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# UNIVERSITÀ DEGLI STUDI DI TORINO

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# **Inactivated Human Platelet Lysate with Psoralen: a New Perspective for Mesenchymal Stem Cell Production in Good-Manufacturing Practice Conditions**

RUNNING TITLE: GMP expansion of MSCs

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## ABSTRACT

**Background:** Mesenchymal Stem Cells (MSC) are ideal candidates for regenerative and immunomodulatory therapies. The use of xenogeneic protein free GMP-compliant growth media is a prerequisite for clinical MSC isolation and expansion. Human platelet lysate (HPL) has been efficiently implemented into MSC clinical manufacturing as a substitute for fetal bovine serum (FBS). As the use of human-derived blood materials alleviates immunologic risks, but not the transmission of blood-borne viruses, the aim of our study was to test an even safer alternative than HPL to FBS: HPL subjected to pathogen inactivation by psoralen (iHPL).

**Methods:** Bone Marrow samples were plated and expanded using 3 culture media,  $\alpha$ -MEM + 10 % HPL or iHPL or FBS, MSC morphology, growth and immunophenotype were analyzed at each passage; karyotype, tumorigenicity and sterility at the third passage. Statistical analyses were performed.

**Results:** MSCs cultivated in the 3 different conditions did not show any significant differences in terms of CFU-F number, immunophenotype and multipotent capacity. Conversely, HPL/iHPL-MSCs appeared smaller, more numerous, had higher proliferative potential and showed a higher Oct-3/4 and NANOG protein expression than FBS-MSCs. Although HPL/iHPL-MSCs exhibit characteristics attributable to higher primitive stemness than FBS-MSCs, no tumorigenic mutations or karyotype modifications were observed.

**Discussion:** We demonstrated that iHPL represents a good, safer than HPL, GMP-compliant alternative to FBS for MSC clinical production which is even more advantageous in terms of cellular growth and stemness.

**Keywords:** Mesenchymal Stem Cells (MSCs), Good Manufacturing Practice (GMP), Human Platelet Lysate (HPL), Inactivation, Psoralen

## **LIST OF ABBREVIATIONS**

a-MEM = alpha-Minimum Essential Medium

ATMP = Advanced Therapy Medicinal Products

BC-PC = buffy coat-platelet concentrate

BM = bone marrow

CAD = Compound Adsorption Device

CFU-F = Fibroblast Colony-forming Units

cPD = cumulative population Doubling

FBS = foetal bovine serum

GF = growth factors

GMP = good manufacturing practice

HPL = human platelet lysate

HS = human serum

iHPL= inactivated **human** platelet lysate

IP = inactivated pathogens

MSC = Mesenchymal Stem Cells

Oct-3/4 = Octamer-3/4

PI = Pathogen Inactivation

PBS = Phosphate Buffer Saline

PDGF = Platelet-Derived Growth Factor

PLT = platelet

SD = Standard Deviation

## INTRODUCTION

Rapid progress in the fields of biotechnology and medicine has led to the development of new treatments and innovative medicinal products. Among them, new cell-based medicinal products (CBMPs), containing viable human cells of autologous or allogeneic origin, have a high potential for cell-based therapies for various severe diseases. In particular, Mesenchymal Stem Cells (MSCs), can be easily isolated from bone marrow (BM) thanks to their capacity to adhere and proliferate and expand in culture (1, 2). They are multipotent stem cells with high immunomodulant proprieties and produce multiple cytokines, growth factors, adhesion molecules: all important factors which influence the hematopoietic microenvironment (3, 4). The particular characteristics and high plasticity of these cells, make them very relevant in the fields of cell therapy, tissue repair and in tissue engineering strategies, as therapeutic products tailored to a number of clinical scenarios: from degenerative to post-traumatic diseases caused by damage or cell loss.(5, 6). The increasing use of MSCs as Advanced Therapy Medicinal Products (ATMP) has led to production processes that need to meet Good Manufacturing Practices (GMP) (7, 8). The regulatory context for ATMPs is established by Regulation (EC) N. 1394/2007 which is designed to facilitate the patient access to these products, while guaranteeing the highest level of safety for patients (9) .

In order to ensure product safety and efficacy, GMP guarantee that products are consistently produced and controlled to the quality standards required for their intended use, from collection to release, including cell harvesting, cell manipulation processes, maximum number of cell passages, combination with other components of the product, filling, packaging etc ... . Although human MSCs themselves are not highly immunogenic, when expanded in xenogeneic sera such as in fetal bovine serum (FBS), they are likely to generate immune responses in some patients after administration (10, 11). It was shown that a single preparation of  $10^8$  hMSCs grown under standard conditions in FBS carry with it approximately 7–30 mg of FBS proteins (10). Thus, in view of a clinical GMP production, the use of xenogenic serum is complicated as there is high lot-to-lot variability and it is associated with a risk of transmitting infectious agents and immunizing effects (12, 13).

On these bases, regulatory guidelines for GMP productions, aimed at minimizing the use of FBS, used in most expansion protocols as a cell culturing medium supplement, have further reinforced an intensive search for safer media supplementation alternatives (14). Serum-free medium is unable to promote MSC expansion unless several recombinant human growth factors (GF) such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF)- $\beta$ , and epidermal growth factor (EGF) are added to the medium. However, 1) defining the optimal amount of GF is difficult, 2) only a few GFs are licensed for therapeutic use, 3) recombinant Human-GFs are expensive, and 4) isolated GF cannot replace the different physiological functions of FBS (15-17).

Interesting works have evidenced the possibility of replacing FBS with autologous or allogeneic platelet lysate obtained from a single-donor or pooled human serum or platelets as they contain a plethora of growth-promoting factors. Moreover, it is being established as a safe and efficient MSC culture supplement for robust MSC cultivation, thus offering certain advantages as potential FBS substitutes (18-21). Analyses of platelet lysates, and subcellular fractions have shown that numerous bioactive molecules are stored within distinct platelet organelles including adhesive proteins, coagulation factors, mitogens, protease inhibitors, and proteoglycans (22). Although the use of human-derived blood materials alleviates the immunologic risks of FBS, the possibility of transmitting blood-borne viruses remains, especially when materials from multiple donors are pooled to provide a sufficient volume for therapeutic-scale MSC expansion and to limit individual donor variability. Pathogen inactivation (PI) technologies are aimed at enhancing blood safety through the inactivation of emerging pathogens, both known and as yet-identified ones, that are not detected by current screening or testing protocols. Since 1990, significant progress has been made in pathogen inactivation technology, which at present is widely available in European blood services, with multiple CE (an abbreviation of Conformité Européenne, French for "European Conformity") marked products to treat both platelets and plasma for transfusion. The CE marking states that the product is assessed before being placed on the market and meets EU safety, health and environmental protection requirements.



The European experience with pathogen inactivated platelets and plasma now numbers millions of units, with a safety record that has been widely reported in the literature (23).

Demonstration of the PI capacity of these techniques is beyond the scope of this publication, however extensively validation studies, using blood products with high titers of added bacteria, enveloped and non-enveloped viruses, and protozoa, have been performed to prove their efficacy (24).

Therefore, the application of PI technique on platelets to be used for the preparation of Human Platelet Lysate (HPL) to supplement culture medium for MSC expansion in a GMP setting, seems to be highly desirable as it might obviate the problem of virus transmission.

In this study, the pathogen inactivation process was performed with Intercept Blood System technology (INTERCEPT, Cerus Europe BV, Amersfoort, Netherlands), that uses a photoactive compound, a derivative of Psoralen (Amotosalem) and long-wavelength ultraviolet (UVA) illumination. Upon exposure to UVA light, Amotosalem becomes reactive and forms a chemical crosslink that locks-up the strands of RNA and DNA, blocking and inactivating the replication of viruses, bacteria and leukocytes in PLT concentrates (25-28).

In our study, to ensure that the inactivation process does not induce changes in the cells, we set up, in parallel, cultures of MSCs in FBS, the standard supplement, HPL, widely used as a cell growth supplement and discussed in the literature, and iHPL. We then compared the effects of the three supplements on cell growth, immunophenotype, multipotent capacity, karyotype, tumorigenesis and stemness protein expression. Both HPL and iHPL batches were prepared according to the blood bank procedures (29), where, in keeping with the current normative provisions, a high standardization is strongly recommended.

## MATERIALS AND METHODS

### Human Platelet Lysate (HPL) preparation

Whole Blood ( $450 \pm 45$  mL) collection from 60 healthy blood donors was performed in a triple bag system (Fresenius Kabi, Bad Homburg, Germany) containing 63 ml of Citrate-Phosphate-Dextrose (CPD) as anticoagulant. According to Italian laws and European guidelines, routine testing of blood donors was performed for the following: ABO blood groups, irregular red blood cell antibodies and infectious markers (Hepatitis B and C, HIV 1-2 and Treponema pallidum). The blood units were centrifuged and separated into Plasma, Buffy Coat and Red Blood Cells using an automated blood component separator (Compomat G5, Fresenius Kabi; Bad Homburg, Germany).

For Buffy Coat-Platelet Concentrate (BC-PCs) preparation, four O-group Buffy Coats were pooled with one AB-group plasma, then centrifuged and automatically separated through a leukoreduction filter using the TACSI system (Terumo BCT Europe, N.V. Zaventem, Belgium).

Twelve BC-PCs were prepared and six experimental replicates were performed. Each replicate was prepared from two BC-PC units using a pool and split process, resulting in 6 test units ( $n=6$ ) and 6 paired control units ( $n=6$ ).

Samples were taken from each unit to evaluate that platelet concentration, platelet content and leucocyte contamination complied with any specifications set for transfusional purposes and for use with the inactivation processing set.

Platelet concentration was determined with a haematology analyser (Sysmex XE-2100) and residual white blood cells contamination with flow cytometric method (BD Leukocount kit; BD Biosciences).

Each control unit remained untreated; the test unit was sterile docked to an INTERCEPT Large Volume processing set (CERUS Corporation, Concord, CA, USA) and processed according to the manufacturer's instructions. The component was treated with  $3 \text{ J cm}^{-2}$  UVA light, in the presence of  $150 \mu\text{M}$  Amotosalen. Following treatment, the BC-PC was transferred to the associated Compound Adsorption Device (CAD) bag and incubated for 16 h, with agitation at  $22^\circ\text{C}$ , to reduce to low level residual Amotosalen and free photoproducts.

Samples were taken from each unit to evaluate platelet concentration, recovery post inactivation treatment and sterility (BacT/ALERT, bioMérieux).

All BC-PCs were frozen at  $-35^\circ\text{C}$  and thawed at  $37^\circ\text{C}$  three times to obtain platelet fragmentation and growth factors release. The BC-PCs were subsequently centrifuged at  $5000g$  for 8 min to remove platelet bodies and collect the supernatant.

To improve standardization and reduce individual donor variations, all six untreated supernatant units were pooled in a single HPL unit, resulting in a batch of 60 different donors. The same was done with the other six treated units. Each batch of HPL was then divided into aliquots of 100-150 ml and frozen again at  $-35^\circ\text{C}$  until use.

To eliminate any traces of residual platelet, HPL was filtered by  $0.2 \mu$  filters before the use.

Each batch of HPL and iHPL was tested for the presence of endogenous and adventitious viruses. DNA extraction was performed starting with 1 ml of HPL and iHPL followed by an extraction step using the automatic extractor easyMAG (Biomérieux, Marcy l'Etoile, France) according to the manufacturer's instruction, and eluted in a final volume of 50 µl. For endogenous viruses, five microliters of elute were added to 20 µl of amplification mix for detection of HCMV and EBV(Q- CMV Real Time Complete Kit, EBV Q-PCR Alert Kit, ELITech Group, Puteaux,), reconstituted following the manufacturer's instructions, and amplified with the following thermal profile: 50°C 2', 95°C 10', 45 cycles of 95°C 15'', 60°C 1'. Twenty microliters of elute were added to 20 µl of amplification mix for detection of parvovirus B19 (Parvovirus B19 Elitè MGB Kit, ELITech Group), reconstituted following the manufacturer's instructions, and amplified with the following thermal profile: 50°C 2', 95°C 2', 45 cycles of 95°C 15'', 60°C 40'', 72°C 20''. For simultaneously detection of 15 mayor respiratory viruses (RSVa, RSVb, Influenza A, Influenza B, Adenovirus, Coronavirus 229E/NL63, Coronavirus OC43, Parainfluenza 1,2,3,4, Rhinovirus A,B,C, Bocavirus 1,2,3,4, Enterovirus, e Metapneumovirus) RV15 OneStep ACE detection (Seegene, Seoul, Korea) was used according to the manufacturer's instruction.

#### Harvest and preparation of MSCs

BM cells were harvested from the iliac crest of adult or pediatric donors who underwent BM collection for a related patient after informed consent. We used a part of the BM initially dedicated to transplant or when available, an unfiltered BM collection bag (Baxter Healthcare Corporation, IL, USA) which was normally discarded before BM infusion. The bag was washed 3 times with Phosphate Buffer Saline (PBS) 1X (Lonza, Versviers, Belgium) and the cells were centrifuged 400 g for 10 minutes, counted and plated directly in different culture media.

First, we tested HPL at a concentration of 10%, 7% or 5% with 20 IU/ml heparin to assess the best result in terms of cellular growth.

Then the whole BM sample was equally spited in 3 cellular culture conditions: alpha-Minimum Essential Medium Eagle (a-MEM) (SIGMA-ALDRICH®, LTO Irvine, Ayrshire, UK) containing 10% of 1) FBS ,

2) HPL or 3) iHPL. The seeding density of starting whole BM was at 10000 cells /cm<sup>2</sup> as previously reported (30).

After 7 days, the non-adherent cells were removed and discarded. The adherent cells were re-fed every 5-7 days and when they reached confluence, they were detached, counted using the fast read® disposable chamber as previously described in Gunetti et al. (31) and re-plated for a further 3-5 passages at 1000 cells/cm<sup>2</sup>. We considered MSCs at Passage 1 (P1) the first which were harvested and re-plated. The cellular condition was maintained from cellular plating of whole BM and during the expansion process. We indicated the following passages with increasing numbers: P2, P3, etc.. On the basis of our experience and our previous reported data (30) we retained that 3 passages suffice for clinical applications. For this reason all data are obtained on MSC expanded until P3.

#### Colony-Forming Unit-Fibroblasts (CFU-F)

In order to quantify MSC precursors, we performed a CFU-F test: the BM cells were plated directly in  $\alpha$ -MEM (SIGMA-ALDRICH®, LTO Irvine, Ayrshire, UK) containing 10% FBS, HPL or iHPL at densities of [10000] cells/cm<sup>2</sup> or [100000] cells/cm<sup>2</sup> in a 6-well plate (SPL Life Science, Eumhyeon-ri, Korea). MSC clonogenic precursors were scored macroscopically after 7-10 days from seeding and clusters of more than 50 cells were considered colonies. All experiments were performed in duplicate and by 2 different operators. The CFU-Fs means were expressed as fibroblastic clones obtained from 1 million BM cells (CFU-F/10<sup>6</sup> cells).

#### MSC Cellular Growth Evaluation

In order to evaluate the cellular growth, the cell growth rate was expressed in terms of population doubling (PD) using the formula ( $\log N / \log 2$ ), where N is the cell number of the detached cells divided by the initial number of seeded cells and the expansion in terms of cumulative PD (cPD).

#### MSC Cytofluorimetric Analysis

To analyze the immunophenotype, flow cytometer analysis was performed on adherent cells at each passage. Briefly, 200000 cells were incubated with the appropriate amount of antibody according the specific antibody titration as described in Rustichelli et al.(32) for 20 minutes with anti CD90 FITC, CD73 PE, CD34 FITC, CD14 FITC, CD45 FITC (Becton Dickinson, San Jose, CA, USA), CD 105 APC and CD146 APC (Miltenyi Biotec srl, Bologna, Italy). The labeled cells were thoroughly washed with PBS 1X and the cells are acquired using FACScanto II (Becton Dickinson) by DIVA software program. The percentage of positive cells was calculated using the cells stained with Ig FITC/ PE/APC as a negative control and mean fluorescence intensities (MFI) was analyzed on the positive cells.

#### MSC Immunocytochemistry

To analyze the stemness protein expression we performed immunocytochemical staining for Octamer-3/4, (Oct 3/4) and for Homeobox transcription factor Nanog (NANOG). Briefly, the cells were fixed and permeabilized with methanol and acetone (1:1) at -20°C for 10 minutes. Non-specific binding was blocked with 5% NHS (normal horse serum) in Antibody (Ab) diluent. The cells were incubated with goat anti-NANOG (1:50 R&D Systems) and goat anti-Oct-3/4 antibodies (1:100 R&D Systems) and then with the secondary antibody Alexa fluor 488-coupled anti-mouse (Molecular Probes, Oregon, USA; 1:200). Incubation with the primary antibody was performed over night at 4°C, while incubation with the secondary antibody was for 1 hour at room temperature. The cells were examined under epifluorescence microscopy (Axiovert 200, Carl Zeiss, AG, Germany) and analyzed by AxioVision Rel 4.2 (Carl Zeiss, AG, Germany). Positive cells were counted and compared with the total cell counts labeled with 4',6-Diamidino-2-phenylindole (DAPI, Molecular Probe).

#### RNA Extraction and Real-Time PCR.

Total RNA was extracted using RNeasy Plus Mini Kits (Qiagen, Austin, Texas). Reverse-transcription polymerase chain reaction (RT-PCR) was carried out using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. RNA and cDNA concentrations were measured with a GeneQuant pro spectrometer

(Amersham Biosciences, UK). We performed Real Time PCR to detect the transcripts for human Oct-4 and NANOG using specific assays (assay ID: OCT-4 (POU2F2) Hs00231269\_m1; NANOG: Hs04260366\_g1; HPRT1:Hs02800695\_m1) and TaqMan Universal PCR Master Mix (Applied Biosystems, FosterCity, CA, USA).

Comparative Cycle threshold ( $\Delta\Delta CT$ ) experiments were performed in accordance with the manufacturer's specifications in a total reaction volume of 25  $\mu$ L. All experiments were performed in three replicates. To normalize the PCR results, we used hypoxanthine phosphoribosyltransferase 1 (HPRT1) as housekeeping gene. We perform the Relative Quantification (RQ) method which is based on the expression levels of a target gene versus a housekeeping gene and allows us to compare different RT-PCR experiments (33) For each set of experiment we analyzed Nanog and Oct-4 mRNA expression levels in MSCs obtained in 3 different culture conditions at P3 and FBS-MSC condition was used as reference sample .

### Potential Assay

In order to analyze the multipotent capacity, MSCs at the 3<sup>rd</sup> passage in each culture condition were cultured in osteogenic, adipogenic and chondrogenic media (Lonza, Cologne, Germany) for 21 days, according to the manufacturer's instructions. Briefly, 5000 and 10000 cells, respectively for control samples and for differentiation experiments, were seeded in a 6-well plate for osteogenesis and adipogenic culture conditions respectively. To induce osteogenesis and adipogenesis, the medium was replaced with specific complete induction medium (Stemcell Technologies). Osteogenic differentiation was demonstrated by the accumulation of crystalline hydroxapatite by Von Kossa staining; and the adipogenic differentiation, by the presence of intracellular lipid vesicles assessed by Oil Red O. MSC chondrogenic differentiation was obtained as previously described (2) and the differentiation was evaluated by Alcian Blue staining which identifies the presence of hyaluronic acid and sialomucin.

## MSC Karyotype Analysis

To exclude cytogenetic transformation during *ex vivo* expansion, MSCs at the 3<sup>rd</sup> passage were detached and then cultivated until approximately 80% of confluence was reached. Karyotype analysis was performed after the cells were arrested at the metaphase by incubation with Colcemid (Invitrogen Corporation, Grand Island, NY, USA). The cells were then maintained in a hypotonic solution (0,075 M KCl), fixed with 3:1 methanol/acetic acid (Merck, Milan; Italy), and stained with Giemsa using standard laboratory protocols for chromosome analysis. A total of at least 50 cells at metaphase were analyzed using MackType software (Nikon Corporation, Japan) according to the ISCN (International System for Human Cytogenetic Nomenclature) (34).

## Tumorigenesis Tests

In order to exclude the possible potential tumorigenicity induced by the *ex vivo* expansion of MSCs we performed soft agar tests.

The cells, at the 3<sup>rd</sup> passage, in each culture condition were harvested, washed and seeded at a density of 1000 cells/well in 24-well plates (SPL Life Science, Eumhyeon-ri, Korea) in duplicate. The test was performed using 0.8% and 0.3% agar in a-MEM +10% HPL/iHPL/FBS (final volume/well = 1ml), arranged respectively at the base and on the surface.

The cells were incubated for 21 days at 37° C in the presence of 5% CO<sub>2</sub>. After incubation, the colonies were counted using the inverted microscope.

A primary line of osteosarcoma was used as the positive control and a sample of whole BM from a healthy donor was used as the negative control.

Microbiological control and Mycoplasma detection.

For microbiological control, we collected supernatants at each passage and an aliquot of them was tested through BacT/ALERT (bioMérieux SA, France) (35) by the Bacteriology Laboratory of City of Science and Health of Turin, S. Anna Hospital.

We conduct the mycoplasma detection test (Mycoplasma Detection Kit, for conventional PCR, Minerva Biolabs, Germany), an aliquot of supernatant at each passage during the expansion process and on an amount of each growth supplement (pure HPL, iHPL and FBS). Briefly, the presence of a mycoplasma contamination of the analyzed samples was detected through a semi quantitative PCR reaction. As the positive control we used a positive control DNA, provided by the kit. To monitor the success of the extraction procedure, we used the internal control DNA of Venor®*GeM*. As negative control, we used the PCR grade water.

### Statistical Analysis

Statistical analyses were performed using SPSS 20 (IBM, Chicago, IL, USA).

Firstly, we investigated whether the distributions of each group were normally distributed by Shapiro-Wilk test. As the distributions were not normal, we used the non-parametrical Friedman Test to compare the different groups. We considered a significant difference if the p value was <0.05.

## RESULTS

### HPL preparation and INTERCEPT treatment

Two batches of Human Platelet Lysate, inactivated (i-HPL) and uninactivated (HPL), were prepared by pooling six paired leuko-reduced BC-PCs as described above. The average starting parameters of platelet content, volume, leukocyte content in the BC-PCs (Table 1) intended to PI treatment were in keeping with the recommended specifications both for the treatment of platelets with the INTERCEPT Large Volume processing system and for transfusional use



Compared with the paired untreated units, inactivated BC-PCs showed a lower platelet count due to the addition of Amotosalen and the consequent dilution. (Table 2) The multiple transfer steps, including the CAD incubation, reduced volume and platelet yield by up to 10%.

Sterility testing was negative in all BC-PC units.

In all batch of HPL and iHPL tested for the presence of endogenous and adventitious viruses only one sample resulted positive for Parvovirus B19 DNA (HPL). All iHPL resulted negative.

### MSC Harvest and Preparation

Nineteen bone marrow samples were collected from healthy donors: 10 over 18 years of age (age range: 39-50 years) and 9 with ages younger than 18 years (age range: 0.5-10 years). The study was conducted according to the Helsinki Declaration.

### MSC isolation and Expansion

Adherent cell clones were observed in all the samples after 7 days' culture and an adherent monolayer was achieved in the following 10-12 days. HPL/iHPL- MSCs but not FBS-MSCs reached confluence 7 days after plating (P1). HPL-MSCs reached a mean  $\pm$  standard deviation (SD) of cPDs after the 3<sup>rd</sup> passage of respectively  $6.88 \pm 0.049$ ;  $8.27 \pm 0.82$ ;  $12.64 \pm 1.11$  when they were expanded in a-MEM + 5%, 7% and 10% HPL respectively. At the 3<sup>rd</sup> passage, the cPDs of 10% HPL-MSCs were significantly greater than those obtained by culturing the cells in -a-MEM + 5%/7% HPL and a-MEM + 10% FBS ( $5.04 \pm 0.19$ ), used as the control ( $p=0.029$ ). On these bases, we chose a 10% HPL supplementation for all the following experiments.

### CFU-F potential and MSC morphology

After 7 days, the number of CFU-F/ $10^6$  cells was significantly greater when the cells were plated at 10000 cells/cm<sup>2</sup> (low density), compared to cells plated at 100000 cells/cm<sup>2</sup> (high density) independently from the cellular condition. The mean CFU-F number was:  $99.3 \pm 11.07$ ,  $106.8 \pm 12$  and  $99.5 \pm 9.6$  at a plating density of 10000 cells/cm<sup>2</sup> and  $33.1 \pm 4.75$ ,  $29.3 \pm 14.7$  and  $30.9 \pm 9.88$  at a plating density of 100000 cells/cm<sup>2</sup> respectively for HPL/iHPL/FBS MSCs (Fig. 1 A). Although the absolute number of colonies counted in the three culture conditions was not significantly different, we observed that MSCs from CFU-Fs grown in HPL and iHPL were smaller than those from FBS CFU-Fs. Moreover, when CFU-Fs from HPL and iHPL–MSCs were detached and analyzed for cell numbers, they were more populated, small and dense (Fig. 1 B; a, b) and reached confluence faster (Fig. 1 B; d, e) than those cultured in FBS (Fig. 1 B; c, f).

Moreover, we compared cells cultured in  $\alpha$ -MEM + 10% HPL vs iHPL vs FBS and we observed that HPL/iHPL-MSCs appeared smaller, had well visible nucleoli, and were more homogeneous in the morphological characteristics (Fig. 1C; a, b) than FBS-MSCs. FBS-MSCs, instead, increased in size and showed a polygonal morphology with a large jagged cytoplasm (Fig. 1C; c). Furthermore, when HPL/iHPL-MSCs were detached and re-plated, after 12 hours' of cell culture, they tended to reform colonies, emit prominent extensions morphologically similar to pseudopodia and, interestingly, create tri-dimensional structures similar to embryoid bodies (Fig. 1 C; d, e) which we had never observed in FBS-MSCs (Fig. 1 C; f).

### MSC expansion and immunophenotype

Cellular growth analysis showed that cPDs at the third passage were  $11.16 \pm 1.01$ ;  $11.00 \pm 0.98$ ;  $7.16 \pm 1.10$  (means  $\pm$  SD) respectively in HPL, iHPL and FBS-MSCs. Significant statistical differences were observed on the cellular growth in term of cPDs of HPL/iHPL-MSCs compared with FBS-MSCs at the 2<sup>nd</sup> and 3<sup>rd</sup> passages ( $p= 0.00041$  and  $p= 0.00032$  respectively) as shown in Fig. 2 which reported

the cPDs of the three passage of all BM-MSCs cultured in each condition with the relative means with standard deviations.

During the first 3 passages, MSCs were analyzed at each passage for the expression of: CD45, CD34 and CD14, hematopoietic surface antigens; CD90 (a membrane glycoprotein, also called Thy-1), used as a stem cell marker; CD105 (endoglin); CD73 (Ecto-5-prime-nucleotidase) and CD146 (cell surface glycoprotein MUC18). At the first passage, MSCs isolated from whole BM and cultured in a-MEM + 10% HPL, iHPL and FBS, were CD45, CD34 and CD14 negative with an antigen expression of under 5%, while they showed a high expression of CD90, CD73, CD105 and CD146. In all culture conditions, during the expansion time, the MSCs were negative for hematopoietic antigens, whereas at each passage, they expressed high percentages of CD90, CD73, CD105 and CD146. No statistical differences were observed among the three different media. Table 3 shows the mean percentage of antigen expression which was analyzed at the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> passages on the cells cultured with 3 different supplemented media. The fluorescence means of the positive markers at the third passage showed a slight increase for CD90 marker and very slight for CD73 in HPL and iHPL-MSC in comparison with FBS-MSCs. In particular, fluorescent mean intensity was for CD90:  $37301.14 \pm 7569.091$ ,  $31360.14 \pm 5628.379$  and  $25385.86 \pm 4410.403$ , for CD73:  $194 \pm 73.347$ ;  $884.43 \pm 703.200$  and  $194.00 \pm 73.347$  and for CD105:  $16834.86 \pm 4153.280$ ;  $17940 \pm 4198.606$  and  $16054.43 \pm 3829.858$  respectively for HPL, iHPL and FBS-MSCs but also here the differences were not significantly differenced (p calculated by Friedman test for CD90 = 0.276; for CD73 = 0.651 and for CD105 = 0.867. We noted a strong correlation between the values obtained in HPL and iHPL in all the marker analyzed, a correlation  $< 0.098$  with  $p < 0.001$  to demonstrated that PI doesn't interfere with the MSC immunophenotype.

#### Pluripotency markers expression analysis

As HPL/iHPL-MSCs when detached and re-plated tended to form aggregates of cells similar, in structure, to embryoid bodies, which were not seen in FBS-MSCs, we analyzed embryonic stem cells markers such

us Oct-3/4 and NANOG by immune-cytochemical technique and real time PCR. We observed positivity of these markers both at proteic and molecular level. In particular, we observed that the intensity of Oct-3/4 and Nanog nuclear protein expression was higher in HPL/iHPL-MSCs compared to FBS-MSCs, in term of number and expression intensity. As shown in Fig. 3, in the cellular structures formed by HPL/iHPL-MSCs the fluorescence is more concentrated in the nucleus (Fig. 3 A; a, b and B; g, h) than that observed in isolated cells, in which both nuclear and perinuclear distribution appeared (Fig. 3 A; c and B; i) . By counting the positive cells we reported that HPL-MSCs were:  $100\% \pm 0$  and  $100\% \pm 0$ ; iHPL-MSCs:  $100\% \pm 0$  and  $97\% \pm 3.1$ ; FBS-MSCs:  $89\% \pm 6.6$  and  $82\% \pm 13$  respectively for Oct-3/4 and NANOG protein expression. These data were confirmed by real time PCR where in all analyzed samples we observed that HPL and iHPL induced a higher Oct-3/4 and Nanog gene expression in comparison with FBS (Oct-3/4 mean RQ values:  $4.2 \pm 2.7$ ;  $4.6 \pm 2.2$ ;  $1 \pm 0$ ; NANOG mean RQ values:  $1.8 \pm 0.6$ ;  $1.9 \pm 1.6$ ;  $1 \pm 0$  respectively for HPL, iHPL and FBS-MSCs). The rights panel in the Fig. 3 A and B showed an representative experiment of MSCs isolated and expanded from the same BM sample in 3 different cellular conditions. Friedman test, both for protein and mRNA expression, showed a statistical significant difference for Oct-3/4 ( $p=0.050$  and  $p = 0.022$  respectively).

### MSC Differentiation Potential Assay

The MSCs obtained in the 3 different conditions showed multipotent capacity because all samples, at the 3<sup>rd</sup> passage differentiated into osteoblasts, adipocytes and chondrocytes as shown in Fig. 4.

### MSC Karyotype Analysis and Tumorigenesis tests

Cells expanded in a-MEM + 10% HPL, iHPL and FBS did not show, at the third culture passage, any chromosome modifications.

The absence of colonies in soft agar allowed us to exclude tumorigenic transformation of MSCs expanded in all conditions as showed in Fig. 5.

#### MSC Viability and microbiological control

At each passage, viability was over 95% in all cell culture conditions and all microbiological analyses, including the mycoplasma test, were negative.

## DISCUSSION

This study shows that HPL itself is a safe and efficient MSC culture supplement for robust MSC cultivation, thus offering certain advantages compared to FBS, especially in terms of cell growth and stemness maintaining. Moreover, it represents a good GMP-compliant alternative to animal serum for MSC clinical production confirming the recent data reported in the literature (18, 36, 37).

HPL preparation was performed according to blood bank procedures where a high standardization is strongly recommended. The development of HPL batches from a multiple source (i.e. from 60 different donors), makes the HPL itself a virtually standardized medium supplement in both a growth factor concentration and in inter-donor variability.

Moreover, as the risk of transmission of infective agents not routinely tested, or for which no tests are available remains, HPL quality and safety had to be greatly improved. This was done with photochemical treatment by Amotosalem and UVA, a technology that is efficient against the vast majority of known pathogens and which might also prevent the transmission of unknown pathogens (25, 38-41)

INTERCEPT technology is routinely used for PI of PLTs and plasma for clinical use as it is able to inactivate a wide spectrum of bacteria, viruses, and parasites, as well as contaminating leukocytes (42-46). INTERCEPT process and nucleic acids targeting PI technology **is** not, however, effective against prion diseases, therefore the risk of prion transmission by treated lysates would remain. It is true that precautionary measures such as donor selection and leucocyte depletion can be taken.

The INTERCEPT Blood System is a Class III medical device, that means a registration which requires regulatory review of preclinical and clinical data. This system is routinely used in many blood centers in Europe, the Middle East and many other countries with at least 700000

INTERCEPT treated units transfused world-wide (38, 40). The manufacturer has supported an extensive program of post-market surveillance and hemovigilance studies to monitor the introduction of INTERCEPT products in Europe. In studies of over 30000 treated PLT units and over 30000 treated plasma units to date in a broad patient population, INTERCEPT PLTs and Plasma have a safety profile comparable with conventional components. The efficacy and safety of INTERCEPT PLTs have been established in 11 trials and over 1000 patients (47).

Moreover the recent Swiss haemovigilance data, reporting two years routine transfusion of 62,500 inactivated Platelet Concentrates, support the improvement safety profile and the successfully prevention of septic transfusion reactions after the introduction of the pathogen inactivation technique on platelet components (48).

INTERCEPT -treated platelets have received additional country-specific regulatory approvals in France, Germany, and Switzerland (23). A new application of this widely used technique might be a new niche for blood banks. The latter, in fact, could use this method to provide safer products for clinical cell culture and transplantation and, by doing so, increase safety for patients.

To our knowledge, no data have been reported about PI on PLT lysate for MSC expansion. Although, a recent study on the use of PI-HS compared with uninactivated pathogen-PI-HS (49) showed that PI does not exert a negative impact on human islets of Langerhans, MSCs, T cells or cell lines and may even have a positive effect by the down-regulation of inflammatory mediators induced by DNA or RNA strands released from damaged cells. We sought to compare simultaneously HPL and iHPL with FBS to exclude abnormalities in MSC isolation and expansion due to the inactivation itself.

The literature has widely reported the efficacy of psoralen-UVA inactivation using blood products with high titers of added bacteria, enveloped and non-enveloped viruses, and protozoa and the demonstration of the inactivation of contaminating pathogens was beyond the scope of this publication. However, we tested on lots of HPL and iHPL a number of viruses that are not required by Regulatory Authority and not routinely tested by Trasfusional Center. The negativity of almost all of the viruses tested show that a good selection of donors by the Transfusion Center is a fundamental requirement to have safe starting blood component. Parvovirus B19 can be transmitted from asymptomatic blood donors to the recipients of their blood components. High rates of seroconversion, as well as a few cases of symptomatic illness and hypoplastic anemia, have been described in patients receiving clotting factor concentrates, which were derived from large plasma pools. The virus is relatively heat-stable, and it resists treatment with solvents and detergents. B19 DNA was found by PCR in plasma-derived clotting factor concentrates from various manufacturers and treated with different virus-inactivation methods by many investigators. Clinically evident transfusion-transmitted B19 infection, however, is infrequent, even in susceptible hosts such as HIV-infected hemophilia

patients receiving clotting factor concentrates. We had only a positivity for Parvovirus B19. virus ... in a lot of HPL which resulted negative after inactivation to testify the PI process also in our hand is efficient and offers more safety.

In our hands, INTERCEPT treatment on HPL resulted in a 10% loss of platelets but these data confirmed those reported by Wagner et al (50), and did not affect cell culture behavior.

We consider that PI treatment carries on the possibility that traces of psoralens may contaminate iHPL. Using the Compound Adsorption Device (CAD), the levels of residual Amotosalem are greatly reduced and the psoralen concentration in platelets is about 0.5 micromolar, that is the approximate concentration in the iHP. We reduced ten times this concentration using HPL at 10% in the cell culture. We don't test the presence of psoralen inside the MSCs expanded in HPL but considering that during extracorporeal photochemotherapy, which is used in variants of cutaneous T cell lymphomas, graft-versus-host disease, systemic sclerosis, in solid organ transplant rejection and Crohn's disease (51), a leukocyte fraction from the peripheral blood is exposed to about 40-50 mM of photo activated 8-methoxypsoralen and reinfused into the patient without collateral effects, we consider the psoralen traces in our MSC clinical product absolutely negligible and safe. However, as for transfusional purposes INTERCEPT products are contraindicated in patients with a history of allergic response to psoralens, we consider to declare in the certificate of analysis that traces of psoralens might be present in the MSCs isolated and expanded in GMP for the clinical application to exclude their use in psoralen allergic patients.

In this work, we found that HPL and iHPL had similar characteristics: they were more advantageous, in terms of cellular growth, than FBS and they did not interfere with mesenchymal phenotypes as they did not modify the mesenchymal marker molecule expression. Moreover, HPL and iHPL did not cause chromosomal alterations or karyotype modifications in cells expanded up to the 3<sup>rd</sup> passage. Interestingly, when whole BM was seeded in HPL and iHPL no significant differences in the CFU-F number were observed compared with FBS-MSC, but they appeared more dense and richer in term of cells/colony. This observation was confirmed in terms of the number of detached cells at each passage because HPL and iHPL-MSCs showed higher proliferative potential. Moreover, they were smaller and more morphologically homogeneous than FBS-MSC. Differences in their CFU-F potential and colony morphology may be representative and possibly predictive of cell fate and function. Recently, Gothard et al. (52) reported that CFU-Fs can be analyzed combining two different pieces of information: diameter and density, which are properties related to and affected by cell proliferation, and mobility and

differentiation potential. On these bases (53), small and dense colonies, which we observed in HPL and iHPL –MSCs, represent the BM-MSC fraction with good CFU-F capacity. These data suggested a higher clonal expansion of HPL/iHPL-MSCs, that might be predictive of greater stemness than FBS-MSCs.

In one batch of HPL and iHPL, we tested the presence of growth factors such as PDGF and FGF. We did not observe any differences in terms of quantity (data not shown), but we maintain that the growth promoting effect of HPL and iHPL is probably due to growth factors or specific compounds which are released from the platelet fraction .

Another important aspect observed in both HPL or iHPL was the formation of spherical structures similar to embryoid bodies which, together with the pseudopodia emission (Fig. 2), suggested that HPL/iHPL-MSCs had a more primitive stemness than FBS-MSC, as shown in MSCs isolated from neonatal tissues compared with adult tissue (54). As reported in the literature, the pseudopodia emission may suggest a trend of the cells to migrate and, consequently, to repopulate damaged tissues and organs with potential implications for regenerative medicine (55). The entity of this occurrence is higher in HPL/iHPL-MSCs compared to FBS-MSCs.

To verify whether these morphological and proliferative characteristics of the HPL/iHPL-MSCs might be linked to more stemness than FBS-MSC, we analyzed the presence of pluripotency markers such as Oct-3/4 and NANOG, which we had already observed in MSCs isolated from neonatal tissue such as the amniotic fluid (56). Oct-3/4, is a key transcription factor essential to the pluripotent and self-renewing phenotypes of undifferentiated embryonic stem cells (ESCs) (57). NANOG is a homeodomain protein present in pluripotent human cells which plays a critical role in the regulation of the cell fate of the pluripotent inner cell mass during embryonic development, maintaining the pluripotent epiblast and preventing differentiation to the primitive endoderm (58). NANOG is thought to function in concert with other factors such as Oct-3/4 itself. Interestingly, the spheroid structures that were observed in HPL and iHPL, not only during the seeding, but also when the MSCs were detached and replaced in the next



passages after the 1st, were highly positive for both Oct 3-4 and NANOG. Moreover, during the expansion HPL and iHPL induce a selection of more primitive MSCs in comparison with FBS.

Although HPL/iHPL-MSCs exhibit increased cell growth, in terms of cPD, and characteristics attributable to higher primitive stemness than FBS-MSCs, we demonstrated that neither the HPL nor iHPL caused *in vitro* tumorigenic mutations or karyotype modifications in cells expanded up to the 3<sup>rd</sup> step. These data are in accordance (only for HPL) with that reported in the literature about GMP-compliant isolation and expansion of BM-MSC (59-61). Moreover, we previously reported that, by plating whole BM at a low cellular density, it was possible to expand high numbers of MSCs for clinical use (30). In the present study, we further showed that, independently from the culture condition, the number of CFU-F/10<sup>6</sup> cells was significantly greater when the whole BM cells were plated at a low density, compared with cells plated at a high density. These results emphasized the advantage of the low seeding density of whole BM to isolate MSCs for large scale use. The use of HPL as an alternative to FBS to isolate and expand MSC confirmed that it is possible to obtain a number of MSCs for clinical doses further reducing the manufacturing time, limiting the passage or reducing the starting volume of BM. Finally, we demonstrated that PI treatment did not modify the characteristics of HPL, it does make it safer and more suitable for MSC isolation and expansion for clinical use and might be a requirement usable for the GMP MSC expansion .

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## Figure Legends

### **Figure 1: HPL and iHPL-MSCs showed differences in CFU-F and cellular morphology in comparison with FBS-MSCs**

**A:** CFU-F numbers of HPL (white triangle), iHPL (grey square), FBS (black square)-MSCs plated at seeding densities of 10000 cells/cm<sup>2</sup> and 100000 cells/cm<sup>2</sup>. Each symbol represents an experiment (N=9) and no significant differences were observed.

**B:** Representative phase pictures at 5X magnification of CFU-Fs in the 3 different conditions: HPL (a) and iHPL (b)-MSC CFU-Fs were more dense, homogeneous and populated of cells than FBS-CFU-Fs (c). Moreover, HPL and iHPL-MSCs (d, e) reached the confluence faster than FBS (f)-MSCs

**C)** Representative phase pictures at 40X magnification showing the MSCs in the 3 different culture conditions after 3 and 12 hours from plating: HPL and iHPL-MSCs emitted prominent extensions (a, b) and formed spherical structures similar to embryoid bodies (d, e). FBS-MSCs showed a polygonal morphology with large jagged cytoplasm (c) and did not form three-dimensional structures (f)

### **Figure 2: HPL and iHPL-MSCs showed higher proliferative potential than FBS-MSCs**

**A, B, C:** Cumulative PD (cPD) of HPL, iHPL, FBS-MSCs (dashed lines). Results are shown as the cPD value at the first three passage of each 16 independent experiments in the three culturing condition. The means  $\pm$  SD are represented in each graph as a black thick line. HPL and iHPL-MSCs showed a higher proliferative potential than FBS-MSCs from the second passage of culture. Asterisks indicate statistically significant differences ( $P < 0.01$ ).

### **Figure 3: HPL and iHPL-MSCs showed more embryonic stem cell markers protein and mRNA expression than FBS-MSCs**

**A and B:** On left part of the panel, Immunocytochemistry analysis of embryonic markers in HPL (left column), iHPL (central column) and FBS (right column)-MSCs (40X magnification); in the right part of the panel a representative experiment of real time PCR: Oct-3/4 (a, b, c) and NANOG

(g,h, i) were more expressed in HPL and iHPL-MSCs in comparison to FBS-MSCs both in term of protein both in term of mRNA. For immunocytochemistry , pictures are representative of 3 experiments and DAPI staining was used to evidence the nucleus of each analyzed cell (d, e, f ,l, m, n). For real time PCR, 5 experiments were conducted but the two panels show the most representative experiment.

**Figure 4: MSC Differentiation Potential Assay after 3 weeks of specific induction in MSCs in the 3 different conditions.**

Van Kossa staining (a, b, c) evidenced the presence of calcium oxalates in osteoblasts, Oil Red showed intracytoplasmatic vacuoles in adipocytes (d, e, f) and Alcian Blue (g, h, i) the hyaluronic acid for chondrocytes respectively in HPL (left column), iHPL (central column) and FBS (right column)-MSCs (N=19).

**Figure 5: Soft agar assay to exclude tumorigenesis potential in MSCs in the 3 different conditions**

(N=3) HPL, iHPL, FBS-MSCs (a, b, c) did not form colonies as whole BM used as negative control (d, e, f) compared to primary osteosarcoma tumor cells used as positive control (g, h, i).

**Table 1:** Characteristic of BC-PCs prior to and after inactivation treatment

	Large Volume Set specifications (range)	Pre inactivation n=6	Post inactivation n=6
<b>Platelet content (<math>\times 10^{11}</math>)</b>	2.5 - 7.0	2.5 – 3.3	2.1 – 2.8
<b>Volume ml</b>	255 - 390	307 - 321	286 - 309
<b>CAD Agitation Duration</b>	16-24 hours	n/a	16
<b>Leukocyte content (<math>10^6</math>/unit)</b>	1	-	0.03- 0.84

The values were expressed as mean  $\pm$  SD

**Table 2:** Characteristics of inactivated BC-PCs and uninactivated BC-PCs

	Inactivated BC-PCs	Uninactivated BC-PCs
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<b>Volume ml</b>	299 ± 9.0	321 ± 8.9
<b>Platelet count (x10<sup>3</sup>/μl)</b>	836 ± 85	895 ± 116
<b>Platelet content (x 10<sup>11</sup>)</b>	2.5 ± 0.3	2.9 ± 0.3
<b>Leukocyte content (10<sup>6</sup>/unit)</b>	0.18 ± 0.3	0.19 ± 0.3

The values were expressed as mean ± SD

**Table 3: Immunophenotypic analysis of HPL-MSCs vs iHPL-MSC vs FBS-MSC**

	HPL-MSCs			iHPL-MSCs			FBS-MSCs		
<b>Passage</b>	<b>P1</b>	<b>P2</b>	<b>P3</b>	<b>P1</b>	<b>P2</b>	<b>P3</b>	<b>P1</b>	<b>P2</b>	<b>P3</b>
<b>CD90</b>	99.1±1.4	100±0.1	99.6±0.1	98.5±2.5	97.8±3.8	99±0.9	98.8±0.6	99±0.4	98.9±0.5
<b>CD73</b>	99.1±1.0	98.5±1.4	97.9±0.1	98.8±1.0	99.1±1.0	99.1±1.0	97.4±1.6	95.5±3.1	98.7±0.7
<b>CD105</b>	93.9±2.1	95.7±2.7	92.7±6.4	92±6.6	88±5.2	95.9±4.6	95±4.3	91.6±5.3	98.1±1.6
<b>CD34/45/1</b>	1.4±1.0	0.44±0.1	0.4±0.3	1.8±0.6	0.6±0.6	0.3±0.8	1.8±1.7	0.5±0.2	0.4±0.4
<b>CD146</b>	92.5±1.9	93.8±2.3	92.2±1.9	85.2±6.0	91.7±6.2	93±1.1	94.2±5.0	94.7±2.7	90.8±4.0

Values are expressed as the mean percentage of fluorescence ± Standard Deviation of MSC antigen expression analyzed at the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> passages for each experimental condition (N=16). No significant differences were observed.