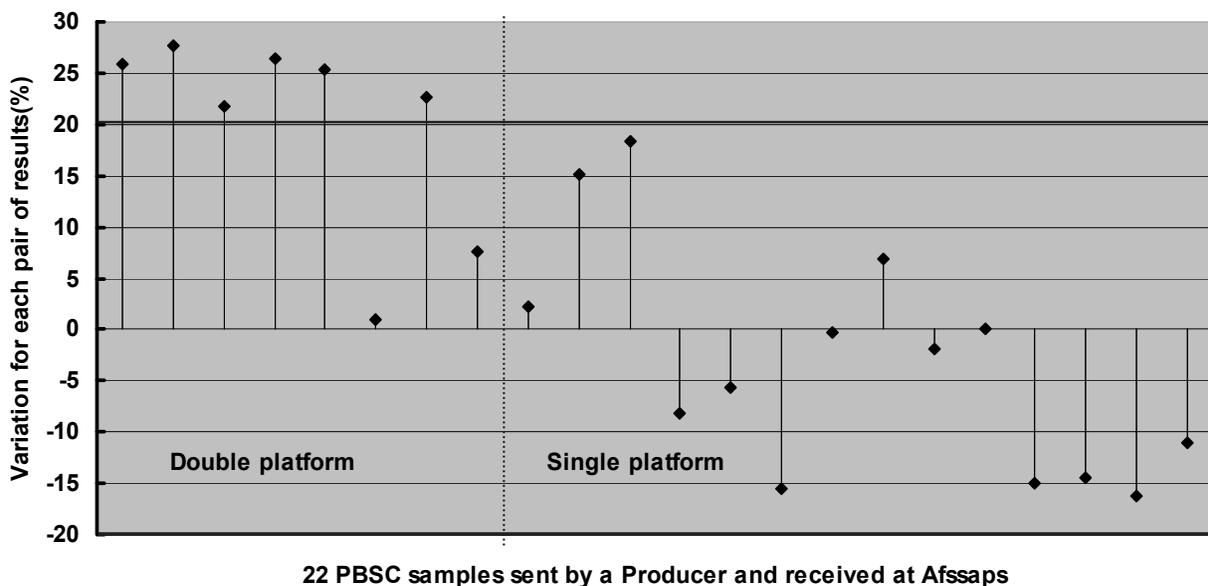


Fig. 1. Distribution of mean deviation between each site and Afssaps for 3 main analyses. Mean deviations for pairs of results between each site and Afssaps (%) and for each analysis is represented by a point (♦) and the mean deviation for all sites is represented by the bar (—).

3.1.3 Site follow-up

As an example, the following graphic shows the CD34 deviations between Afssaps and one French producer of PBSC. As the mean deviation for PBSC samples received in 2009 is equal to $11\% \pm 6.8$, we used this result as a target value and looked at the CD34 delta repartition for each site so that the results are expected to be between -20% and $+20\%$. A deviation equal to 0 means that Producer and Afssaps results are equal, a positive delta means that the Afssaps CD34+ result is higher than that of the producer whereas a negative one is lower. In this example (Figure 2), the mean deviation between the results from Afssaps and the producer for the 22 PBSC samples addressed for control is equal to $13\% \pm 9$. The mean deviation when a double-platform method was used by the producer was $19.8\% \pm 10$ ($n=8$) and was reduced to 9.3 ± 6.6 ($n=14$) when the producer changed for a single-platform method. These 2 results are significantly different ($p=0.02$). Indeed, since the use of a single-platform by the producer, no deviation higher than 20% was measured.



22 PBSC samples sent by a Producer and received at Afssaps

Fig. 2. Deviations between Afssaps and a French Producer site for the CD34+ cell determination in the 22 PBSC samples addressed. Each point (♦) represents the deviation (%) obtained for one pair of CD34+ results and allows to follow the evolution of results for that site along the time (from 2000 to 2009).

We also followed the results for CFU-GM assay site by site and sent personalised control map for that to each of them. For this previous site with the same fresh PBSC, even we observed a coefficient R^2 equal to 0.79, the mean deviation for the number of CFU-GM/kg between this French site and Afssaps remained quite high and was $43.6\% \pm 21.3$ ($n=19$). However it was not the case for some other ones showing that different practices (sample preparation, targeted number of CD34+ cells per dish, medium choice and/or colony estimate) could influence the CFU-GM number result.

3.2 Standardization of CD34 evaluation through the French external quality control – Results from 2000 to 2010

On the basis of international recommendations (Sutherland et al., 1996), the first kits from manufacturers appeared whose objective was to bring a reliable and reproducible measurement, even for products with a very small percentage of CD34+ cells. Using this single-platform method, the DLC evaluated reproducibility of the CD34+ cell numerations by determining the difference expressed as a percentage between measurement of the producer and the measurement carried out in Afssaps for a fixed sample. Without a target value, this variation analysis made possible the establishment of a "normative" reference mark insofar as for approximately 80% of the controlled cellular samples, the difference between producers and Afssaps is lower than 20%. Also, the elements able to explain a higher deviation than 25% were explored, it can be elements related to the technique and/or the nature of the product, such as for example, problems of cellular stability. Thus, progressively with these controls, parameters are identified like being responsible of a lower reproducibility. Groups of defined samples according to these parameters are thus compared. At that time, as shown in table 2, mean deviation for fresh PBSC ($n=789$) between Afssaps and French facilities is equal to $14.7\% \pm 11.8$ with a good correlation ($R^2=0.88$) but higher and around 25% for thawed products. For bone marrow, the mean deviation is equal

to $19\% \pm 16.4$, this higher result is probably due to the different approaches for the CD34+ region according to the more heterogeneous CD34+ population taking in account all the CD34+ cells (dim to bright cells) or only the brightest ones and also due to the different techniques as shown below. For all techniques, the mean deviation for CD34+ cells in thawed products between Afssaps and laboratories was $27.2\% \pm 21$. To analyse the observed differences between Afssaps and producers according to the different kinds of products, we examined these results regarding the use of a single or a double-platform (DP) by laboratories to numerate CD34+ cells (see Table 3). For thawed PBSC, when producers have used a single platform, it was equal to $22\% \pm 16.5$ for viable cells and only $14.7\% \pm 10.9$ for total CD34+ cells.

Products	Mean deviation between Afssaps and Producers for the determination of CD34+ cell number	R ²
PBSC (n=789)	$14.7\% \pm 11.8$	0.88
Bone marrow (n=105)	$19\% \pm 16.4$	0.85
Thawed PBSC (n=150)	$27.2\% \pm 20.7$ (viable CD34+ cells) $20.5\% \pm 15.4$ (total CD34+ cells)	0.73 0.83

Table 2. Mean deviation \pm SD between Afssaps and Producers for the CD34+ cell evaluation according to different types of controlled haematopoietic products for this ten year period. A significant correlation between the participating sites and Afssaps has been obtained for each category with $p < 0.05$.

Regarding the CD34+ cell recovery observed at Afssaps (after transportation), the mean was $55\% \pm 24$ (n=59) whereas it was equal to $72.6\% \pm 29.3$ (n=60) for the producers. Moreover, the double-platform method could lead to recoveries after thawing higher than 100%, because of a fluctuating relative value for CD34+ cells. For PBSC, it is noted, indeed, that the mean CD34+ cell recovery was equal to $90.8\% \pm 28.6$ (n=24) when a DP method is used whereas it was equal to $60.5\% \pm 23$ with the SP method, coherent and in agreement with the data of the literature when robust tools are used (Calmels et al., 2007 ; Dauber et al., 2010) For bone marrow, even the difference between the 2 evaluations is lower when a single-platform is used (15.9 ± 12), there is no significant difference with that obtained when producers used a double-platform method. As said before, because of a more heterogeneous CD34+ bone marrow population, some variations may persist regarding the limit of CD34+ region where dim cells could be taken in account or not. However, with single-platform, the mean variation observed is a satisfactory result. In the same way, no significant difference exists between SP and DP methods for viable CD34+ cells in thawed PBSC because of a certain loss of viability due to the delay between the analyses, but also, the difference with single-platform is lower. Finally, the mean deviations according to the use of a SP or a DP method are significantly different for total CD34+ cells and reduced with the SP method.

Products	Mean CD34 deviation between Producers and Afssaps (%)		p
	Double-Platform	Single-Platform	
Fresh PBSC	17.0 ± 14,9 (n=396)	12.9 ± 9,7 (n=386)	<0.0001
Bone marrow	20 ± 18 (n=57)	15.9 ± 12 (n=30)	0.2
Thawed PBSC (Viable CD34 cells/Prod)	27.5 ± 21,7 (n=87)	22.3 ± 16,5 (n=55)	0.1
(Total CD34 cells/Prod)	21.2 ± 15 (n=69)	14.7 ± 10,9 (n=36)	0.015

Table 3. Mean deviation ± SD between Afssaps and Producers for the CD34+ cell evaluation according to different types of controlled haematopoietic products for this ten year period gathered according to the flow cytometry method (DP or SP) used by those producers.

The good evolution of these results was noted by the follow-up of CD34 variations for fresh PBSC year per year: whereas it was equal to 19% ± 15 in 2000, it is no more than 11% ± 7 today (with p=0.0002) as shown in figure 3. The part of PBSC showing a difference between the 2 evaluations bigger than 20% is now lower than 15% of the products received from all French sites.

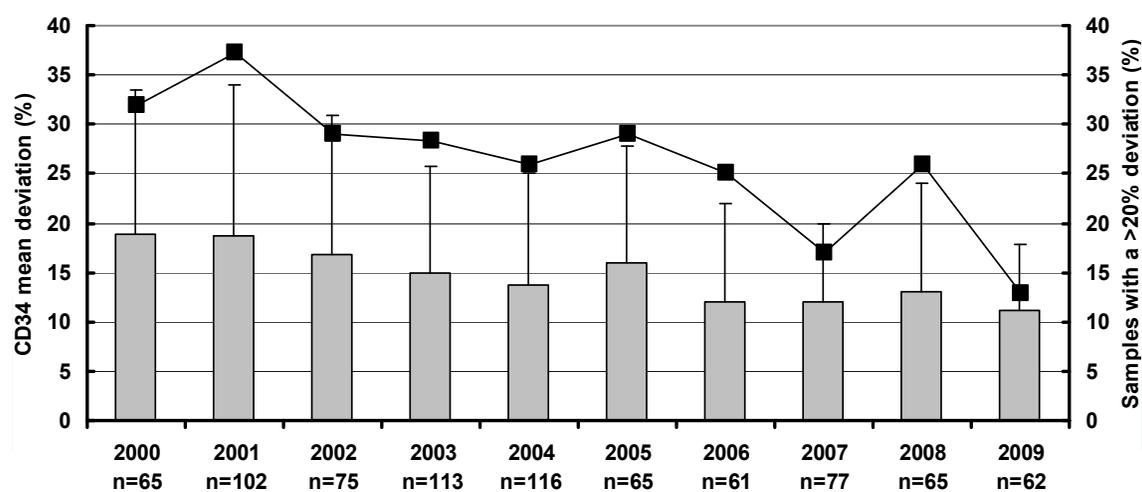


Fig. 3. Evolution of CD34 deviations between Afssaps and Producers for 10 years of external control. CD34 mean deviation (%) for each year since 2000 (grey bar) and part (%) of PBSC controlled samples with a difference Afssaps-Producer higher than 20% for CD34 evaluation (■).

3.3 Standardization of CD34 evaluation through a multi-centric study for cord blood products

In the same time, as the use of haematopoietic stem cell graft from cord blood increased and reached almost 20% of allogeneic grafts in 2009, the question of the thawed cord blood unit

(CBU) quality has been asked. Indeed a lot of cellular therapy facilities have started to thaw these products without being implied in their preparation which is done in cord blood bank. Thus, very little information of the resulting quality was known. In this context, we have organised a study with the French Society of Cell and Tissue Bioengineering (SFBCT) to ensure the inter-laboratory reproducibility of the quality controls practised by the banks during thawing. The cellular recoveries were analyzed according to the thawing techniques, according to the method used in flow cytometry: single-platform versus double-platform, or the product nature, i.e. in total blood or reduced volume. Concerning CD34+ cells numeration, the average deviation between the participating laboratories and Afssaps was $29\% \pm 23$. When dividing laboratories depending on flow cytometry method used, the average deviation was $21\% \pm 16$ for the laboratories using a SP method against $47\% \pm 25$ for those using a DP method.

The CD34+ recoveries are equal to $82\% \pm 60$ in J0 for the participating sites against $52\% \pm 20$ for Afssaps. For the sites using a DP method, it is stressed that this output is particularly high with a rate of $126\% \pm 90$ (n=15) whereas it is $62\% \pm 20$ (n=32) for the sites using a SP method. There exists a significant difference between SP and DP with $p < 0.05$ and, whereas dispersion is weak when a single-platform method is used, dispersion is increased with the DP method. Interestingly, it is noted that the average recovery on the sites using the simple-platform method, although superior, get closed to that found in Afssaps whereas with the double-platform the average recovery is higher than 100% with a very high dispersion as published (Laroche et al., 2005).

Methods	All techniques (n=48)	Double-platform method (n=15)	Single-platform method (n=33)
CD34 deviation between French sites and Afssaps (%)	29 ± 23	47 ± 25	21 ± 16
CD34 output after thawing at the participating sites (%)	82 ± 60	126 ± 90	62 ± 20

Table 4. Mean deviation \pm SD between Afssaps and Producers for CD34+ cell evaluation in thawed cord blood and mean deviation \pm SD for CD34+ cell recoveries after thawing obtained at the participating sites according to the technique used in flow cytometry.

As shown on figure 4, the strong difference for total cell viability (42.5%) and that of CD34+ cells (80%) because of a better resistance of these cells to cryopreservation, is one of the reason why double-platform method can generate over-estimate of CD34+ value and as a consequence to CD34+ cell recovery higher than 100%.

To illustrate the excellent quality of thawed cord blood transmitted to Afssaps, an example of CFU-GM is given on figure 5 showing very large colonies, the mean clonogenicity (CFU-GM number/ 100 CD34+ cells) in Afssaps was $14.9\% \pm 5$ (n=44) and 15.6 ± 9.7 (n=36) for participating sites which are satisfactory results for thawed products. Finally, we analysed the different correlations obtained between the quantity of cells before cryopreservation

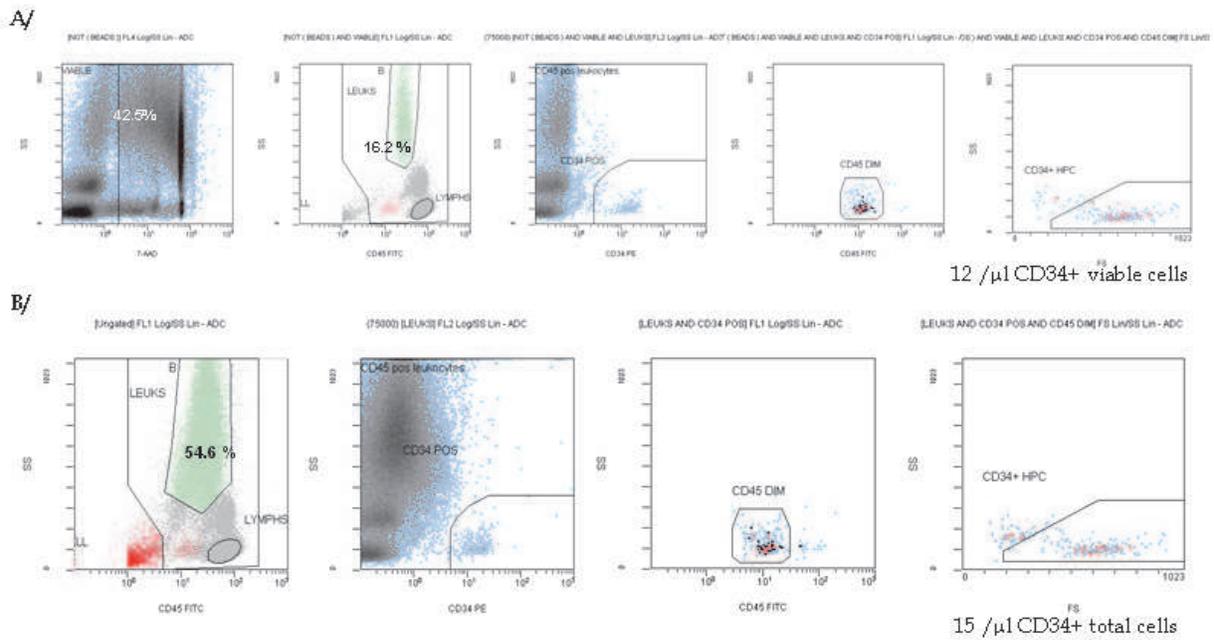


Fig. 4. An example of the analysis of a thawed CBU at Afssaps with StemKit labelling procedure and ISHAGE gating is done: A/ with 7-AAD. B/ without 7-AAD. On this CBU, the total viability of nucleated CD45+ cells was 42.5% whereas that of CD34+ cells was 80%.

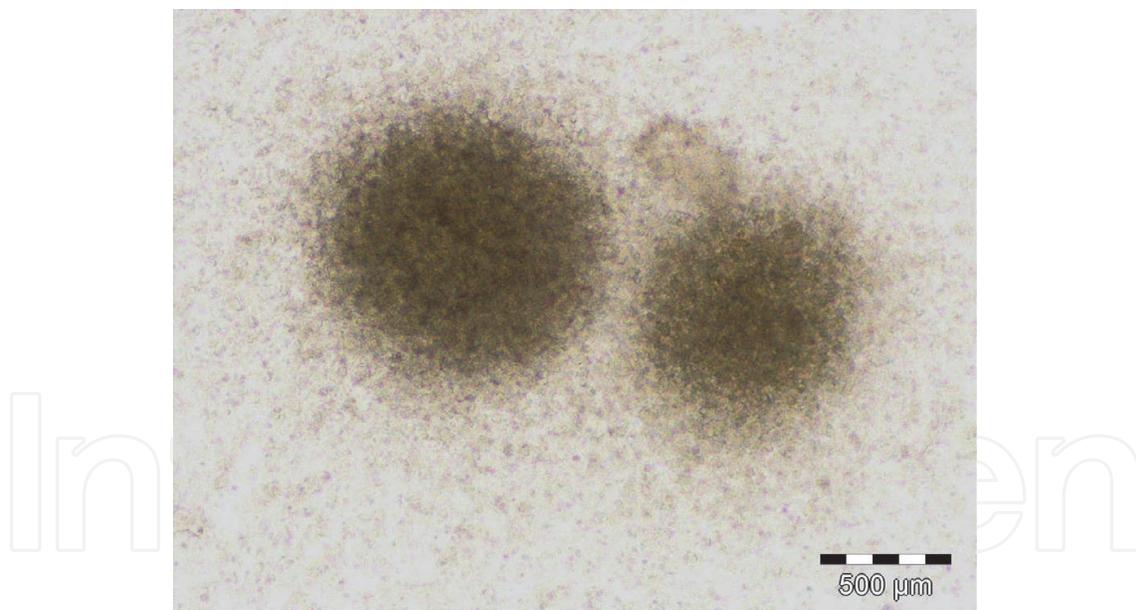


Fig. 5. CFU-GM grown on semi-solid media without Epo, H4535 from StemCell Technologies (Vancouver, Canada).

(determined by the cord blood bank AICT) and CD34+ cell number after thawing obtained by the 14 participating sites. It has been shown that content of total nucleated cells correlates with engraftment (Grewal et al., 2003 ; Rocha & Gluckman, 2009) but also that there exists discrepancy for CD34+ cell and CFU evaluation between the laboratory which have received the cord blood and the cord blood banks which have provided that material (Wagner et al., 2006). Here, in our multi-centric study as shown in figure 6, the linear regression obtained

between TNC before cryopreservation (AICT) and CD34+ cell counts after thawing (participating sites) gave a quite low coefficient of determination with $R^2=0.42$. A similar linear regression was observed between CD34+ cell counts before cryopreservation (AICT) and CD34+ cell counts after thawing obtained by all the 14 participating sites with $R^2=0.46$. On the contrary, when the CD34+ counts obtained before cryopreservation were compared with the CD34+ counts obtained after thawing by the sites which used a SP method, a higher correlation was observed with $R^2=0.81$.

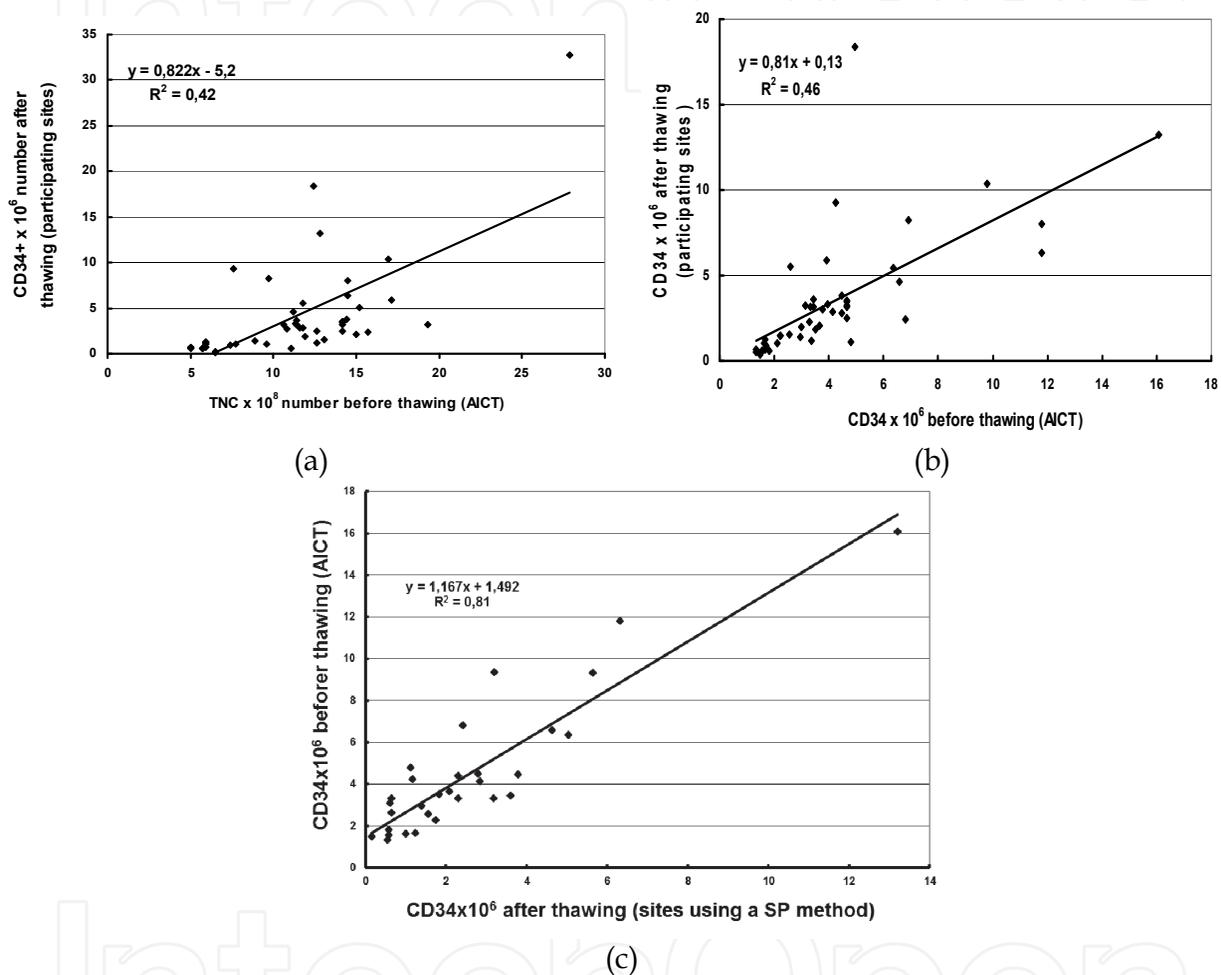


Fig. 6. Linear regression has been done for different contents of cells before and after cord blood cryopreservation. A: between total nucleated cell number before cryopreservation (AICT results) and CD34+ cell content after thawing (n= 46). B: between CD34+ cell number before (AICT results) and after cryopreservation with all the results from the participating sites (n=49). C: between CD34+ cell number before (AICT results) and after cryopreservation with the results from the participating sites who have used a single-platform method (n=30).

These good correlation between CD34+ cell numbers before and after cryopreservation is supported by the results of Lemarie et al. (Lemarie et al., 2007) as well as those of Yoo et al. (Yoo et al., 2007). All together, these results underline a good stability of viable CD34+ cells and a greater reliability of the single-platform methods for the CD34+ cell numeration for these thawed USP (Panterne et al., 2010) supported the CD34+ value as an important criteria of choice for CBU.

3.4 Standardization of CD34 evaluation through comparison of procedures (mono-centric study)

However, even when using a single-platform method to numerate CD34+ cells in cord blood cellular products, a relatively high variability persists (Moroff et al., 2006; Rivadeneyra-Espinoza et al., 2006). These variations could be related to technical variability between cytometers or to biological variability between different reagents. While many recommendations already exist, and have improved CD34+ cell enumeration standardisation (Barnett et al., 1999; Brocklebank & Sparrow, 2001), additional recommendations are necessary for cellular products with low viability such as thawed cord blood units (CBU).

On thawed CBU, it is known for many years that inclusion of a viability dye is necessary (Brocklebank & Sparrow, 2001). However, Brand et al (Brand et al., 2008) recently showed that many different techniques are still in use (manual vs automated; 7AAD vs Trypan Blue), producing inconsistent results. Reproducibility and robustness of quality and potency controls on fresh and thawed CBU could be better standardized by using single-platform techniques and automated or semi-automated procedures to pilot cytometer setups and analyse results.

A recent study searching for a parameter to predict which CBU will engraft in double CBU transplant (Scaradavou et al., 2010) showed a correlation between CD34+ cell viability and engraftment when viability was measured with 7AAD but not with Trypan Blue. Of interest, this study also suggests the importance of using a threshold value for cell viability, which could be a better predictor of hematopoietic reconstitution than the viable CD34 + cell dose.

Currently, three complete diagnostic kits that include a viability dye are commercially available: these are the Stem-Kit™ enumeration kit (Beckman-Coulter) and SCE kit™ (BD Biosciences), used with equipment from the same company, and the CD34 Count kit (Dako). We compared CD34+ cell counts in CBU, obtained 1) with 2 single-platforms diagnostic kits and 2) with manual vs automated procedures. Viable CD34+ cell enumeration in thawed CBUs was equivalent using SCE kit or Stem-Kit ($R^2= 0.98$; Mean deviations between techniques : $13\% \pm 10$; $n=5$). Gating was manual (Cellquest software), based on the adapted ISHAGE guidelines for thawed samples (Brocklebank & Sparrow, 2001). These comparisons were done by the same technologist, on the same sample, on the same cytometer (FACS Calibur BD Biosciences). Comparisons previously performed on 44 fresh apheresis, fresh marrows, fresh CBUs and thawed apheresis samples were also equivalent (Lemarie et al., 2009). For both kits, samples were prepared following the manufacturer's recommendations, except for thawed products to which no erythrocyte lysis reagent was added (based on previous preclinical tests).

We then compared manual vs semi-automated procedures; for the latter, a dedicated software application proposes a grid for cell analysis. Viable CD34+ cell enumeration in thawed CBUs was equivalent using manual or semi-automated procedures to analyse results and set cytometers ($R^2= 0.95$; Mean deviations between techniques : $6\% \pm 15$; $n=6$). Again, these comparisons were done by the same technologist, on the same sample, with the same reagents (Stem-Kit™ Beckman-Coulter). We compared a manual technique using FACS Calibur cytometer with Cellquest software and manual settings (PMT and compensations) vs FC500 cytometer with semi automated Stem CXP software and auto standardization for parameters settings. Comparisons performed on 28 others fresh apheresis, fresh marrows and thawed apheresis were also equivalent (data not shown).

Similarly to what is observed for fresh bone marrows, thawed CBUs samples automated analysis often leads to overestimate CD34 cell counts, including in the count cells with unspecific CD34+ binding. For these kinds of samples, a negative sample helps to correctly position the CD34 gate. Software optimization in these specific samples could help to standardize CD34 enumeration.

3.5 Identification of parameters influencing cellular therapy product quality

Within the framework of this external quality control, we took particular interest to check parameters that could influence the cell viability and HSC recovery to evaluate the quality of the different products. Indeed, without apparent change in transportation, some samples can lose several points of viability. So, we studied different factors such as preservation duration and granulated cell fraction rate. For this study, we analyzed 271 fresh PBSC with a granulated fraction defined in a SSC/CD45 region. The mean viability at collection (producer evaluation) and the mean viability after transportation (Afssaps evaluation) were determined for those PBSC and analyzed according to product composition and storage time.

3.5.1 Viability and granulated cell quantity

As shown on the figure 7, as it is known, viability decrease between Producers (day of collection) and Afssaps (after transportation, mean delay equal to 16H) with a mean viability of $98.6\% \pm 1.5$ before transportation and of $92.7\% \pm 6.4$ after. A significant difference is observed between mean cell viability at day 1 post-collection ($94.3\% \pm 5.3$) and that of PBSC received at day 2 post-collection ($89.9\% \pm 7.3$) with $p < 0.0001$. The cell viability at the time of collection (producer results) before transportation has also been analyzed to define the mean viability for these same groups of products. However for the quite same conditions of transportation, some PBSC have shown a viability loss higher than others. As the granulocytes are known as fragile cells, we looked at the viability according to the percentage of granulated leukocytes (PBSC containing mature and immature granulocytes) corresponding to about twice the median level of the granulated cell fraction ie 40% for those received at day 1 post-collection. Indeed, a significant difference is observed between mean cell viability for PBSC containing less than 40% of granulated cells ($94.9\% \pm 3.4$) and that of PBSC containing more than 40% of granulated cells ($91.7\% \pm 9.3$) with $p = 0.002$. Moreover, when we looked at PBSC received at 48H post-collection ($n = 94$), a stronger loss of viability was observed for the fraction with more than 40% of granulated cells with a mean viability equal to only $85.4\% \pm 9.1$ whereas the mean viability for those with less than 40% of granulated cells was still equal to $91.4\% \pm 6$; a significant difference was also observed with $p = 0.0005$.

The determination of granulated cells in haematopoietic products appears now necessary since side effects have been associated with this cellular fraction during the re-injection according to its richness (Calmels et al., 2007 ; Milone et al., 2007 ; Fois et al., 2007 ; Cordoba et al., 2007). However, in order to follow the haematopoietic products with this criterion, it is required to have a robust determination of this cellular fraction. Indeed, it is necessary to establish a threshold allowing a better product management to limit the possible side effects. A study with inter-laboratories comparison and practices investigation have been done to evaluate this granulated cell numeration and a rather good correlation ($R^2 = 0.8$) has been obtained between Producers and Afssaps.

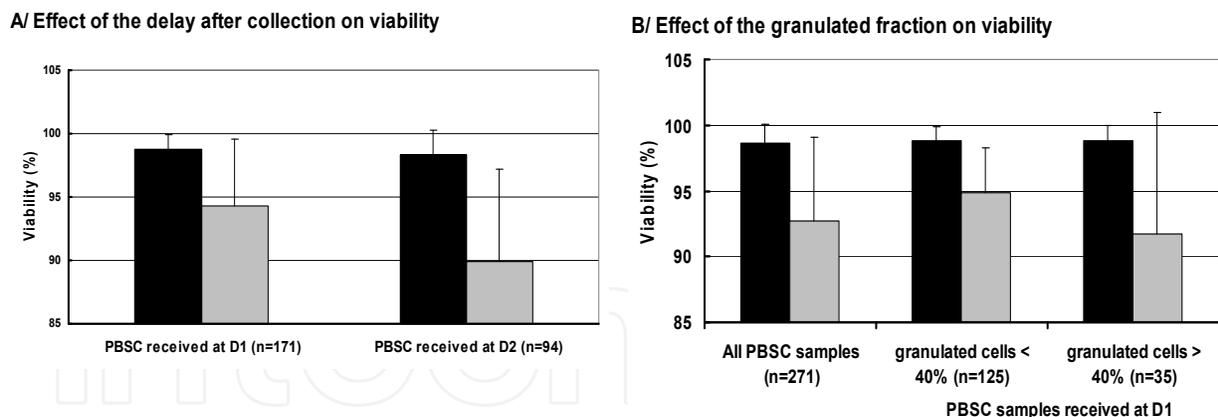


Fig. 7. Mean viability \pm SD of PBSC is done before transportation by black bars corresponding to the producer viability at the day of collection and after transportation by grey bars corresponding to the viability observed at Afssaps for each group studied. A/ the mean viability at a day 1 delay post-collection is compared with that of a day 2 delay post-collection. B/ the mean viability for PBSC containing less than 40% of granulated cells (n=125) is compared with that of PBSC which contain more than 40% (n=35), those 2 groups are composed by PBSC received at day 1 post-collection.

3.5.2 Practice investigation

During leukapheresis performed with classical apparatus, leukocytes are separated from the erythrocytes, the platelets and the plasma of the donor or patient. The population of interest for the graft is contained in the mononuclear cell layer and leukapheresis permits to recover this fraction. Currently, it is still difficult to separate mononuclear cells from polynuclear cells without performing a density gradient. Even if new softwares allow a better recovery of the mononuclear cells compared to the semi-automated system, PBSC are still contaminated by granulocytes whatever the software used (Ravagnani et al., 1999). On the other hand, automated haematology analyzers still have difficulties to determine precisely the blood formula in a product obtained by leukapheresis after a mobilization by a growth factor. Indeed, these products can contain immature granulocytes like granulated metamyelocytes counted as non-MNCs because of their granularity. In this situation, there is a lack of robustness. Because of the need for a better evaluation of the granulated cell fraction, we have organized an investigation to evaluate the techniques used in 2009. Thirty producers were questioned and 26 questionnaires were returned leading to a participation rate of 86%. These cell producers observed a median rate for this cell fraction ranging from 15 to 45% for autologous cells corresponding to a median of 24.5% for all sites. For allogeneic cells, median rates were ranged from 12 to 30% corresponding to a median of 24.5% for all sites whereas the observed median rate for this cell fraction at Afssaps is equal to 22.6% (determined in SSC/CD45 region). Different techniques are still used by producers ie: manual cytology for 57.7% of them ; haematology analyser, 19% ; SSC/CD45 region, 38,5% ; and immunophenotyping for 8%. Finally, even the use of these different techniques, a significant correlation is observed between Afssaps and producers for this cell fraction evaluation with $R^2=0.76$ and $p<0.05$.

Considering the side effects observed with PBSC containing a high quantity of granulated cell and considering the loss of viability as soon as day 1 post-collection, taking in account the percentage of granulated cells as a quality marker is now a current practice in French cell

facilities. This information leads to apply specific procedure for those products meeting a high quantity of granulated cell for example no cryopreservation after 24H and enhanced monitoring at administration for PBSC with a granulated fraction from 30 to 40%. Our results showed that increase in preservation duration, and granulated cell rate had a bad effect on viability. From this observation, we can think that samples with a minor granulated cell fraction can be stored for a longer time than the others.

3.6 Microbiological control of cellular products

To implement the external microbiological control, and to better know the techniques used, an investigation of practices has been done in 1998. This report led to the set up of a working group who prepare national recommendations for this control. In parallel, a validation of automated blood culture was led for these products. This technique enables us to use very low volumes for the inoculation of the bottles and can detect very weak contaminations HSC (1 to 10 germs/ml). The kind of germs able to contaminate, being essentially skin commensal germs (PBSC, bone marrow) or digestive flora (umbilical cord blood), automated blood culture systems allows a better detection than the classical European Pharmacopoeia 2.6.1 method for sterility testing. Our results are confirmed by the comparative study published by Khuu and his collaborators (Khuu et al., 2004) as well as that from Genzyme group for chondrocytes (Kielpinski et al., 2005) showing the superiority of blood culture system. Then, we organized collaborative studies by sending cellular products contaminated at various levels on one hand to know the performances of the methods used, and on the other hand to measure the impact of the recommendations. A panel of samples was sent since 2001 once or twice per year to each participant who performed the analysis according to its own procedure. Results are collected and analyzed by Afssaps. These studies allowed us to propose French recommendations for this control published in 2002. They deal with these different aspects: staff, environment, sample, analysis, results and methods validation. The size of the sample to control is probably the main problem due to the very small quantity of product available for the bacteriological control in most cases. They showed their usefulness by the improvement of performances when they are applied. This work was proceeded and allowed us to propose recommendations at the European level through the European Pharmacopoeia (EP) and a new chapter, 2.6.27., "Microbiological control of cellular products" came into force in January 2007. In this chapter, a recommended list of germs has been established to validate the technique used, and it is particularly adapted for haematopoietic products because of their main therapeutic use. It may be necessary to modify this list depending on the cell origin and on the micro-organisms previously found or associated with the type of cells:

Recommended micro-organisms for validation in 2.6.27 EP chapter

- *Aspergillus brasiliensis*
- *Bacillus subtilis*
- *Candida albicans*
- *Clostridium sporogenes*
- *Propionibacterium acnes*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*
- *Streptococcus pyogenes*
- *Yersinia enterocolitica*

Other approaches to validation may also be used such as interlaboratory comparison. On this basis, we proposed to the French cell therapy banks to participate to collaborative studies organised by the Laboratories and Control Department at Afssaps.

3.6.1 Validation stage at Afssaps

Validation of automated blood culture system was first led for haematopoietic products. A first one has been done on a Vital apparatus and the following list of germs has been tested:

<i>Staphylococcus aureus</i> (ATCC 6538)	<i>Escherichia coli</i> (ATCC 8739)
<i>Staphylococcus epidermidis</i> (ATCC 49461)	<i>Pseudomonas aeruginosa</i> (ATCC 9027)
<i>Streptococcus pneumoniae</i> (ATCC 6303)	<i>Corynebacterium jeikeium</i> (AGMED)
<i>Enterococcus faecium</i> (CIP 5432)	<i>Bacillus cereus</i> (ATCC 10876)
<i>Streptococcus pyogenes</i> (ATCC 19615)	<i>Acinetobacter baumannii</i> (ATCC 19606)
<i>Clostridium sporogenes</i> (ATCC 19404)	<i>Propionibacterium acnes</i> (ATCC 11827)
<i>Bacillus subtilis</i> (ATCC 6633)	<i>Mycobacterium smegmatis</i> (CIP 103599)
<i>Aeromonas hydrophila</i> (AGMED)	<i>Aspergillus brasiliensis</i> (ATCC 16404)
<i>Yersinia enterocolitica</i> (ATCC 9610)	<i>Candida albicans</i> (ATCC 2091)

This technique enables us to use very low volumes for the inoculation of the bottles and can detect contaminations of about 1 to 10 germs/ml. Specificity, reproducibility and sensitivity were checked for 18 germs (bacteria and fungi) chosen because of their representativeness (usual germs found in these products when a contamination happens), for their different type of metabolism and according to the chapter 2.6.1 of the European Pharmacopoeia for the environment germs. Because of a change of the automated blood culture system, a second validation was performed with a comparison of results obtained by Vital apparatus which was the first automated growth-based system we used with the new apparatus BactAlert (BioMérieux). For aerobic bacteria, 100% of positive results were obtained as for the smallest contamination tested (46 UFC/ml), a correlation of 97% was obtained between the 2 systems and no false positive was observed. For anaerobic bacteria, all the contaminations were also detected in the condition that an anaerobic SN media is used to detect *Propionibacterium acnes* which is a very fastidious growing germ. A better detection of anaerobic bacteria with BactAlert apparatus was obtained for the low contaminations (<50 UFC/ml). The sensitivity threshold is around 10³ micro-organisms per bag (ie at least 100ml) when 1% of the bag volume is tested.

3.6.2 Collaborative studies

The decision to organize collaborative studies for the French Microbiology laboratories that perform sterility testing of cell products was taken by the working group "Bacteriological control of cellular products" at Afssaps. The aim of these studies is to evaluate the performances of each laboratory to enhance the use of a standardized and validated control. At least, 30 laboratories participated in each proposed study on a voluntary basis and 12 studies have been organised since December, 2001. The first one was performed with cell samples contaminated by a rapid growing germ (*Staphylococcus epidermidis*) sent to each laboratory participant. This study was designed as a feasibility study where the detection of this germ should normally not be difficult. A negative control was also sent to validate the transportation and manipulation property. Ninety-six % of good answers were obtained and feasibility was demonstrated. The second one took place in 2002 and this time, a more

fastidious growing germ *Propionibacterium acnes* was sent as well as dendritic cell samples contaminated by *Enterobacter cloacae*. The observed results were the following ones:

<i>Propionibacterium acnes</i> 100 UFC/ml	Blood culture media (n=23)	2.6.1 Pharmacopoeia media (n=7)	Agar medium (direct inoculation) (n=8)
Positive result (%)	82.6	71.4	25
Negative result (%)	17.4	28.6	75

Table 5. Positive and negative results obtained by the participants to the collaborative study in 2002 according to control method used.

For the false negative results, 83% were associated to a non respect of the recently diffused recommendations. For *Enterobacter cloacae*, 100% of good results were obtained. At Afssaps, we compared the time to detection obtained with blood culture system equal to $10 \text{ H} \pm 0.9$ which was shorter than that obtained with the 2.6.1 Pharmacopoeia media equal to $24 \text{ H} \pm 0$ (n=8). The same observation was made for *Propionibacterium acnes* with respectively $106 \text{ H} \pm 8$ and 147.4 ± 20 ($p < 0.002$).

Another example is done with the study performed in 2005 where the different blood culture methods used by the participants were compared (Table 6). Here, we showed that the mean time to detection, for all the 4 germs, was significantly shorter ($p = 0.037$) with automated blood culture method ($35.5 \text{ H} \pm 34.6$) than with manual one ($59 \text{ H} \pm 83$). Important standard deviations of the detection time were observed principally with the manual blood culture method.

Mean detection time (H)	<i>Candida glabrata</i>	<i>Acinetobacter</i> 15 UFC/ml	<i>Acinetobacter</i> 150 UFC/ml	<i>Bacteroides</i> <i>fragilis</i>
BactAlert (n=13)	26 ± 12.8	30 ± 13.2	26 ± 11.8	49 ± 20
Bactec (n=8)	102 ± 72.3	18 ± 6.4	14 ± 6.7	18 ± 4.8
Manual blood culture (n=7)	39 ± 36.7	101 ± 141	85 ± 127.4	43 ± 38.9

Table 6. Evaluation of the detection time for the 4 germs sent in 2005 towards the 31 participating laboratories according to the different blood culture method used by 28 of them.

Mean times to detection for *Acinetobacter* at 15 and 150 UFC/ml observed with automated blood culture were also significantly different and shorter of those obtained by manual culture in inter-laboratories conditions.

A summary of all the collaborative studies is shown on the following figure 8 from 2001 to 2009 and their interest taking into consideration the 2.6.27 chapter has been noted. In particular, these studies allow the bacteriology laboratories implied in the control of the cellular products to validate their method by inter-laboratory comparisons. From now on, all the germs recommended by the 2.6.27 E.P. monograph were addressed from 2001 till now through these studies. However, some laboratories could not take part in all the studies or failed to detect some germs, in particular, such as *Propionibacterium acnes* (60% of

satisfactory results with the weak concentration) and *Aspergillus brasiliensis* (82% of satisfactory results).

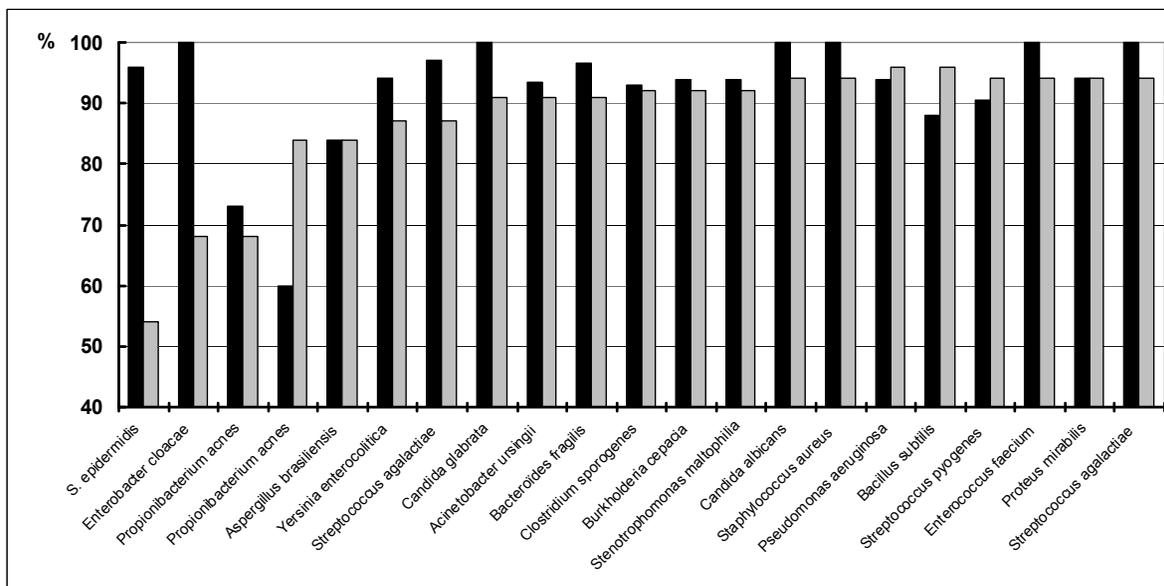


Fig. 8. Percent of satisfactory results obtained by the participant laboratories to collaborative studies performed from 2001 to 2009 (black bar) according to the germ in the contaminated samples sent by the DLC - Afssaps. Percent of blood culture media users for the microbiological control of cellular products (grey bar).

This figure shows that 17 germs chosen for the collaborative study (81% of the total), the percent of good results was superior or equal to 90% and that the users of blood culture media has increased from 54% to 95%. Nowadays, these studies continue in proposing germs which were not found yet by all the participants (either not detected or because the laboratory could not take part in a previous study) such as the germs which could be detected in an irregular way according to the technical requirements (temperature incubation for example for germs like *Pseudomonas fluorescens*). For the last one, we wish to have an inventory of technical conditions applied in the laboratories controlling the products of cellular therapy in France in order to have some information during the re-evaluation of the 2.6.27 monograph.

In order to make possible the use of these results by the laboratories at the time of audit or inspection for example, a laboratory by laboratory synthesis is in preparation.

3.6.3 Haematopoietic stem cell contamination collection

To better know the contaminations found in haematopoietic products and their frequency with an aim of defining new studies and also to analyze the different situations where contaminations happen, in 2004, we decided to collect the information about the contaminated products among the microbiological laboratories implied in the microbiological control of haematopoietic products (n=30). The following graphic (Figure 9) shows the rate of contamination for the different haematopoietic products in France from 2006 to 2009. With this follow-up, it is noticed that the contamination rate of the PBSC remains constant and from approximately 1% since 2006, that of bone marrows decreases since 2006, perhaps as a result of a better sensitizing of the teams for the harvesting whereas

that of the Mononuclear Cells (MNC) exceeds 2% in 2009. The national contamination rate for bone marrow in 2007 and 2008 was the same as published by a mono-centre study (Vanneaux et al., 2007) and it was shown that contaminations came significantly from the harvesting and not from transformation in the cell facility. It is also observed a significant decrease ($p < 0.0001$) of the contamination rate of cord blood units between 2008 to 2009, which could be explained by an improvement of the practices with the implication of new maternities in 2009 (in link with the openings of new cord blood banks) where enabling to the puncture of placental blood were recent (training, sharing experience between midwives, sensitizing to the procedures of disinfection...). For the cord blood units collected for banking, all units with a positive microbiological control at harvesting are discarded.

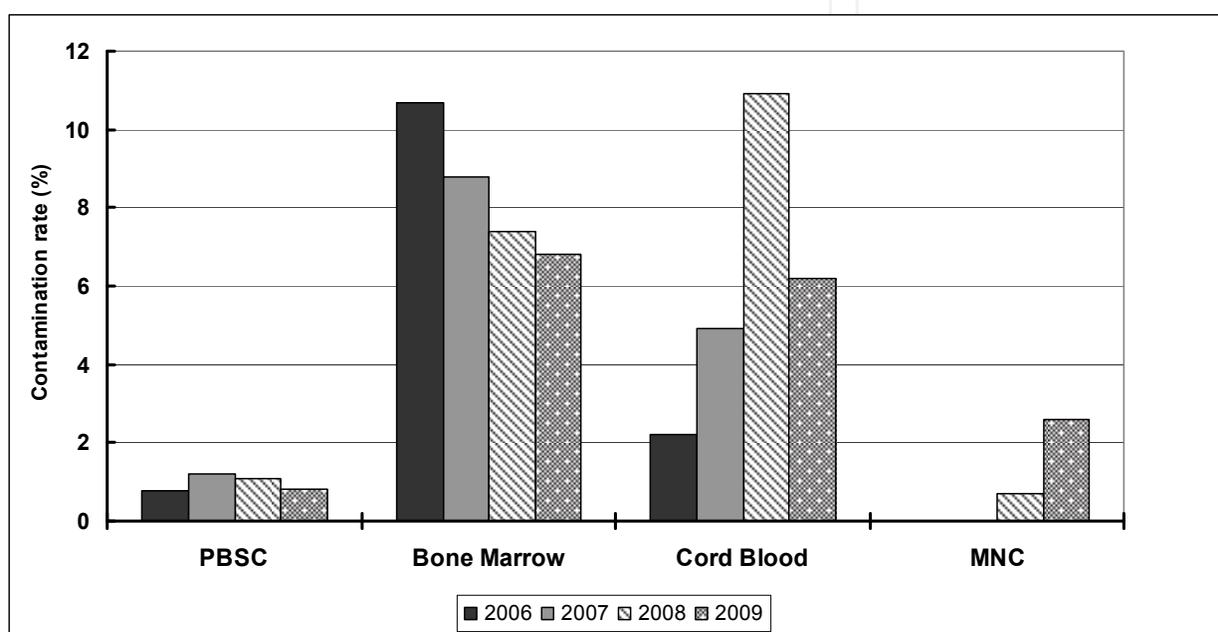


Fig. 9. Contamination rates are represented according to the year and to the haematopoietic product in 2006, 2007, 2008 and 2009.

It is also noted that the contamination rate of PBSC remains constant and of approximately 1% since 2006 and that of bone marrow decreases since 2006, perhaps the result of a better sensitizing of the collection teams whereas that of the MNC exceeds 2% in 2009.

The germs found in the 1132 contaminated products for this 4 year period (for 54432 controlled haematopoietic products in the same time giving a global rate contamination equal to 2%) have been analysed regarding to the genus and as shown in table 7. The most frequent germs are, for PBSC and bone marrow, germs from skin as *Staphylococcus* and *Propionibacterium*; for umbilical cord blood, most frequent germs are from digestive tractus.

It is noted some homogeneity in the distribution of the germs by type of products with close rates from one year to another. This is probably the reflect of the exhaustiveness of the collection with a 90% feed-back for contamination declaim from sites preparing HSC as well as the sensitivity of the techniques implemented.

In this collection, we observed that 6.2% of the contaminations (70/1132) were detected after a 7 day incubation: where, for 70% of these cases, it was a contamination by *Propionibacterium acnes*, a very fastidious growing germ, the used technique was not an

automated blood culture system. Moreover, we didn't have all the details concerning these cases, for example, the inoculum size being perhaps too small or the medium used wasn't the more adapted. Indeed, the performances of different blood culture media need to be checked because this germ doesn't grow with the same speed according to the medium. However, even if this type of micro-organism has a weak clinical relevance (Kamble et al., 2005) it is important to be able to detect all the contaminations to have a microbiological risk as low as possible, to check the process and to improve it, if necessary, in particular according to the contamination origin.

Year	PBSC	Bone Marrow	Umbilical cord Blood
2006	69% <i>Staphylococcus sp.</i> 8% <i>P.acnes</i>	57% <i>Staphylococcus sp.</i> 31% <i>P.acnes</i>	38% <i>Bacteroides sp.</i> 18% <i>Staphylococcus sp.</i> 13% <i>Corynebacterium</i> 10% <i>Streptococcus sp.</i>
2007	64% <i>Staphylococcus</i> 11% <i>Propionibacterium sp</i> 8% <i>Enterobacter</i>	50% <i>Staphylococcus</i> 34% <i>Propionibacterium sp</i>	39% <i>Bacteroides sp</i> 15% <i>Staphylococcus</i> 9% <i>Corynebacterium sp</i>
2008	62.6% <i>Staphylococcus sp.</i> 10.8% <i>Propionibacterium sp</i>	52.8% <i>Staphylococcus sp.</i> 35.8% <i>Propionibacterium sp</i>	28% <i>Bacteroides sp.</i> 26.3% <i>Staphylococcus sp.</i> 13% <i>Corynebacterium</i> 10.7% <i>Streptococcus sp.</i>
2009	61% <i>Staphylococcus sp.</i> 9.3% <i>Propionibacterium sp</i>	43.8% <i>Staphylococcus sp.</i> 32.8% <i>Propionibacterium sp</i>	28% <i>Bacteroides sp.</i> 21.9% <i>Staphylococcus sp.</i> 9.6% <i>Corynebacterium</i> 9.6% <i>Streptococcus sp.</i>

Table 7. Distribution according to main genus identified in the 1132 contaminated products for a 4 year period.

3.6.4 Recommendations for the microbiological control of cellular products

All these results allowed us to propose French guidelines† for this control nationally diffused to cell banks and bacteriological laboratories by Afssaps in 2002, and they showed their usefulness by the increase in performances throughout the various investigations. They deal with these different aspects: personnel, environment, sample, analysis, results and method validation. The size of the sample to control is probably the main problem due to the very small quantity of product available for the bacteriological control in most cases. The organization of collaborative studies by sending cellular products contaminated at various levels allowed to know the performances of the used methods and to measure the impact of

† Recommandations pour le contrôle bactériologique des produits de thérapie cellulaire- Produits hématopoïétiques. Afssaps, Direction des Laboratoires et des Contrôles - Afssaps /DLC. Référence GH/BP 2002-100 - Avril 2002.

the recommendations; we showed that improvement of the performances was in pair with the application of the recommendations. This work continued with new collaborative studies and a positioning of these recommendations at the European level (working group "Cell Therapy Products" near the European Pharmacopoeia) on the basis of 4 years of external control activity for cellular products. The chapter 2.6.27 "Microbiologic Control of cellular products" was published in the addendum 5.6 of the European Pharmacopoeia and is in application since 2007, January, the 1st. The objective is to promote standardized methods to guarantee the quality of the cellular products.

4. Conclusion and perspectives

Thirty dedicated synthesis have been prepared and sent to each site, so that they can look at their own results among all others obtained in the whole 30 sites. For the numeration and the percentage of CD34, a lower deviation than 20% with the Afssaps value is obtained for all the sites.

This assessment made it possible to appreciate some characteristics of this control and to evaluate the PBSC prepared in France by the 30 producing sites. This result follow-up can be integrated into the Quality Assurance System to attest the participation to the External Quality control. Moreover, these data can be provided at the time of a national request for authorization of a cellular therapy process/product in order to inform of the control validations obtained in inter-laboratory conditions. Finally, all these results show the good quality of the PBSC prepared in France and show the reliability of the used methods for the quality control of these haematopoietic products.

In conclusion, these works conducted by DLC at the French Health Product Agency, have left to increase technical standardization. External quality control allows comparing performances between various French facilities and constitutes an excellent indicator for CT laboratories. The single-platform methods are also relevant for the measurement of CD34+ cells in the thawed products where the sample brittleness makes the double-platform use more delicate (without beads) as well as for products from bone marrow because of the presence of erythroblasts (which are CD45 negative cells). Thus, external control results contribute to develop the use of standardized tools by showing their superiority as it confirms by several publications (Barnett et al., 2000 ; Gratama et al., 2003 ; Chang et al., 2004). Indeed, whereas single-platform methods were used only by 30% of French producers of HSC in 2000, there are now 75%. Finally, external control contributes to improve standardization of the used techniques, thanks to the comparative results established for cellular products received from all the production sites; and to guarantee the reliability of controls and decisions which result from this (particularly for the follow-up of the production systems). This control allowed the establishment of a follow-up for the variations site by site in the form of "control cards", though these cards not being of any lawful nature are used today by the producers in the files of requests for a process/ product authorization which they file in Afssaps in order to reinforce the data of quality.

The multi-centre laboratory study on thawed cord blood assesses the impact of the method used in flow cytometry to determine the number of viable CD34 cells. Finally, single-platform methods are especially useful for CD34+ cell enumeration in thawed CBUs. These methods are the only way to standardize sample preparation, viability measurement, gating and cytometers settings, and finally to reduce intra and inter laboratory variability. It is of

particular interest in CBU transplantation, where CBUs are selected on cell counts produced by CB banks, and where clinical outpoint and thawed cellular products cell counts are correlated in multicentric studies.

The study about the granulated cell fraction effect on cell viability has taken a particular impact with the work done by the French biovigilance at Afssaps. Our findings allowed identifying product characteristics which could influence the product quality. The description of noxious effects linked to the granulated cell fraction on the PBSC quality (Calmels et al., 2007; Milone et al., 2007 ; Fois et al., 2007 ; Cordoba .et al., 2007 ; Martin-Henao et al., 2010) led to identify products which could present some risks at the administration. Following biovigilance declarations, highlighted the implication of this fraction in serious side effects of neurological type after the re-injection, an information and recommendation letter[‡] from Afssaps was sent to all the French producers. The evaluation of the granulated cells in PBSC thus becomes a new quality indicator. This interaction biovigilance/quality control results in emitting “warning statements” in the certificates of analyses when the products are detected as fragile. As a consequence, it led to a particular management for these products on the sites (ie: cryopreservation at D0 only, premedication of the recipient...) and to sensitize to declare undesirable effects. Particular follow-up of that “risk” products could be done with additional control, precautions at the time of the administration, follow-up of the recipient. At last, as a good result, these measures led to a reduction in the declarations concerning this type of side effects on the national plan.

As a consequence, this work allowed us to propose recommendations at the European Pharmacopoeia to promote standardized methods to guarantee the quality of cellular therapy product. These recommendations are now validated and give rise to several monographs ie: 2.3.23 “Human haematopoietic stem cells”; 2.7.23 “Numeration of CD34/CD45+ cells in haematopoietic products”; 2.7.24 “Flow cytometry”; 2.7.28 “Colony-forming cell assay for human haematopoietic progenitors cells”; 2.7.29 “Nucleated cell count and viability” and 2.6.27 “Microbiological control of cellular products”

Another important example is given with the implementation of a standardized microbiological control using blood culture system in more than 90% of the French cell banks leading to the monograph 2.6.27. The type of contaminated germs which can contaminate the HSC being primarily commensal germs, the blood culture media allow a better detection than media used with the method of the European Pharmacopoeia 2.6.1 and it is often more adapted because of the low volume available for this control. Our results are confirmed by the comparative study published by Khuu and collaborators (Khuu et al., 2004) showing the superiority of automated growth-based method. Those results are also confirmed by the complete validation data of this automated system done for chondrocytes (Kielpinski et al., 2005) leading to an approval of the use of this technique for the bacteriological control of this licensed product by FDA. At our level, a study for the bacteriological control of chondrocyte preparations initiated at the producer request made possible to test the use of the BactAlert apparatus in parallel of the use of TS/TR media. The results showed better results with the automated method ($p < 0.05$).

All these results support the general use of automated growth-based methods for sterility testing of cell therapy products. They could be useful for the evaluation of cellular products at the European level. Indeed, several points should be considered:

[‡] <http://www.afssaps.fr/content/download/555/5823/version/3/file/csh.pdf>

- Need for a rapid and sensitive control at the producer level even if the result is known after the release and the injection to the patient, it helps to better monitor performances of the production facilities if necessary. However, to improve this control, a revision of the 2.6.27 chapter is currently in progress with the development of rapid methods for sterility testing like PCR, flow cytometry or the detection of microcolonies. The Paul Erlich Institute (an Official Medicinal Laboratory Control for biological products) in Germany has already validated flow cytometry for the microbiological control of platelets (Karo et al., 2008) and it is in progress for the control of cell products.
- Considering the rare and single character of some products and the benefit/risk, the challenge is to have the best suitable test to be sure to take the decision even if a contamination has been found.
- Need for a method as growth-based one to allow identification
- Clinical sequelae following infusion of a microbial contaminated progenitors cells are rare (Padley et al., 2007 ; Kamble et al., 2005 ; Lowder & Whelton, 2003 ; Schwella et al., 1998 ; Klein et al., 2006)

All these considerations are in agreement with those of Padley and colleagues (Padley et al., 2007) who have reviewed the product culture results and clinical outcomes from 1998 to 2006 representing 7233 haematopoietic stem cell collections. Finally, we have done a study in 2009 with the French cord blood bank to evaluate performances of automated culture system after the thawing of the cord blood units. This system has already been validated for these products by an umbilical cord blood bank in Australia (Sparrow, 2004). All these results would allow us to complete these 2.6.27 recommendations.

In the future, we propose to study apoptosis, to establish CFU-GM recommendations for the assay validation which could complete the 2.7.28 European Pharmacopoeia chapter, to evaluate new methods for rapid germ detections and to initiate other analysis on human cells used in immunotherapy and regenerative therapy.

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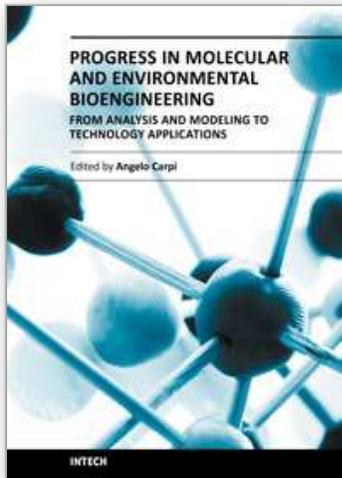
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This book provides an example of the successful and rapid expansion of bioengineering within the world of the science. It includes a core of studies on bioengineering technology applications so important that their progress is expected to improve both human health and ecosystem. These studies provide an important update on technology and achievements in molecular and cellular engineering as well as in the relatively new field of environmental bioengineering. The book will hopefully attract the interest of not only the bioengineers, researchers or professionals, but also of everyone who appreciates life and environmental sciences.

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