Characterisation of the immuno-inflammatory response following operative adolescent idiopathic scoliosis and biological properties of osteoprogenitor cells harvested from vertebral bodies compared to iliac crest.

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Submitted in accordance with the requirements for the degree of Doctor of Medicine

The University of Leeds
School of Medicine

November, 2016

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly authored presentations has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Jointly authored podium presentations

1) Mesenchymal stem cells from vertebral body and iliac crest bone marrow: comparison of two tissues and two harvesting techniques.
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Presented at:
1) 5th International Satellite Symposium AICC-GISM, Advances in Mesenchymal Stem Cells research**.** 14/11/2014, Verona, Italy
2) 100th National Congress SIOT. 7-10/11/ 2015, Rome, Italy

2) Mesenchymal stem cells harvested from vertebral body and iliac crest bone marrow: Are they equally good? Comparison of two tissues and two harvesting techniques.
Evangelos M. Fragkakis, Elena Jones, Ippokratis Pountos, Peter Giannoudis
Global Spine Congress and World Forum for Spine Research Dubai, United Arab Emirates, April 13–16, 2016

Work attributed to Evangelos M. Fragkakis - Design of the study and the experiments, patients recruitment, collection of data and samples, performing the experiments, data analysis and interpretation, first and final drafts, literature review, composition of the figures, submission.
Work attributed to other authors - (EJ) Designing experiments, data analysis and interpretation, final draft (EJ, IP, PG)

Abstract

In patients undergoing adolescent idiopathic scoliosis (AIS) surgery the yield and differentiation capacities of mesenchymal stem cells (MSCs) from vertebral body (VB) and iliac crest (IC) bone marrow (BM) were investigated comparing the effectiveness of two MSC-harvesting procedures [density gradient centrifugation (Lymphoprep, LMP) and red cell lysis (Ammonium Chloride, AC)]. The burden of surgery of 3 surgical approaches (posterior, posterior-costoplasty, antero-posterior), for AIS correction was also quantified.

In 18 patients 10ml of BM were aspirated intraoperatively from IC and VB and MSCs were isolated using AC and LMP harvesting procedures. MSCs were enumerated and phenotypically characterised using colony-forming-unit-fibroblast (CFU-F), flow cytometry and trilineage differentiation assays. For the immuno-inflammatory study 23 patients were recruited. ELISA-assays were performed on serum from 4ml of peripheral blood, at 6 peri-operative time-points (T), measuring 4 cytokines [(Interleukin) IL-6, IL-10, IL-18 and Procalcitonin (PCT)]. Surgical parameters and gender variability were also analysed.

Both IC and VB exhibited similar concentrations of MSCs, however AC yielded significantly more MSC/ml by 1.8-fold (IC, $p=0.012$) and 1.5-fold (VB, $p=0.043$), compared to LMP. Calcium and glycosaminoglycan (GAG) concentrations were both significantly higher from VB MSCs, compared to IC [Calcium: AC 8.7-fold ($p=0.001$), LMP 1.2-fold, GAG: AC 3.8-fold ($p=0.0005$), LMP 1.9-fold ($p=0.003$)]. Adipogenesis did not show significant differences. VB AC-harvested MSCs presented a significantly smaller colony area by 1.9-fold ($p=0.039$), compared to IC AC.

The post-operative concentrations of IL-6 and IL-10 were always significatintly elevated at T2 and T3 points, in all 3 approaches ($p<0.01$) with antero-posterior approach presenting the highest levels followed by posterior-costoplasty and posterior approaches. Although prolonged procedures and gender type affected cytokine concentrations, their levels were not statistically significant.

In summary, AC isolated more effectively MSCs, without affecting their function. VBBM MSCs demonstrated higher osteo- and chondro-genic differentiation capacities, representing a valid source of MSCs in spinal surgery. The surgical stress reaction quantified with immune-inflammatory markers, was higher in more aggressive and extensile procedures, suggesting greater vigilance in identifying and preventing complications.

Acknowledgements

I would like to thank my main supervisors Prof Peter Giannoudis and Dr Elena Jones for their constant guidance, encouragement and support. Prof Peter Giannoudis for believing in me and giving me this precious opportunity and also for supervising and helping me with the clinical and logistic aspects of this research. Dr. Elena Jones has been my main guidance in organising my experimental studies and has supervised each and every part of my research. Their combined knowledge and mentorship it was precious and without their help and efforts this work would not have been possible as it stands. Moreover, I would like to express my appreciation to my third supervisor Dr Ippokratis Pountos for the valuable learning opportunities he provided.

I would like also to thank the Leeds General Infirmary spinal team for their enthusiasm and generosity in providing the bone marrow samples. In particular I am grateful to Mr Robert Dunsmuir, Mr. Peter Millner and Mr Abhay Rao, for their constant support and encouragement throughout my study period, as well as their vision in the transitional aspects of the project. Their contribution in optimisation of the bone marrow aspiration technique, their help with recruitment and logistics, were fundamental aspects for the outcome of this research.

Moreover, I would like to thank; Dr Filomena O.G. Esteves for her help in chondrocyte pellets staining techniques; Dr Jehan El-Jawhari for her valuable assistance with flow cytometry and Dr Yasser El-Sherbiny for his help with ELISA assays. Also I am grateful to Karen Henshaw for helping me to build my confidence with tissue culture lab techniques, providing unsurpassed training and support.

Special thanks goes to Dr. Wanyi Wang, for her great help and suggestions in the statistical analysis of the ELISA and biochemical results.

I would also like to thank for their valuable contribution the Leeds General Infirmary minors' pre-assessment team for their help in the challenging patients' recruitment, and also the nurse staff in wards L24, 25, 39, 40, 41 for their help in peripheral blood and clinical data collection.

I wish to acknowledge the Italian Society Of Trauma and Orthopaedics (SIOT) and the Yorkshire Children's Spine Foundation for their financial support, which mainly included laboratory bench fees and reagents.

I wish to express my gratitude to Prof Pasquale Bianchi, Chairman of the Orthopaedic clinic in the Second University of Naples, for his support, valuable suggestions and training during the years of my specialization. Without this background it could not have been possible to stand where I am now.

I would like also to thank all the patients and their families for taking part in this study.

Last but not least I wish to thank my family and friends for their ongoing support and understanding during the entire period of this work.

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Abbreviations

(AC)	Ammonium chloride
(AIS)	Adolescent idiopathic scoliosis
(ALP)	Alkaline phosphatase
(AP)	Antero-posterior approach
(AR)	Alizarin red
(ARDS)	Acute respiratory distress syndrome
(BM)	Bone marrow
(BMP)	Bone morphogenetic proteins
(BP)	Blood pressure
(BSA)	Bovine serum albumin
(CFU-F)	Colony forming unit - fibroblast
(COX-1)	Cyclooxygenase-1
(CPC)	Cresolphtalein complex-one
(CRP)	C-reactive protein
(CV)	Coefficient of variation
(D)	Days
(DMEM)	Dulbecco's modified eagle's medium
(DMSO)	Dimethyl sulfoxide
(ELISA)	Enzyme linked immunosorbent assay
(F)	Female
(FDA)	US Federal Drug Administration
(FSC)	Fetal calf bovine serum
(G.C.P.)	Good clinical practice
(HB)	Haemoglobin
(HLA-DR)	Human leukocyte antigen - D related
(HSC)	Hematopoietic stem cell
(IC AC)	Iliac crest ammonium chloride
(IC LMP)	Iliac crest lymphoprep
(IC)	Iliac crest
(ICBM)	Iliac crest bone marrow
(IL)	Interleukin
(IL-1RA)	Interleukin 1 receptor antagonist
(INF)	Interferon
(L1)	Lumbar 1st vertebra
(LGI)	Leeds General Infirmary Hospital
(LMP)	Lymphoprep
(M)	Male
(MACS)	Magnetic-activated cell sorting
(MEA)	2-aminoethanol buffer
(MFI)	Median florescent intensity
(min)	Minutes
(mln)	Millions
(MNCs)	Mononuclear cells
(MODS)	Multiple organ dysfunction syndrome
(MSCs)	Mesenchymal stem cells
(N., **n., #)**	Number
(n/a)	Not available
(NK)	Natural killer
(NSAIDs)	Non steroidal anti-inflammatory drugs
(OCT)	Optimal cutting temperature compound
(OD)	Optical density

(P)	Posterior approach
(p(s))	P value(s)
(P+Cost)	Posterior approach + costoplasty
(PB)	Peripheral blood
(PBS)	Phosphate buffered solution
(PCT)	Procalcitonin
(PD)	Population doubling
(PMN)	Polimorphonuclear
(PR)	Pulse rate
(PT)	Prothrombin
(PTT)	Partial thromboplastin
(RR)	Respiratory rate
(RT)	Room temperature
(SCs)	Stem cells
(SEM)	Standard error of the mean
(SIRS)	Systemic inflammatory response syndrome
(T)	Time point
(TGF)	Transforming growth factor
(TNF)	Tumour necrosis factor
(V11)	Thoracic 11th vertebra
(V12)	Thoracic 12th vertebra
(VB AC)	Vertebral body ammonium chloride
(VB LMP)	Vertebral body lymphoprep
(VB)	Vertebral body
(VBBM)	Vertebral body bone marrow
(WBCs)	White blood cells or Leucocytes
(4-PL)	Four parameter logistic analysis

1. Introduction

1.1.1 Description of adolescent idiopathic scoliosis (AIS)

Scoliosis is a three dimensional deformity of the spine. It is characterised by a lateral deviation in the frontal plane, rotation and torsion of the vertebral bodies, associated also with progression over the years, in a percentage of patients. It is a relatively common clinical finding, especially in the less severe grades[1]. Based on a demographic analysis performed by the American National Scoliosis Foundation, spinal deformity affects approximately 2-3% of the general population, corresponding to an estimation of 7-9 million people in the United States. In the vast majority of cases, is not possible to identify a cause and so it is classified as idiopathic. The treatment can vary from physiotherapy and use of special braces, to surgical correction when scoliosis progression can not be otherwise controlled. From the affected population, 38.000 patients undergoing spinal fusion surgery (Figure 1a,b,c) every year. Similarly in the UK, one thousand surgical procedures for spinal deformity correction are approximately performed anually[2].

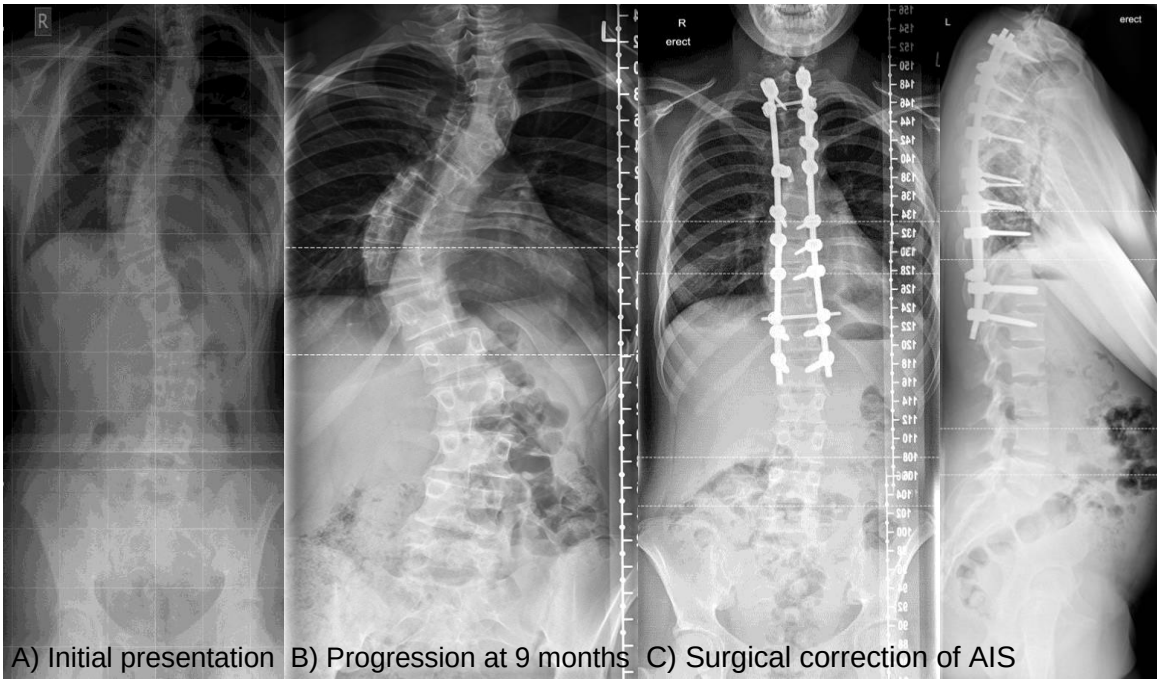

Figure 1: Standing X-Rays of patient presenting scoliosis deformity: A) At initial presentation, B) Progression after 9 months of follow-up, C) Antero-posterior and lateral projections at post-operative control. The metalwork used in this case was bars, pedicle screws and cross connectors.

The surgical correction of AIS is a well established procedure, able to deliver substantial improvement and subsequently produce dramatic changes in patients' quality of life[3].

The surgical procedure for AIS is characterised by two fundamental steps: correction of the deformity and fusion of the vertebrae involved (arthrodesis), in order to preserve the surgical correction. Spinal fusion is a fundamental element of the surgical procedure and its success is achieved by two components; the use of metallic implants (immediate) and the biological fusion (long term) of the vertebrae involved. Metalwork secure the correction achieved intraoperatively, whilst biological fusion ensure the preservation of the correction, virtually for the rest of patients' life. Implants are made from different metal alloys, to achieve adequate mechanical behaviour and desired strength for the human body[4,5]. Routinely, implants remain in situ for patient's lifetime and the only reason to be removed, is in case of complications, such as infections, pain or failure of the metalwork[2,6].

The instrumentation is lacking of "self-repairing" possibilities and can resist for a relatively limited period of mechanical stress cycles. Whilst the biological fusion is achieved via the iatrogenic micro-fractures of the posterior elements of the vertebrae, in combination with the addition of graft material which incorporates with the patient's skeleton.
A fast biological fusion will ensure a better preservation of the intraoperative correction and also will limit the rate of pseudoarthrosis associated with failure of metalwork[1]. For this reason it is crucial to guarantee the earliest bony fusion of the vertebral bodies involved within the scoliosis correction construct. Moreover, a fast biological fusion will improve the rehabilitation outcome and the prompt return of the patients to the previous life standards. A satisfactory bone callus formation is radiologically evident within 6-12 months approximately from the date of the surgical correction[1].

In order to enhance the healing process autograft material is traditionally used from the iliac crest (IC) which is considered the gold standard source due to its properties. This practice, despite being in use for many years, is not devoid of risks and early or late complications. One of the biggest complication, which affect approximately 40% of the patients, is the pain from the donor site[7,8]. Other complications are related to superficial or deed infections, formation of seromas or haematomas, neurologic

injuries, structural weakness of the iliac wing and delay of the rehabilitation process[7,8].

In recent years new advances in the field of cellular and molecular mechanisms involved in the biology of fracture healing, provided with new solutions able to improve the spinal fusion process. These include the use of BM-MSCs combined with scaffold materials and also growth factors, like bone morphogenetic proteins (BMPs). The use of which has become recently controversial[7,9,10].
Both ICBM and VBBM are rich of stem cells and can be used for augmenting vertebral arthrodesis, due to the high potential of proliferation and osteogenic differentiation. The potential of BM and in particular of VBBM, as a valid alternative to enhance bone fusion in combination with graft[8,11-13], is still not adequately explored in spinal field.

Scope of this study is to shed more light among the differences between IC- and VB-MSCs, isolated with two different methods and also focus on the effects of the surgical treatment on the immuno-inflammatory system.

1.1.2 Bone tissue

Spine, due to its nature and function, combines two apparently opposite biomechanical concepts, flexibility and stability[14]. The skeleton, and in particular spine, has a fundamental role in human evolution[14]. From a biomechanical point of view, provides mechanical support, locomotion and protection of vital organs (chest cage, brain, medullar canal)[14,15]. Moreover, from a metabolic point of view, its high calcium content, makes it extremely important for calcium homeostasis. Furthermore, the bone marrow contained within the cancellous bone, represent the main site for the hematopoietic and the mesenchymal stem cells production.

The skeleton, is a connective tissue and its metabolic activity is influenced by a great variety of biochemical, biomechanical, cellular and hormonal factors. Under physiological conditions, the harmonic balance of these factors play an important role in the maintenance of bone turnover and they become key players in the reparative process[16], when a fracture occurs.

A bone fracture is defined as a high concentration of forces able to exceed the strength/resistance of the bone, causing disruption of its continuity[14-16]. Given the spectrum of fractures there are numerous classification systems, providing a common language to describe them accurately. Fractures can be defined based on their anatomic location (metaphysis, diaphysis, epiphysis), their relation with joints (intra- or extra-articular), based on fracture's three-dimensional configuration of the fragments (comminuted displaced, undisplaced, transverse, spiral, etc.). Following a fracture, the bone can be exposed to the outside environment (open fracture), which is associated with a higher risk of infection and non-union, or in case the skin remains intact (closed fracture) the bone is protected and the sterility is maintained[16].

From an aetiological perspective fractures can be classified as: 1) caused by a sudden injury applied directly or indirectly on a healthy bone, which are the most common, and 2) pathological fractures, occurring in subjects where bone tissue is already weak, mainly due to local or systemic metabolic condition (fatigue fractures osteoporosis, tumours), or also to genetic predisposition[14,16]. Iatrogenic fractures are performed intentionally to correct axial malalignments, or in case of spinal surgery, micro-fractures in addition to bone-graft can guarantee the spinal fusion via reactivation of the bone healing process (Figure 2).

1.1.3 Physiology of the fracture healing

Following a fracture, the bone tissue activates physiologically reparative mechanisms regulated by the coordinated action of a variety of mediators and cells. This process is characterised by the unique feature of healing without scarring, due to continuous remodelling and bone homeostasis, compared to other tissues[14-16]. Conventionally, this process consist of three stages: initial inflammatory response, followed by osteogenic/angiogenic repair (soft and hard callus) and finally bone remodelling[15,16,18], known as indirect (secondary) process (Figure 2). With the advances in the orthopaedic field and the use of metalwork in fracture reconstruction, through a rigid construct and minimal gaps between bone ends, it was achieved a more direct (primary) type of fracture healing minimising the second stage of callus formation[16,17]. In the spinal field, however, the presence of bone callus is paramount in order to achieve the desirable biological fusion. In this study will be mainly described the secondary fracture healing process.

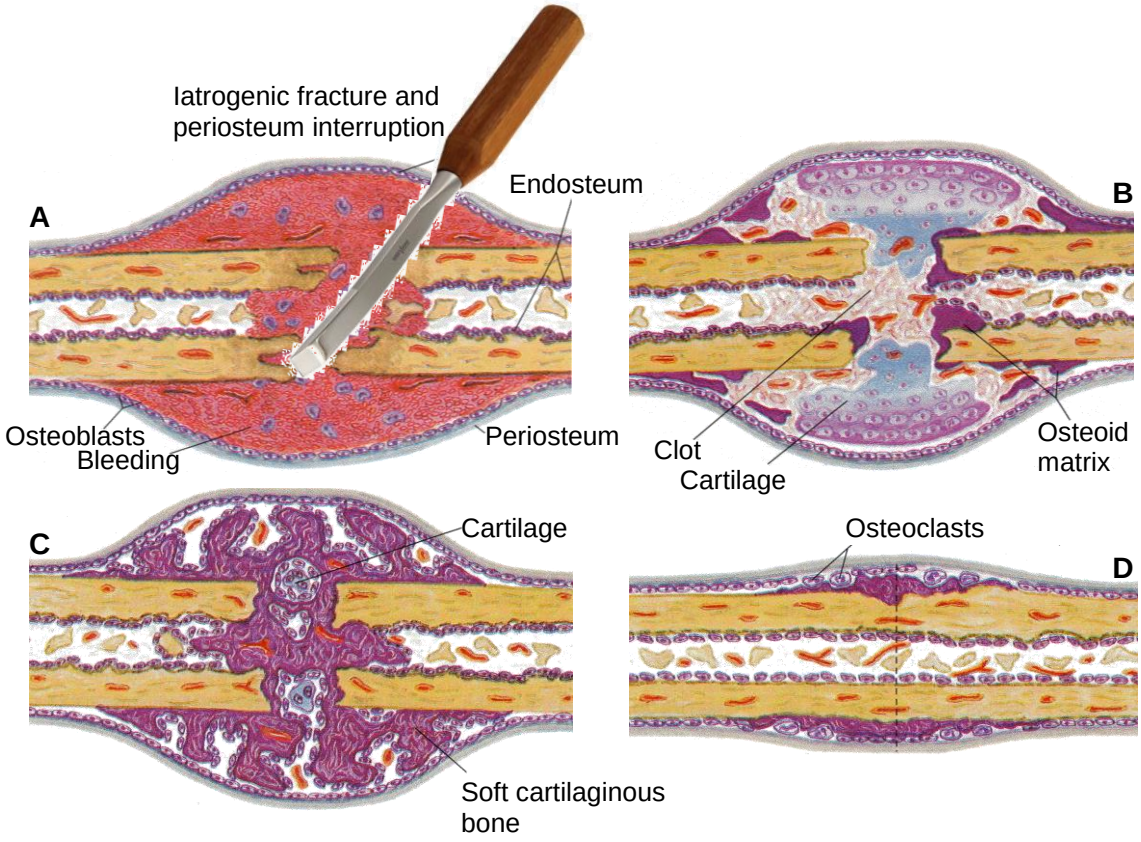

Figure 2: Secondary bone healing process. Schematic representation, following iatrogenic fracture. (Adapted from Netter's Orthopaedics, Italian edition, 2007, following explicit permission from Elsevier.)

The secondary fracture healing process is the most common type of fracture repair mechanism and is characterised by the production of callus. It simulates both intramembranous and endochondral ossification, depending on the type of fracture and the degree of stability, in a multi-step process with many similarities to embryogenesis during skeletal development[16].

During endochondral ossification, MSCs differentiate into chondrocytes, producing a cartilaginous structure, which becomes the "foundation-stone" for the following angiogenetic and osteogenetic steps of the healing process. Contrarily, the cartilaginous phase is missing during intramembranous ossification (clavicle) and the new bone is formed in a more direct manner following inflammation[16,17].

Following a fracture, the hematoma between and around the fracture ends, triggers the inflammatory response which peaks within 24 hours and lasts approximately a week[19] (Figure 2a).

During this stage, phagocytes and macrophages are attracted to the fracture site removing in a first place the debris, preparing subsequently the ground for the MSCs' homing and the beginning of the reconstruction stage. During the inflammatory process, the release of mediators, grow factors and cytokines (TNF-α, IL-1, IL-6, IL-11 and IL-18), promote further the recruitment of more inflammatory cells stimulating angiogenesis, MSCs and the extracellular matrix formation[18-20]. BMPs 2 and 7, but also 4, 9 and 14, have a fundamental role in this process, acting on different types of cells, including MSCs. BMPs, are known to be responsible for MSCs homing, MSCs chondroblastic and later osteoblastic differentiation, following the dynamic pathway of the healing process[21].

Gradually, at the end of the first week, the inflammatory stage is succeeded by the repair stage (Figure 2b, c). MSCs, under the effect of BMPs, signalling molecules and growth factors, are differentiated into chondroblasts, which consequently become chondrocytes, providing a soft callus[22]. This offers a temporary mechanical support, as well as a scaffold to use during the angiogenesis process. Adequate blood supply is a critical phase for the further progression of the healing progress, which leads to the transformation of the soft callus into hard callus, through the mineralization of the extracellular matrix. This stage lasts approximately 4-6 weeks[16,18-21].

The remodelling of the callus represents the final stage of secondary bone healing process. It is performed though the coordinated action between callus resorption from the osteoclasts and lamellar bone deposition from the osteoblasts[18-21]. This is a slow process, which may take years to be completed and is influenced by a variety of parameters including age, biomechanical stability and metabolic factors at local and systemic level.

1.1.4 Risk factors affecting bone union

Despite the fact that bone tissue is physiologically predisposed to heal following a fracture, sometime fracture healing mechanisms fail. Given the multiple factors that can influence the fracture healing process, there is no yet international consensus regarding the cut-off point (in terms of timing) between fracture union and non-union. However, the US Federal Drug Administration (FDA) council, defines a non-union as a fracture which does not present radiological and clinical signs of healing nine

months after the initial injury and without signs of further biological activity for the last three months[22].

On the other hand, fracture union is characterised by the absence of motion and tenderness at the fracture site during physiologic biomechanical load. Moreover, from a radiological perspective, union is diagnosed when a callus formation is bridging the fracture site in a satisfactory manner to ensure stability[14-16]. Between union and non-union, lies the delayed union which is the period between the expected time to union, for the anatomic site of interest, and the development of a frank non-union[14-16].

Several systemic and/or local factors are known to be associated with possibility of a fracture progressing to non-union. General factors such as age, dietary habits, diabetes and osteoporosis[23] can influence the reparative mechanisms contributing to the development of non-union[14-16]. Contrarily, children and young adults, due to a better blood supply at local level and grater MSCs potential tend to heal faster[18,22].

Another factor affecting negatively bone healing is smoking. Nicotine, present in tobacco, apart from the known vasoconstriction effect at local level, is able to interfere with osteoblast proliferation during fracture healing[24,25]. Furthermore, the non-union and the post-operative complication rates in spinal surgery is high in smokers[26,27] and even higher if smoking is associated with excessive alcohol consumption[28] and poor diet[23,29]. Alcohol-induced neuropathic pain associated with nutritional deficiency is a well known combination, which can increase the rate of pre- and post-operative complications[30] (pain, infection, pseudoarthrosis) and compromise the post-operative compliance with rehabilitation[31], in spinal field. Additionally, alcohol can delay osteogenesis affecting negatively MSCs function[32] and angiogenesis[28].

Despite the diffuse use of non steroidal anti-inflammatory drugs (NSAIDs) on daily basis, their administration following a fracture is a double-edged sword solution; has the benefit of the pain relief, but also the inconvenient of a higher risk of complications. NSAIDs can affect MSC chondrogenic and osteogenic differentiation activity, interfering with prostaglandin synthesis and possibly with cyclooxygenase-1 (COX-1) activity[33]. Additionally, prolonged administration of NSAID, for more than four weeks period, associated with smoking[33] can delay the healing progress and significatively increase the non-union risk. Another commonly prescribed drug which can be of a great utility in spinal field, but also can interfere with the bone metabolism

are the steroids[34], which are considered the first line drugs in many rheumatic/ autoimmune conditions. However, they can delayed bone healing[35], lead to vertebral body avascular necrosis[34,36] and osteoporosis followed by vertebral fractures[37], in a wide age range of patients and not exclusively on elderly population.

Other local factors including, deep spinal infection, length of the surgical wound and soft tissue status, can influence the healing progress and increase the non-union risks.

To ensure spinal fusion, a concept similar to "diamond concept", characterized by a biomechanically stable environment, bone graft, presence of MSCs and growth factors, from the micro-fractures, are necessary to ensure fusion[38]. Since healing requires an adequate blood supply any interference with angiogenesis (smoking, diabetes, multiple revision surgeries) may interfere with the fracture healing process. On the other hand, considering the close relation of the bony component of the spine with the neurological structures, it is fundamental to achieve the right amount of fusion, but without an exuberant callus or ectopic bone formation, which can compress the neurological structures surrounding the spine[39]. Moreover, a genetic susceptibility to fracture non-union, may exist, though inhibition of the signalling pathways between BMP and SMAD-6, acting synergically to other risk factors[40].

1.5 Mesenchymal Stem cells

1.5.1 History and definitions of MSCs

In 1867, the German pathologist Cohnheim demonstrated the presence of a non-haematopoietic stem cell population in bone marrow. In his experiment, he first created wounds in animals for after injecting in bloodstream an insoluble dye observing the appearance of dye-containing cells on the wounds' edges. The author observed that the great majority of those cells were coming from bone morrow, initiating in this way, the idea of bone marrow as a reservoir of fibroblasts capable of collagen generation by the wound site[41]. Two years later, author Goujon (1869) documented the presence of mesenchymal progenitor cells with osteogenic differentiation capacity within the rabbits' bone marrow, performing heterotopic transplantation experiments[42]. Goujon's experiments were confirmed in the transplantation field, by Biakow (1870) and later by Danis (1960), using diffusion chambers containing BM in animal models, showing that bone marrow itself presented osteogenic characteristics and was not only an inductive or chemoattractant factor for osteogenic cells[42].

At the beginning of 19th century, the Russian histologist Alexander A. Maximow, proposed the existence of a common precursor cell, within the BM, which generates different types of blood cells, the hematopoietic stem cell (HSC)[42,43]. Later on, experiments similar to Goujon, Danis and Biakow, but this time guided by authors Petrakova (1963), Friedenstein (1966) and Bruder (1990), using diffusion chambers, reinforced the concept of BM being capable of osteogenesis and chondrogenesis[42]. In parallel Ernest A. McCulloch and James E. Till, from experiments on mice in the 1960s, revealed the clonal ability of marrow cells[44]. Friedenstain's group in 1973 identified a non-hematopoietic BM-subpopulation, with colony-forming abilities, transplantable and able to growth in basic culture medium showing adherent capacity on glass flasks. Later in 1980, Friedenstein and his group, observed that every single cell was able to generate a colony with fibroblastic-like characteristics. Moreover, the colonies were transplantable, with self-renewal abilities and also capable of producing, in adequate environment (recipient animals), specific tissues like bone, fat, and cartilage indicating their stem cell nature[42,43]. The term "colony forming unit fibroblast, CFU-F" (referring to stromal cells) was coined in that work, and is in use till today.

In 1978, Owen formally expressed first the concept of BM-derived stem cell. Later on in 1985, Owen and his group proposed a model for the "new" non-hematopoietic stromal lineage that contained terms like "stem cell", "committed progenitor", "maturing cell", with "fibroblastic", "adipogenic", and "osteogenic" end-stage phenotypes[42]. In the same period, Caplan and Prockop were among the first to recognize the important discoveries of the Russian scientist Friedenstein in the Western world. In 1991, Caplan expanded further the concept of multi-lineage differentiation of the CFU-F cells, having the capacity to differentiate into multiple mesenchymal phenotypes including adipose, tendon, ligament, and bone coined the term "mesenchymal stem cell".

For the following fifteen years an "explosion" of interest and application of MSCs in different fields has been observed, changing consequently the MSCs' terminology according to new knowledge discovered every time, leading into misunderstandings and confusions between the scientific community. To ensure clarity and a standardized use of the MSCs' terminology, in 2006 the Mesenchymal and Tissue Stem Cell Committee, of the International Society for Cellular Therapy, published a position statement[69] to define MSCs' features, including; their tri-lineage differentiation nature, the adherence to plastic surface, positive expression of surface antigens (CD105, CD73, CD90) and lack of expression of others (CD34, CD45, CD14, CD11b, CD19, CD79, HLA-DR) which will be discussed in the 3.6, 4.7 chapters of this manuscript.

The terms "mesenchymal stem cell" and "marrow stromal cell" have been used interchangeably in different circumstances. The mesenchymal stem cells, derived from mesenchyme, can differentiate into hematopoietic cells, however the term is often used to describe "marrow stromal cells"[42,45]. However, this is not entirely accurate because the term "stromal cells" defines supportive and structural function rather than cells with reparative function, able to differentiate into bone, fat and cartilage. Hence, a better term to describe this type of cells, could be "multipotent stromal cells"[45]. Mesenchyme is a form of loose reticular connective tissue, made mainly by collagen type III. It derives from mesoderm, and protects and supports the cells, and reticular fibers within an embryo. Cells within mesenchyme can develop into haematopoietic (circulatory and lymphatic system) or non-haematopoietic (bone, cartilage, connective tissue) mature cells[42]. Nevertheless, the term "mesenchymal stem cell" gained such global usage that it became the most accepted description.

Although several different attempts were made, to create a model including all the known characteristics of "multipotent stromal cells", prevailed Gimble's model (1996) which expressed the stromal and reparative properties of these cells in an overlapping manner[42] (Figure 3).

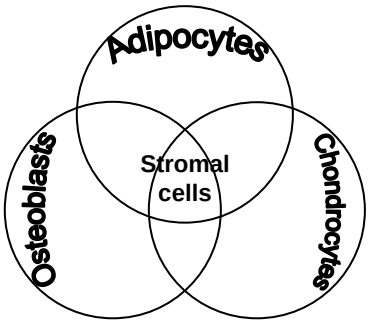

Figure 3: Gimble's modified model (1996), summarising MSCs features.
Illustration adapted from Stem cells handbook, Humana Press Inc., 2004.

However, Gilbert's model and the terminology used to describe MSCs are all based on the BM tissue. Nonetheless, with the advancement of knowledge in the field and the discovery of MSCs or MSC-like cells in different tissues (muscle, fat, hair follicles, tooth root, placenta, dermis, articular cartilage, umbilical cord, tendon, etc)[42], Gilbert's model is not expressing satisfactorily any more the MSCs' multipotentiality[42] nor plasticity[42,43], based also on their heterogeneity according to their topography[46,47]. Moreover, tri-lineage differentiation capacity of MSCs, is expressed differently, influenced to a certain extent, by their anatomic origin.

The Stochastic Activation/Repression Model was then proposed by Dennis and Charbor in 2002[48], describing better the plasticity, predisposition and multipotentiality of MSCs. MSCs are characterised by the plasticity to trans-differentiate or de-differentiate and re-differentiate into other cell types, generating a "blurring of the boundary" effect between phenotypes, based on the cells' predisposition and the stimulus received from the surrounding environment[42,43]. Moreover, in this model, the multipotential events are considered to occur within an "MSC compartment", which is not intended as physical space, but as a "genetic state" of expression of their multipotential nature. Cells within the MSC compartment can exhibit a range of multi- or mono-potential differentiation characteristics[42,48], while cells in the differentiation compartment are expressing those potentials in response to external stimuli[42,48] (Figure 4).

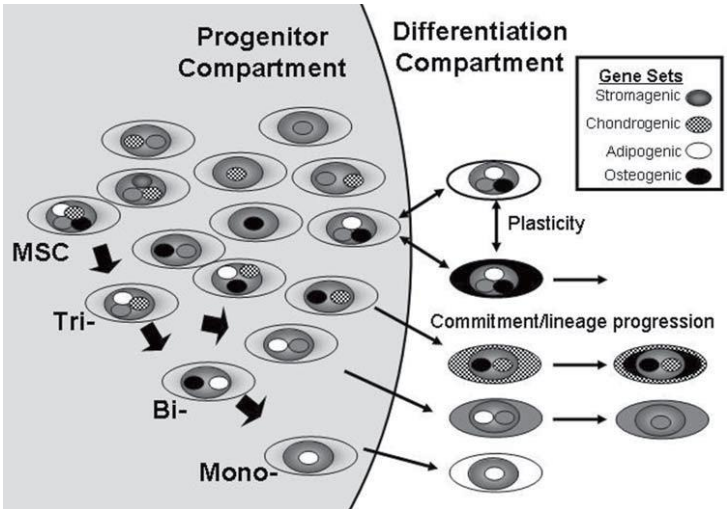

Figure 4: Stochastic activation-repression model, by Dennis and Charbord, in 2002. Illustration adapted from Stem cells handbook, Humana Press Inc., 2004.

1.5.2 Bone marrow harvesting and MSCs isolation techniques

In the orthopaedic field, MSCs are traditionally isolated from BM, nonetheless they can be isolated from a great variety of tissues. In this research will be described the BM-harvesting and MSCs isolation procedures from ICBM and VBBM. The stem cells are typically harvested directly from the iliac crest. Often BM-MSCs may also be taken from the sternum, while tibia/heel and umbilical cord are mainly used for infants and newborns sampling. MSCs can be harvested from the. BM-MSCs can be also harvested from vertebrae, but given the difficult access this can be performed only during spinal surgery.

As it will be discussed more in details in the next paragraphs, in terms of aspiration technique, the principle of "small volumes in small syringe", validated by Hernigou[49], was adopt in order to maximize the BM-MSCs harvesting. The majority of authors consider 1-4ml as the ideal aspiration volume per sample[50-53]. Nevertheless, from slightly bigger BM volumes (10ml approx.), it is possible to obtain a higher number of MSCs[51,53], without affecting significatively the quality of the sample. Indeed, according to Muschler, is possible to have up to 70% donor to donor variability, even by using the same technique, comparing to only a 20% variability between different aspirated volumes[51].

MSCs can be isolated from BM aspirates mainly by two techniques: the red cells lysis with ammonium chloride (AC) followed by gravity centrifugation and leucocytes isolation, or by density gradient centrifugation, using Lymphoprep (LMP), and mononuclear cell (MNC) isolation. As it will be discussed more in detailed in the appropriate section, AC is a salt solution, causing the lysis of the red cells and concentrates the remaining white cells (WBCs) composed by lymphocytes, monocytes and granulocytes[54]. Whilst, LMP solution has a specific density (1.077 ± 0.001 g/ml), which is similar to MNC (monocytes and lymphocytes) and is able to "capture" cells within that density range[55-57]. Despite the wide use of LMP for MSCs-isolation, for at least 3 decades, its effectiveness, in terms of maximal MSC yield, has been recently questioned, in favour of the AC[56,57].

Following BM purification, the majority of modern culture techniques are still using the CFU-F assay to select MSCs and count the colony-numbers. Additionally, flow cytometry can be used to select MSCs based on specific surface and intracellular markers, which will be described in the flow cytometry section 3.5, 4.7. An alternative method for MSC-isolation, is by magnetic-activated cell sorting (MACS). This method utilize magnetic nanoparticles coated with antibodies against particular surface antigens of MSCs. With the help of a strong magnetic field, MSCs attached to the magnetic nanoparticles expressing the antigen, will stay within the magnetic field, while the rest will flow through. In this way its possible to isolate a particular group of cells depending of their magnetic charge.

1.5.3 *In vitro* expansion of MSCs for therapeutic purposes

MSCs populations are rare and often culture expansion is fundamental for regenerative medicine and bone tissue engineering, in order to produce therapeutic concentrations of MSCs to guarantee an effective healing outcome[58-60]. Nonetheless, tissue culture conditions (oxygen concentration, seeding density, medium composition, doubling rate), can determine the "in-vitro-age" and affect the way MSCs behaving when transplanted in vivo[42,43,61]. Moreover, the amount of tissue culture stress and consequently their chromosomic stability[61], can affect the safety of MSCs. Additionally, MSC passaging, though trypsinisation, is a laborious and hard to control procedure, which put MSCs under extra stress.
Concerning media composition, the most common ingredient contained in the MSCs expansion media is the bovine fetal calf serum (FCS). However, the use of FSC can

contain prions and generate unexpected immunological reactions, and MSCs behaviors leading currently to extensive research on FSC alternatives[62]. A possible serum alternative can be patient's own serum, combined with autologous MSCs. However patient's serum presents donor-to-donor variability and can be affected by patient's immuno-inflammatory underlying conditions, influencing consequently MSCs behavior and making more difficult comparisons between cultures[63,64]. For all the above reasons, ideally, MSCs should undergo the minimum number of population doublings, required to achieve a therapeutic concentration, which is directly related to the initial sample concentration and the anatomic/therapeutic requirements.

The use of fresh, autologous and minimally-manipulated MSCs, without been subjected to culture expansion, is an attractive alternative. Firstly, because cells preserve their relevant biologic characteristics, hence are virtually risk-free for the recipient organism; and secondly because are framed under a more flexible human tissues legislation, compared to expanded MSCs. United States FDA and also European bodies, identifies as "minimally-manipulated MSCs" criteria, only cells cryopreservation, centrifugation and separation procedures[65]. The advantage of minimally manipulated MSCs, apart from the medico-legal aspect, is related to the recipient's protection from accidental prions contamination, less complex clinical management and consequently more cost- effectiveness. Nonetheless, donor-to-donor variability and potentially low MSCs numbers, are the possible challenges to face. However, the intra-operative use of portable volumetric flow cytometry devices, cell concentrators and the possibility to harvest vertebral body bone marrow from all vertebras included within the spinal instrumentation and additionally access to the posterior iliac crests, if needed, are all important elements in an attempt to overcome or minimize the possible challenges.

1.5.4 Clinical application of MSCs

The ability of MSCs to be expanded in culture, together with their potential to differentiate, makes MSCs an excellent source of cells for tissue repair strategies. Also the relatively easy harvesting, represent an additional advantage. On the other hand, given their physiological presence in the injury site, as part of the healing process, their use without further manipulation, is safe and they do not increase the risk of tumour formation[61], unless subjected to excessive stresses during tissue culture expansion. IC remains the gold standard autograft material, given its anatomic features, however bone graft harvesting is not devoid of risks and BM aspiration is not always assured[66,67]. Therefore, alternative approaches and MSCs sources have been proposed[51]. VBBM is rich in MSCs which can be used for graft augmentation in vertebral arthrodesis[7,68] and harvested while preparing the pilot holes for pedicle screw implantation; it can therefore open new possibilities in spine surgery and minimize the use of ICBM aspirate. Regarding the therapeutic concentration for the autologous bone-marrow grafted MSCs, in order to be both effective and safe an answer was given by Hernigou[70,71], suggesting 50.000 MSCs as optimum concentration, at least in relation to atrophic tibial diaphyseal non-union. Moreover, the author highlighted the importance of cell concentrators, in order to increase MSCs numbers in the recipient site. However, the therapeutic activity of MSCs is not only limited to the tri-lineage differentiation capacity within the graft, but also though their trophic action which can influence the growth of the surrounding structures[72]. Another point for reflection is that although in orthopaedic field is always highlighted the importance of the osteogenic differentiation of MSCs, in some cases bone formation may also be inappropriate such as in spondyloarthropathies (ankylosing spondylitis)[73,269].

In orthopaedic field, a post-operative non-union, is the result of an impaired biology in combination with local and/or systemic risk factors. Spinal non-union is often associated with significant morbidity, which represents not only a therapeutic challenge for the surgeon, but is also an expensive complication for both the health care system and consequently the society[41,43]. The increasing demand for higher health-care standards and the higher life-span of the population, increases ultimately the clinical challenges. In order to promote bone-fusion, different procedures have been developed in an attempt to improve the local biology[38] (neo-osteogenesis and -vascularisation) and reduce the risks factors, maximising the clinical outcome and reducing subsequently the health-care-cost[43]. The strategies adopted include

optimisation of the mechanical stability, use of scaffolds[74,75], growth factors (BMP 2,7), and a better utilization of the MSCs which can be harvested directly from VB or from IC if needed.

Other orthopaedic conditions where the potential therapeutic properties of MSCs can be applied are the age-related musculoskeletal disorders such as osteoarthritis and fragility fractures from osteoporosis. Both conditions represent a great challenge from a medical and socio-economic point of view[43,75]. Furthermore, in pioneering experiments MSCs have also been tested in cartilage and tendon repair models. Although is well known the chondrogenic ability of MSCs, hence the potential use for tissue repair, it is also notorious the difficulty of articular cartilage to heal, especially in adults[43]. Similarly following a tendon injury, MSCs loaded on collagen scaffolds and implanted within the damaged tendon, showed integration with the native tissue and maintenance of the original tendon architecture, given the arrangement of the collagen fibers[76].

MSCs were also tested as a therapeutic option for peripheral nerve and brain injury repair. The promise of MSCs as neuro-regeneration, is the hope of many patients with a permanent impairment. Many trials have shown improvement of the neurological condition or at least protective role[43,77]. Nonetheless, because of the complex neuro-biology, the restoration of the function depends from many factors, including the initial nerve damage, the type on neurological structure involved, as well as the local and systemic biology. Although there are several trials covering the main categories of diseases (demyelisation/spinal cord injury, amyotrophic lateral sclerosis, stroke, Parkinson/Huntington, macular degeneration and peripheral nerve diseases), there is no MSCs therapy approved yet for routine use[77].

Due to their anti-inflammatory/immunosuppressive characteristics, culture-expanded MSCs have shown promising results in the transplantation field, to control the graft versus host disease and potentially, to treat rheumatoid arthritis[78]. MSCs are capable of suppressing T and B cell proliferation in a dose dependent manner. Additionally, allogenic MSCs are not targeted as "non-self" by T cells and natural killers[79], thus, they can be administered to patients, even with an intact immune system[80]. Bone marrow can be used from a compatible donor to another (allogenic) or harvested and returned into the same original donor at a later stage (autologous). Systemic BM transplantation is mainly used in case of severe bone marrow diseases, such as autoimmune diseases or malignancies and the procedure is usually associated with

radiotherapy and chemotherapy with scope to neutralize recipient's BM and his immune system response before transfusion[43].

However, a fundamental obstacle to overcome is to retain their physiological characteristics during the expansion in artificial culture conditions[42,43]. Furthermore, a second difficulty, is the delivery of MSCs to the site of repair, and the local integration and promotion of differentiation toward the appropriate mesenchymal phenotype. In orthopaedic field is common practice the direct injection or placement of MSCs into the affected site, rather than intravenous transplantation, where MSCs could be sequestered mainly by the lung filter. Another important technical limitation with the intra-operative use of autologous BM-MSCs, is the lack of possibility to know prospectively the amount of MSCs delivered locally. Currently, the way to count the MSCs' concentration is retrospectively in the tissue-culture lab, via a BM-sample. In order to optimize the therapeutic potential of MSCs, new portable devices using the volumetric flow cytometry method, may bridge the current technical gap offering a real-time intraoperative quality/quantity control on the BM-sample.

It has been entrusted to regenerative medicine and bone tissue engineering disciplines, both using the MSCs' therapeutic potential, to provide solutions in order to replace or repair damaged tissue through activation, stimulation or correction of reparative mechanisms with scope to restore the function. Although the research into MSCs biology and the derived applications play a key role in medicine, is still in its infancy. Some of the therapies are already available (osteogenesis in orthopaedic field), however for the great majority of them, more work is needed before they can be routinely applied. Moreover, there are still several unanswered questions about MSCs control in vivo, delivery to the target and their effectiveness. Studying how these cells work and interact within the body, is critical for the development of effective treatments in the future.

1.3 Cytokines and Immuno-inflammatory response

1.3.1 Host defence response in adults and adolescents

When a traumatic or surgical insult takes place, a series of complex mechanisms are activated in order to control and restore the physiological homeostasis under the principle of the minimal enthalpy status[81]. These events can trigger a diversity of immuno-inflammatory responses which can gradually increase its complexity and grade of severity in response to the amount of pathological or surgical conditions involved (second hit phenomenon)[82-88].

In particular, a surgical insult can elicit a characteristic response, involving increased circulating concentrations of stress hormones (e.g. cortisol and catecholamines) and synthesis and release of various humoral mediators (e.g. pro- and anti-inflammatory cytokines)[89]. Moreover, acute-phase proteins like C-reactive protein (CRP) and metabolic changes (e.g. lipolysis or hyperglycaemia) can occur[90].

More specifically, a traumatic or surgical insult can induce an early hyperinflammatory response inducing high concentrations of TNF-α, IL-1a, IL-1b, IL-6, IL-8 as well as neutrophil and macrophage activation. This condition in turn, leads to the activation of anti-inflammatory mediators, such as prostaglandin E2, IL-10, IL-13, TGF-β, INF-α, INF-γ and a series of endogenous soluble receptor antagonists (e.g. IL-1Ra). Inducing in such way, a state of immunosuppression, which can interfere with monocytes' activity and shift the Th1/Th2 ratio, to the Th2 dominated cytokines pattern[94,95]. Due to the complexity of these mechanisms and the individual response of each patient, the fine balance between pro- and anti-inflammatory systems can be compromised, predisposing the host to infections, Systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS) and ultimately death[96]. Another element which can influence patients response, is the immuno-protective role of female steroids and the immunosuppression effect of android hormones[91,97]. Thus, to restore the fine balance between inflammatory and anti-inflammatory forces, a holistic clinical approach, has become fundamental[94,96].

Host defence response has been mainly studied in polytrauma patients, but only few publications involve patients with associated spinal injuries[91-93]. In these studies, authors underline the severity of the clinical picture and the additional immuno-

inflammatory involvement when a spinal lesion is present. However, it is difficult to quantify its additive effect, in isolation[91-93].

Host defence responses following a major elective surgery, such as scoliosis operation in adolescents is not as widely studied as in adults. Additionally, the available literature is often performed retrospectively, increasing up to 3 times the possibility to miss short term risks/complications during the data collection, which can potentially affect studies' outcome[98].

Suzuki *et al.*, analysed the pre- and post-operative cytokines from blood samples of adolescents undergoing AIS surgery and compared them to a control knee arthroscopy group[99]. The results showed a decrease of the B-Lymphocyte population and transient increase of NK and cytotoxic-T-Lymphocytes in scoliosis group. Production of INF-α, INF-γ was also suppressed for 3 weeks and the overall postoperative effects lasted up to 3 weeks when the surgery was particularly prolonged. In addition, an extensive surgery could lead to a greater incidence of infection occurring even 4 years postoperatively[98,99]. Moreover, Krohn *et al.*, concluded that cytokines respond differently at local and systemic level following AIS surgical correction[100].

In severe forms of scoliosis with structured curves, antero-posterior surgery is required in order to release the spine, via anterior discectomies, achieving a better correction of the deformity. Therefore it was investigated whether the intervertebral disc material can contribute to the overall immuno-inflammatory status, considering that physiologically, the intervertebral disc is a privileged structure with a low interaction with the immune system, due to its anatomy[101]. Herniated disc per se, can cause immuno-inflammatory effects even before the surgical intervention (e.g. discectomy)[101]. Several studies correlated herniated disc material with disc material harvested during scoliosis operation, showing higher concentration of TNF-α, IL-1, IL-6, IL-8, monocyte chemoattractant protein 1, nitric oxide, metalloproteinases and prostaglandin E2 in the herniated disc, compared to scoliosis group. However, the data did not always correlate with the clinical symptoms[102-107].

Considering the above, it becomes a prerogative to investigate further the immuno-inflammatory aspects of this major elective operation, in a prospective manner, in order to be able to implement our knowledge and patient's care, especially in a paediatric setting.

1.3.2 Essential cell types and mediators involved in the immuno-inflammatory response

A dynamic interaction exist between cytokines, complement degradation products, eicosanoids, platelet activators, coagulation factors, leukocytes, endothelial cells and numerous other chemical factors regulating the inflammatory response[108].

Cytokines are markers of trauma and key mediators of host defensive reactions. They influence the haemodynamic, metabolic and immune responses[97,109]. The cytokines mainly involved in the response to a traumatic or surgical insult, include TNF-α, IL-1, IL-2, IL-6, IL-8, IL-10, IL-17 and IL-13[110]. Cytokine cascade forms a complex biochemical network with various effects on the injured host[111,112]. Moreover, a significant overlap exist in terms of the bioactivity between different cytokines[108,110].

Cytokines interfere with several cell types including macrophages[113], neutrophils, mast cells, endothelial cells[114], T and B cells[108] and NK[115]. Furthermore, a mutual inter-relationship between cytokines and non-immune cells such as MSCs exists, considering that MSCs express surface receptors for cytokines and respond to its stimulation[116-118]. The available literature, analyzing these immuno-biologic effects in children, is rather limited.

1.4 Proinflammatory and antiinflammatory cytokines

Cytokines can be classified as proinflammatory (TNF-α, IL-1, IL-6, IL-8, IL-18, procalcitonin) and antiinflammatory (IL-10, IL-13). Their balance contribute to the restoration of the natural homeostasis, promoting the healing process following an insult[81,94,96,119]. For the purpose of this study, the kinetics of IL-6, IL-18, procalcitonin and IL-10 were analysed and will be described in detail in the next paragraphs. The characteristics of TNF-α, IL-1, IL-8 and IL-13 will be briefly described herein.

TNF-α has a 20 minutes half-life, and following an insult reaches the highest concentration within 4 hours. It is produced by activated macrophages, mast cells, T and B cells, NK and also hepatocytes and splenocytes[115]. Trigger molecules for its secretion are principally IL-1, IL-6, INF-γ and TNF-α itself. Also its involved in the

activation of the coagulation cascade[95]. TNF-α can increase in case of disc herniation[106,107], but not necessary following scoliosis operation[102].

IL-1 has a 6 minutes half-life, and to a certain extent, presents similar effects with TNF-α, having a prominent role in the host defence system. In low concentrations can trigger a local inflammatory reaction, activating and promoting the homing process, inducing also IL-6, IL-8 and TNF-α secretion[120]. In high concentrations, i.e. in case of traumatic insult or infection, it is able to generate a systemic inflammatory response. IL-1 is mainly produced by neutrophils and macrophages and is directly involved with bone metabolism[113]. The cellular receptors for IL-1 are divided in two categories, the IL-1RI characterised by a better biological activity and the IL-1RII which seems to behave as a decoy receptor neutralising the biological effects of IL-1 itself. The activity of IL-1 is also controlled by other interleukins like IL-4, IL-10 and IL-1 receptor antagonist (IL-1Ra)[95,114].

IL-8 increases rapidly following an injury and reaches the highest concentration within 24 hours. Alongside with IL-6, it is considered to be a key mediator in SIRS and ARDS[121]. IL-8 is produced by endothelial cells and promotes chemotaxis for both neutrophils and lymphocytes, especially in the lung[103]. Moreover, increased levels in the peripheral circulation have been reported in trauma and also a 100-fold increment, has been documented in patients undergoing scoliosis operation[88,102].

The role of IL-13 is to provide and maintain a balance between pro-inflammatory and anti-inflammatory functions[122], impairing the expression of pro-inflammatory cytokines (TNF-α, IL-1, IL-6) and up regulating IL-12, which has a principal role against a broad range of intra cellular pathogens[123]. A functional relation between the anti-inflammatory cytokines IL-4 and IL-13 has been observed, however several aspects of this interrelationship need to be further analysed[124].

1.4.1 Interleukin-6

Interleukin-6 (IL-6) is a pleiotropic proinflammatory cytokine with a broad range of humoral and cellular effects related to inflammation, host defence, and tissue injury[125]. IL-6 is induced by viruses, LPS, IL-1, INF-γ and TNF-α[125] and it is produced by a variety of cell types including fibroblasts, monocytes, macrophages, hematopoietic cells, endothelial cells, and T and B cells[108]. IL-6 is the major mediator

of the acute-phase response, leading the synthesis of C-reactive protein[125] by hepatocytes. It is also crucial for the thermoregulation and the mucosal production of IgA[108].

IL-6 apart from its proinflammatory function, can self-control its action in an antiinflammatory manner, mediated through its inhibitory effects on TNF-α and IL-1, and activation of IL-1Ra and IL-10[108]. Moreover, IL-6 has a local antiinflammatory action at muscle level (myokine). IL-6 is elevated during muscular activity, acting like a hormone, facilitating delivery of energy substrates to the myocytes[126]. Its increment during muscular exercise, however, is controlled by the antiinflammatory actions of IL-1Ra and IL-10, and defers from infection/inflammation, which is preceded by a TNF-α[126]rise.

IL-6 levels from fresh peripheral blood samples in healthy adults population range between 0.01 and 11.5 pg/ml[125] or according to other studies between 5-15 pg/ml[127]. Its levels are constantly elevated during infection, in polytrauma patients and also following scoliosis surgery[102]. IL-6 presents a long half-life and correlates to the degree of systemic immuno-inflammatory reaction and outcome. Therefore it is less transient and more readily measurable marker than IL-1 or TNF-α and can be considered a reliable marker of the magnitude of a traumatic or surgical insult[91]. In addition, IL-6 has a regulatory role in bone metabolism balancing the function between osteoblasts and osteoclasts. Moreover, estrogens can down regulate IL-6 function, limiting osteoclasts' action and this can be used in the treatment of postmenopausal osteoporosis[91,97].

It was previously hypothesised, that IL-6 together with MMP3 and MTNR1B genes could predispose for AIS[128-130]. Considering that BMP4 and leptin have a key role in bone metabolism, and also are directly interacting with melatonin, IL-6 and MMP3, it was thought that these genes could be potentially "AIS predisposition genes"[128-130]. Additionally, an association between IL-6 gene expression and bone mineral density in the lumbar spine was observed[131]. However, despite this evidence, all authors tried to correlate specific gene patterns with aspects of AIS which are not necessary unique to the scoliosis etiopathogenesis itself[125]. Moreover, factors like demographics, geographic location, dietary habits and lifestyle of the populations examined, could have influenced these correlations. On the other hand, different genetic patterns can be found between different populations, making it difficult to replicate and extend this hypothesis to all patients presenting with AIS. Furthermore,

in contrast with that hypothesis, other authors[129,132] did not identified any correlation between IL-6, MMP3, and MTNR1B with AIS, at least at a single gene level. Nonetheless, a possible synergistic effect may exist between combination of genes and AIS[129], however, further studies are needed to rule out this potential association.

1.4.2 Interleukin-18

Interleukin-18 (IL-18) is a proinflammatory pleiotropic cytokine, structurally similar to IL-1 and its biological activities overlap with those of IL-1 and IL-12. It is produced mainly from activated macrophages, Kupffer and dendritic cells, but also T and B cells, playing a major role in the inflammatory cascade. Additionally, IL-18 is constitutively present in many tissues as an inactive precursor (e.g. keratinocytes, epithelial and glial cells)[133].

IL-18 amplifies the expansion of Th1 cells and also enchases the activity and proliferation of NK[134]. It was originally described as an IFN-γ–inducing factor, because it appears to be essential for IFN-γ production, in synergy with IL-12[135]. IL-18, due to its pleiotropic nature, presents unique characteristics which are important for its protective role in sepsis, mainly due to its ability to induce IFN-γ, but also TNF-α.

IL-18 in serum of healthy adults was reported to range between 64±17pg/ml[135] and 126 pg/ml in healthy adults, or 260pg/ml in healthy adolescents[135]. Its levels can be significantly increased in patients with traumatic injuries associated to lethal sepsis[137], otherwise trauma-induced systemic inflammation itself seems to not increase significatively IL-18 concentrations[137]. Moreover, IL-18 levels correlate with the magnitude of injury, the severity of the clinical condition and can represent an early predictive marker for MODS and lethal outcome of postoperative sepsis[95].

IL-18 has a key role in many human diseases, including pancreatitis[138], myocardial insult, arthritis, bowel disease and hepatic/renal failure[135]. Moreover, IL-18 plays an important role in interstitial lung disease and pneumonia in adults[139] and adolescents[136], which can represent an important post-operative complication in spinal surgery. Particularly in case of costoplasty and antero-posterior scoliosis

correction, with iatrogenically controlled pulmonary collapse and consecutive direct approach to the spine through the thoracic cavity.

1.4.3 Procalcitonin

Procalcitonin (PCT) is an inflammatory marker produced in response mainly to bacterial infections, but does not usually rise significantly with viral or low grade non-infectious inflammation[140-143]. Determination of the presence or absence of bacterial infection is fundamental for patients' management and appropriate use of antibiotics[141].

Synthesis of PCT is regulated by gene CALC-1 and is the precursor of the hormone calcitonin (CT)[144]. Under normal metabolic conditions PCT and CT are present in the thyroid gland C-cells and are secreted in response to hypercalcemia inhibiting bone resorption by osteoclasts, minimizing oscillations in serum calcium concentration and calcium loss. Moreover, hepatocytes can also produce large amounts of procalcitonin following stimulation by TNF-α and IL-6[142,145]. Although during severe systemic inflammation, related particularly to bacterial infection, PCT is secreted in large quantities, CT levels do not necessary rise[142,145].

PCT serum level in healthy individuals is generally low[146,147] (adults and children ≤0.15ng/ml) and its directly related to the severity of a bacterial infection. Thus when levels exceed 0.25ng/ml, is indicative of minor systemic bacterial infection or infection at local level. Contrary, when concentrations rise above 0.5ng/ml, could indicate a important systemic bacterial infection[146-149]. PCT level increases from 3 to 4 hours, reaches a peak at about 6 hours and then plateau in 24 hours. In blood serum, PCT has a half-life between 25 and 30 hours. In contrast, C-reactive protein (CRP) levels rise between 12 and 18 hours after a bacterial challenge[146-149].

Higher PCT concentrations are more likely related to bacterial infections, thus, PCT is useful in differentiating bacteria from other infectious agents (viruses)[150-157]. Non-infectious inflammatory stimuli, like cancer (thyroid), major trauma/surgery, acute respiratory distress syndrome, graft-versus-host disease, cardiogenic shock, and burns, need to be extremely severe to increase PCT levels[150-157]. In these conditions, PCT has a greater sensitivity and specificity for differentiating patients with Systemic

Inflammatory Response Syndrome (SIRS) from those with sepsis. Furthermore, it is a more specific indicator of a severe bacterial infection rather than other inflammatory markers (IL-2, IL-6, IL-8, CRP and TNF-α) and could be considered one of the earliest and most specific markers of sepsis[145,158,159] .

Moreover, as suggested by the national guidelines[141], PCT in combination with other diagnostic tools can help to distinguish between an early infection or non-infectious condition, leading consecutively to a better rationalise use of antibiotics and potentially to a reduction of hospital stay[141]. Even though these data are mainly related to adult patients, there is limited evidence suggesting that similar effects may apply to children presenting a bacterial infection. Additionally, recent studies concluded that children with bacterial pneumonia had significantly higher procalcitonin levels than those with a viral aetiology[150,153,156,159]. However, due to a certain degree of overlap this test should not be used as a primary "stand alone" diagnostic tool, but has to be interpreted in correlation with clinical findings and additional complementary tests. Nevertheless, when diagnosis is established can be utilized as a monitoring tool[141].

1.4.4 Interleukin-10

Interleukin-10 (IL-10) acts as a pleiotropic antiinflammatory cytokine, in both innate and adaptive immunity, producing mainly immunosuppressive, but also immunostimulatory effects on various cell types[123]. IL-10 is structurally similar to INF-γ, even though acts as a "brake" to inflammatory responses targeting principally macrophages, neutrophils, eosinophils and mast cells, by inhibiting genes responsible for TNF-α, IL-1, IL-6, IL-8 and IL-12 synthesis. Also can self-regulate its production by destabilising its own mRNA[115,123,160]. IL-10 can also down-regulate Th1 cells which trigger the production of IL-2, IL-3, IFN-γ and TNF-β, rebalancing the Th1/Th2 ratio[95,161]. Additionally, IL-10 presents an important protective function against conspicuous cytokine release during endotoxic shock[123].

IL-10 synthesis can be triggered not only by pathogens and their products, but also by proinflammatory cytokines. Several cell types can secrete IL-10 (hepatocytes, mast cells, eosinophils, B cells) but principally it is produced by activated monocytes, macrophages and Th2 cells[123,162].

IL-10 levels in serum of healthy adult individuals[162] range from 3-13.7pg/ml or 0-8 pg/ml according to other studies performed on adolescents[100]. IL-10 concentrations can rise within 3 hours from the insult in polytraumatized patients and in scoliosis operations, however IL-10 concentration tend to become significative after the first post-operative day[163]. Nonetheless, IL-10 concentration correlates with the severity of injury and also the degree of the post-insult complications, in comparison to patients with an uneventful post-insult course[102,113,164].

1.5 Coagulation cascade and inflammatory response

The pathophysiology of inflammation and haemostasis are interrelated through a bidirectional relationship, and both represent different arms of the host defence system. A local or systemic insult is able to activate the inflammatory system as well as the coagulation cascade. The close interaction and reciprocal control of these two systems, under physiological conditions, restores the tissues physiology following a traumatic or infectious insult[165,166]. The inflammation activates the coagulation cascade, and is able to shift the haemostatic balance towards a pro-coagulative state, increasing fibrogen concentration (higher risk for thrombotic disease) and decreasing the activity of the anti-coagulant and fibrinolytic mechanisms[165,166]. Major inflammatory activators of the haemostatic system at the initial stage, are the pro-inflammatory cytokines like TNF-a, IL-6 and IL-8[165].

The coagulation cascade and the clotting process can enhance the inflammatory response, releasing mediators from platelets and also through an inter-cellular interaction, promoting in this way the inflammatory response[165]. Within the coagulation cascade, the fibrinogen and the thrombin have a strategic importance and represent key molecules of the intrinsic, extrinsic and common pathways. The partial thromboplastin time (PTT) is a performance indicator of the intrinsic and common pathways (coagulation factors XII, XI, IX, VIII, X, V, prothrombin (II), fibrinogen (I), prekallikrein, high molecular weight kininogen), whilst prothrombin time (PT) is an indicator of the external and common pathways (coagulation factors VII, X, V, prothrombin (II), fibrinogen (I)) [165,166]. The evaluation of these two indicators is a way to monitor bleeding and clotting disorders, and also represent an indirect way to understand better the dynamic evolution of the inflammatory cascade.

2. Hypotheses and Aims

2.1 Hypotheses

The effectiveness of density gradient concentration technique (LMP) for MSCs isolation will be lower compared with red cells lysis (AC) technique, for both ICBM and VBBM.

The number of MSCs in VBBM of scoliosis patients is similar or even higher, compared ICBM of the same patients.

MSCs isolated from VBBM are expected to show similar or even better proliferation and differentiation potentials compared to MSCs from ICBM.

Following scoliosis surgery, a correlation exists between the type and duration of the surgical insult with the kinetics of the proinflammatory and antiinflammatory markers, as well as with the clinical and biochemical parameters.

2.2 Aims

To quantify MSCs in ICBM and VBBM samples, in donor-matched patients.

To compare the effectiveness of MSCs isolation following density gradient technique (LMP) and red cells lysis technique, from both ICBM and VBBM in donor-matched patients.

To confirm MSCs' nature and to study their characteristics performing enumeration, proliferation and trilineage differentiation (chondrogenesis, osteogenesis and adipogenesis) assays in addition to flow cytometry for both VBBM- and ICBM-MSCs, isolated using two different techniques from donor-matched patients.

To define the correlation between pro-inflammatory and anti-inflammatory markers with clinical and biochemical parameters as well as the duration of the surgery.

To measure, for one week, the proinflammatory cytokines (IL-6, PCT, IL-18) and the antiinflammatory cytokine (IL-10) in serum of 3 groups of patients, undergoing AIS operation with: a) posterior approach, b) posterior approach and costoplasty, c) antero-posterior approach.

3. Materials and Methods

3.1 Patient cohort

3.1.1 Recruitment of patients

Patients undergoing Adolescent Idiopathic Scoliosis (AIS) correction in the spinal department of Leeds General Infirmary were invited to participate in the study.

External ethics committee approval (06/Q1206/127) has been granted from the NRES Committee Yorkshire & The Humber – Leeds East (Appendix), followed by Paediatric ethics committee approval, and Research and Development approval of Leeds General Infirmary hospital. Given the young age range of the patients recruited and the relative legislation, three different categories of informed consent were created for patients and parents: a) patient assent document for ages 13-16y, b) parental agreement for same age group (13-16y) and c) a separate consent document for patients 16-17y. Additionally, according to Good Clinical Practice guidelines, a specific pathway has been put in place for patients recruitment (Figure 5).

Inclusion criteria were considered: 1) age range between 13 to 17 years old, 2) indication for the surgical correction of AIS and 3) their willingness to participate in the study.

Exclusion criteria from the study were: 1) age below 13 or above 17 years old, 2) presence of abnormal hepatic or renal function on routine laboratory analyses, 3) patients treated with known immunomodulatory agents (e.g. corticosteroids) and with known bleeding disorders.

Considering the young age of donors, it was not-ethically acceptable to recruit a group of healthy donors with similar age-range and without AIS, as a control group, for peripheral blood harvesting or for bone marrow harvesting. Therefore, for both cases, the internal control concept has been applied. Specifically, pre-operative peripheral blood sample and iliac crest bone marrow aspirate have been considered as reference values for the post-operative peripheral blood analysis and the comparison with the vertebral body bone marrow, for each single patient.

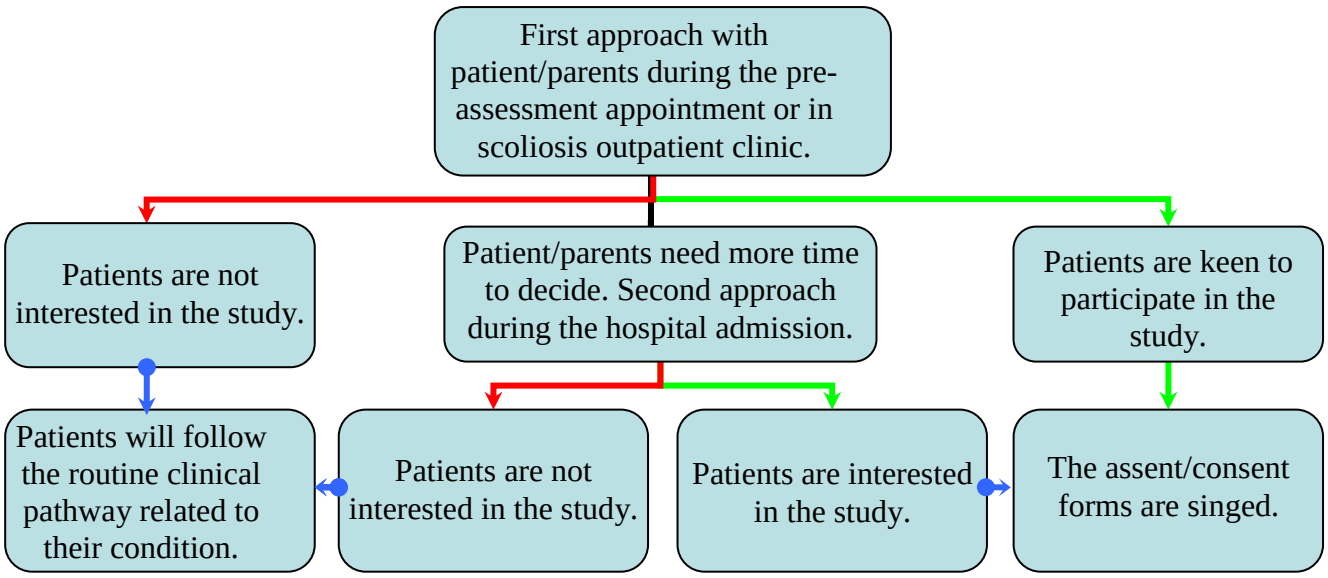

Figure 5: Algorithm used for patients consent. The process of informed consent, in a paediatric setting, is a more complex act, compared to adults and must follow a more rigorous structure, as per guidelines.

3.1.2 Patients' cohort: MSCs study

From a cohort of 37 patients, randomly recruited, undergoing elective AIS surgery, a total of 46 bone marrow samples were harvested from 19 patients (12 females and 7 males, median age 15.4 years, range 13-17). From all patients 10ml of bone marrow were aspirated intraoperatively from the posterior elements of pelvis (iliac crest, IC) and the 12th thoracic vertebra (V12), using validated and publish techniques[49-53] (Table 1), as it will be described in section 3.2.

3.1.3 Patients' cohort: immuno-inflammatory response study

The same cohort of 37 randomly recruited patient, (29 females and 8 males, median age 15.4 years, age range 13-17) undergoing AIS surgery, was divided in three groups, based on the type of surgical approach (Tables 1, 2). The posterior approach (group1) was predominant, as it was sufficient to correct the deformity in the majority of the cases, followed by the less frequent posterior approach with costoplasty (group2) and the antero-posterior approach (group3) which are indicated for more severe scoliotic deformities. Lenke classification for the AIS was used[3]. From all patients an average of 8ml of peripheral blood (PB) was withdrawn at specific time-points and following centrifugation serum and plasma were isolated and cryopreserved accordingly, as it will be explained in sections 3.2.1, 3.3.

Table 1: Overview of all the patients recruited and the main experiments performed.

N.	Patient Code	M/F	Surgical approach	ICBM	V12 BM	BM from other vertebra	Serum/ Plasma from PB	CFU-F	Tri-lineage Differentiation	Flow Cytometry	ELISA
1	AF 01	F	Posterior				√				√
2	AF 02	F	Posterior				√				√
3	AF 03	F	Posterior				√				√
4	AF 05	M	Antero-Posterior				√				√
5	AF 06	F	Posterior				√				√
6	AF 07	F	Posterior				√				√
7	AF 08	M	Posterior				√				√
8	AF 09	F	Posterior				√				
9	AF 10	F	Posterior				√				
10	AF 11	F	Posterior				√				
11	AF 12	F	Post.+Cost.				√				√
12	AF 14	F	Post.+Cost.				√				√
13	AF 16	F	Posterior				√				
14	AF 17	F	Post.+Cost.				√				√
15	AF 18	F	Posterior				√				√
16	AF 19	F	Antero-Posterior	√	√		√	√			√
17	AF 20	F	Posterior	√	√		√	√			
18	AF 21	F	Posterior				√				
19	AF 22	F	Post.+Cost.				√				√
20	AF 23	F	Posterior	√	√	V11*	√	√			√
21	AF 24	F	Posterior	√	√	L1*	√	√			
22	AF 26	M	Posterior	√	√		√	√			√
23	AF 27	M	Posterior	√	√		√	√			√
24	AF 28	M	Antero-Posterior	√	√		√	√	√	√	√
25	AF 29	M	Posterior	√	√		√	√	√	√	√
26	AF 31	F	Posterior	√	√		√	√			
27	AF 32	M	Posterior	√	√		√	√			
28	AF 33	F	Posterior	√	√		√	√			√
29	AF 34	F	Posterior	√	√	L1*	√	√			
30	AF 35	F	Posterior	√	√	L1*	√	√			√
31	AF 39	F	Post.+Cost.	√	√		√	√	√	√	√
32	AF 40	F	Posterior	√	√		√	√			√
33	AF 43	F	Posterior	√	√	L1*	√	√			√
34	AF 47	F	Posterior				√				
35	AF 48	F	Antero-Posterior	√	√	L1*	√	√			√
36	AF 49	F	Antero-Posterior	√	√		√	√			√
37	AF 50	M	Antero-Posterior	√	√	V11*, L1*	√		√	√	√

* V11, V12, L1 are used as abbreviations of 11th and 12th thoracic, and 1st lumbar vertebrae, respectively.

Table 2: Distribution of the three groups of scoliosis, based on the different surgical approach.

Group	Group size	Type of approach
Group 1	(n=26)	Posterior
Group 2	(n=5)	Posterior + Costoplasty
Group 3	(n=6)	Anterior + Posterior

3.2 Collection of blood and bone marrow

3.2.1 Blood sampling/processing and clinical/biochemical data collection

Peripheral blood was withdrawn at the following seven time points: pre-operatively baseline (Time 0), 2 hours after the onset of the surgery (Time 1), at the end of surgery (Time 2), 24 hours after the surgery (Time 3), at 3 days (Time 4), at 5 days (Time 5) and at 7 days (Time 6), post-operatively.

It is well known that patient position during prolonged procedures, is associated with predictable changes in organs' physiology[167,168], but also with a number of possible cardio-pulmonary and abdominal complications, that could be related to the direct or indirect compression from the spine and the surgical table to the patient's organs[6,7]. These conditions are usually reversible by posture changing, however, may be more common than published[167,168], suggesting further evaluation. For this reason an additional time-point for PB sampling was added (time-point "Turn") only for the antero-posterior approach group, to investigate possible immuno-inflammatory changes immediately after intraoperative change from lateral to prone position.

As control group it was considered the Time 0 (post-anaesthetic induction, pre-surgery) of each patient. Moreover, as an additional level of control, an extra time-point (Time 0a), (pre-anaesthetic induction, pre-surgery) was considered, for four patients, to investigate whether anaesthetic procedure can affect interleukins' (IL-6, IL-10, IL-18, PCL) baseline.

Post-operative time points were designed to coincide with the routine blood sampling for the clinical monitoring, in order to minimise patients' discomfort. Additionally, during the same period, patients' clinical course, blood results, transfusions, and drug

prescriptions were recorded, as well as local and systemic complications. Due to the early hospital discharge of the majority of the patients, the blood sampling of the 5th and 6th time points was often applicable only to a few donors with longer length of in-hospital stay.

3.2.2 Plasma and serum isolation and storage

At each time point, the 8 ml of PB collected, were equally divided into 2 vacutainer tubes (Vacuette K2EDTA for plasma and Vacuette Serum Clot Activator for serum). Plasma contains: dissolved proteins, antibodies, clotting factors, electrolytes, hormones, enzymes and products of metabolism, while serum is similar to plasma, but devoid of clotting factors. Following blood collection, tubes were gently inverted few times to ensure adequate mixing of the blood with the additive contained in the tube. Blood was processed within 2 hours of collection. Samples collected for serum were allowed to stand for at least 30min., to ensure clotting. Next centrifugation was performed for both vacutainer tubes at 446xg for 15min.[169]. Serum and plasma were extracted, aliquoted in 1ml cryovials and cryopreserved in -80°C.

3.2.3 Bone marrow sampling

Considering the young age of donors, to establish the optimum volume of bone marrow aspirates, in terms of patient's safety and experimental needs, the relevant literature[3,7,51-53,167-174] and the World Marrow Donor Association guidelines[175,176], were taken in account. Two bone marrow samples of 10ml each, were collected from 19 patients, during scheduled operation for AIS, from IC, and 12th thoracic vertebra (V12), as a standard procedure. In addition, from 7 patients an extra bone marrow sample of 10ml was aspirated from the thoracic 11th (V11) or the 1st lumbar (L1) vertebra, in order to assess possible changes in cells concentration between vertebras (Table 1).

The bone marrow aspiration, did not affect the surgical procedure which was carried out using the classical posterior approach and midline incision, associating thoracotomy or costoplasty according to the indication. The only variation to the standard technique was the use of a 13 gauge bevel-tipped trocar (Stryker), under fluoroscopic control, to create the typical pilot hole for screw insertion instead of the

blunt probe instrument which is commonly used. The trocar was inserted at 3-4cm depth from the posterior part of the pedicle, in order to just penetrate the cortical bone and reach the periphery of the cancellous bone of the vertebral body (Figure 6). Subsequently, a 10ml syringe was used to aspirate 10ml of VBBM, using established technique validated and published by authors Hernigou *et al*[49] and McLain *et al*[52]. During the same stage of the operation, 10ml of ICBM from the posterior elements of pelvis, were also harvested with the same technique. ICBM aspirate was the only additional step during the operation, however, its often used in spinal surgery, when BM aspirate is needed for scaffold enrichment to enhance the bone healing process[177].

The left or right side for the bone marrow aspiration, for both IC and VB, was decided based on the convenience of the surgeon, considering also that there is no evidence of any anatomic difference[7,62,68,170-173].

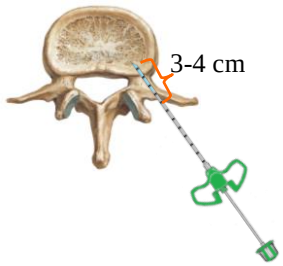

Figure 6: Schematic representation of the vertebral harvesting technique. The 13 gauge bevel-tipped trocar (in green), was inserted in the vertebra for 3-4cm via the pedicle, till the periphery of the vertebral body. Next, a 10ml syringe was connected to the trocar, to aspirate 10ml of VB BM (Adapted from Human Anatomy Atlas, Netter 3rd edition, 2006, following explicit permission from Elsevier.)

3.2.4 Bone marrow processing, with LMP and AC

All the procedures related to BM processing were performed under aseptic conditions at the cell-culture facility laboratories using biological safety cabinets, Class II. The BM aspirates from IC and VB were placed in vacutainer tubes containing K2EDTA, to prevent clotting, and immediately processed at the end of each surgical procedure. BM from each anatomic region was subsequently divided in two equal portions and cells were isolated using density gradient separation (LMP) and red cells lysis (AC) techniques. In case of LMP, the principal is to generate a thin interlayer, after centrifugation, which isolates monocytes and lymphocytes. At the end of the centrifugation the remaining BM components (red cells and granulocytes), are lying on the bottom of the falcon tube used. In contrast, AC solution lyses erythrocytes and following centrifugation, the remaining WBCs (lymphocytes, monocytes and granulocytes) are concentrated within the cell pellet*.

For cell separation using density gradient separation technique, BM was initially diluted with PBS (1:1) and subsequently layered over the LMP solution (10ml of diluted BM for 15ml of LMP) (Figure 7a). Following centrifugation at 600xg for 20min. at RT, the interface, denoted by an arrow (Figure 7b) and part of the supernatant plasma layer was aspirated using sterile pipettes and the rest discarded, (Figure 7c,d). Subsequently the solution was centrifuged for 4min. at 600xg to concentrate the cell pellet, and the supernatant was discarded. The cell pellet was then resuspended, washed twice with 5ml of PBS and finally cells were counted in 1ml of PBS solution (Figure 7e).

For cell separation using red cells lysis techniques, a 0.86% AC solution it was prepared fresh every time, by dissolving 4.3g of high purity AC salt in 500ml of distilled and sterilized water. In a 50ml falcon tube, 8ml of 0.86% AC solution was added for every 2ml of BM, (Figure 8a). The solution was then gently mixed and placed into an incubator at 37°C in 5% CO_2, for 10min. to lyse the erythrocytes. The purified cell pellet was obtained following 3 cycles of centrifugation (washing) at 600xg for 4min. each, (Figure 8b,c) and then cells were counted in 1ml of PBS solution (Figure 8d).

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*Acronyms LMP and AC, will be used in this manuscript as abbreviations of lymphoprep and ammonium chloride, respectively, but also to refer to density gradient separation and red cells lysis techniques.

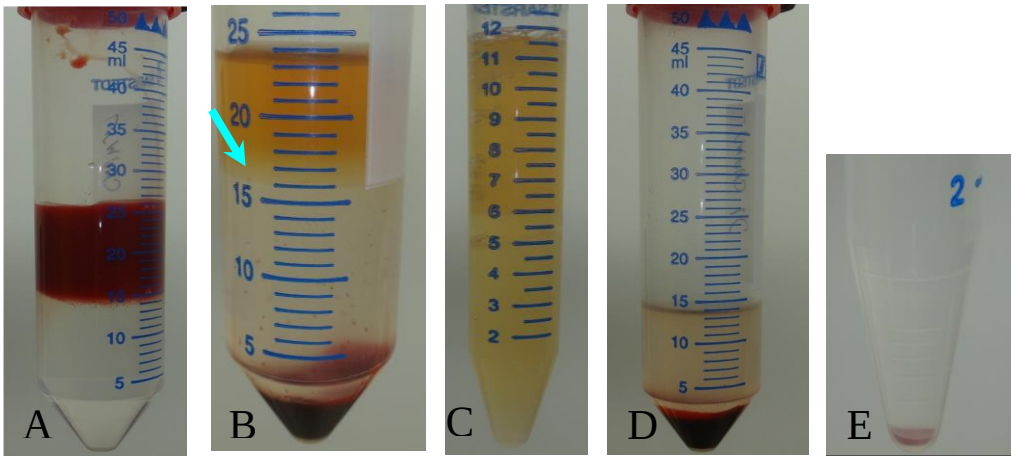


Figure 7: Overview of BM processing using LMP. A) Diluted BM laid over LMP, B) Supernatant and interlayer (arrow) containing the cells of interest (top). LMP and discarded component as appears following centrifugation (bottom), C) Supernatant and interlayer containing the cells of interest isolated and ready for further wash, D) Discarded component and E) Final cell pellet.

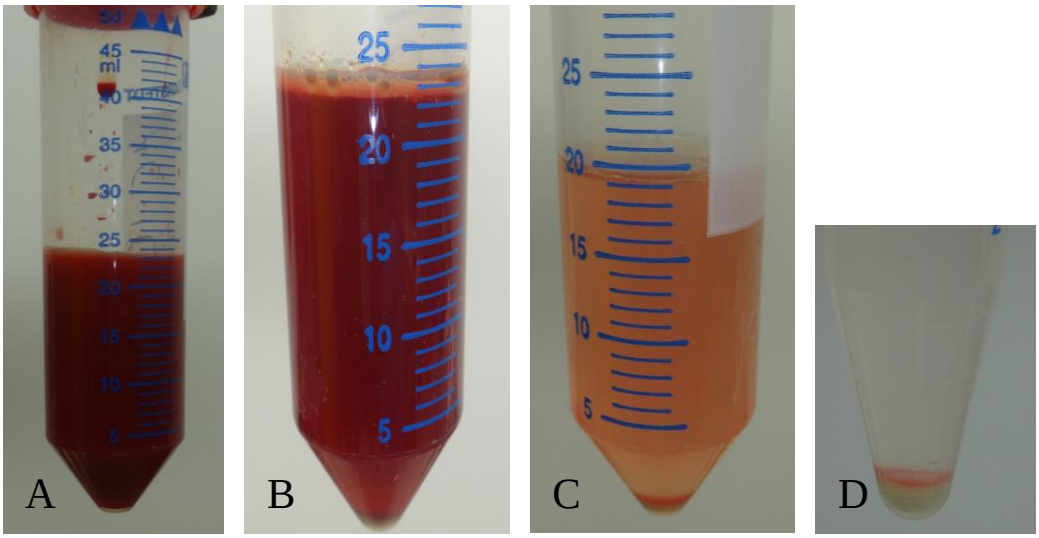


Figure 8: Overview of BM processing using AC. A) BM mixed with AC, B) Following 10min. incubation, C) Cell pellet is visible following first centrifugation, D) Final cell pellet following thee cycles of wash.

3.3 Tissue culture techniques

3.3.1 Cell counting and cells' viability estimation, with trypan blue dye

The cell pellet, following centrifugation, was resuspended in 1ml of PBS and cells were counted in 0.4% trypan blue solution, loaded on a haemocytometer (improved Neubauer type), under an inverted microscope (Leica DMIL090). As per counting method protocol, 1:2 dilution (10μl of cell solution were diluted with 10μl of trypan blue) or 1:10 dilution (10μl of cell solution were diluted with 90μl of trypan blue) were performed according to the number of cells[178], before counting.

The viability of cells using trypan blue dye, works based on the integrity of the cellular membrane, the dye can not penetrate intracellularly when the membrane is intact, therefore the cells appear as a transparent spheroid surrounded by a bright crown (Figure 9a), otherwise they appear as a dark-blue disc (Figure 9b). Cell concentration was calculated using the following mathematic formula:
Cells/ ml = Mean cell count from 4 large grid squares x 10^4 x dilution factor[178].

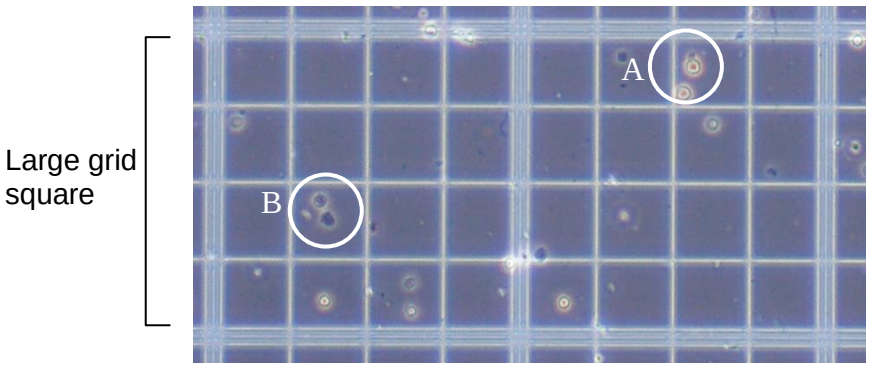


Figure 9: Microscopic view of haemocytometer during cell counting. a) Viable cells with intact cellular membrane excluding the dye, b) Non-viable cells absorbing the dye. 0.4% trypan blue solution was used. Magnification x40.

3.3.2 Cells' cryopreservation

Freezing medium solution was prepared, under sterile conditions, consisting of 10% Dimethyl sulfoxide (DMSO), 45% Fetal bovine serum (FBS) and 45% Dulbecco's modified eagle's medium (DMEM).

Following counting, 10-20x10^6 of mononuclear cells or at a later stage 1x10^6 of MSCs, were frozen in each cryovial, appropriately re-suspended into 1ml of freezing medium. The cryovials were placed into a freezing container, containing isopropanol which allowed cells to freeze gradually, preserving the cellular membrane integrity. Subsequently, following 24hours stay in -80°C freezer, cryovials were transferred to -150°C freezers or into liquid nitrogen for long-term storage[178].

3.3.3 Cells' resuscitation

DNAse ready solution, Sigma, was prepared by dissolving DNAse in 2.75ml of PBS and frozen in aliquots of 20μl at -20°C. This was then added to the medium to prevent the "clumping" effect of the DNA released from the dead cells, which can aggregate the viable cells together, resulting in cells' loss. To the medium 10% FCS was also added, which has a protective and nutrient function, at the first stages of cells thawing[178].

The frozen cryovials were placed in water-bath at 37°C and as soon as the solution was defrosted, the content of a vial was quickly transferred into a 15ml falcon tube containing 5ml DMEM, supplemented with DNAse (20μl/10ml of medium), 10% FCS and Penicillin/Streptomycin (5ml/500ml medium). Cell pellet was subsequently gently re-suspended and washed twice in PBS by centrifugation at 200g for 10 min. At the end of the second cycle of wash, the supernatant was discarded and cell pellet was resuspended in 1ml of DMEM/FCS. At this stage cells were counted, as described above[21] and utilized accordingly for CFU-F assay, tri-lineage differentiation or for flow cytometry as described in sections 3.5, 3.6, 3.7, respectively.

3.3.4 Establishment of MSCs cultures

Following thawing and counting, cells were seeded in flasks with a density of $1x10^5$ per cm^2, using NH® MSCs expansion medium and incubating at 37°C in 5% CO_2. Cells were left for 2 days to adhere and fed twice weekly by half culture medium exchange[178]. All cultures were regularly examined under the microscope to monitor the culture progress and the early detection of colonies.

3.3.5 MSCs passaging, trypsinisation

When cells reached 80-90% confluency (passage P0), they were trypsinised for further expansion or freezing. Before trypsinisation, medium was discarded and the culture was washed using sterile PBS. Next, 0.1% trypsin solution was added in the culture, incubating for 2-3min. at 37°C in 5% CO_2 (Figure 10a,b)[178]. Cultures were examined microscopically and once cells were lifted from the plastic surface, cell suspension was transferred in equal amount of DMEM/10% FCS solution. Next, cells were counted, following two washing cycles (centrifugation/re-suspension at 200xg for 5min. in 5ml of DMEM/10% FCS solution).

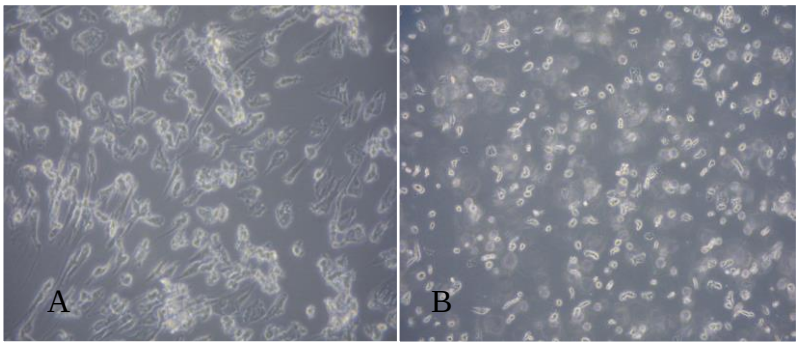


Figure 10: Microscopic view of trypsinisation process. A) MSCs partially detached from the plastic surface of the flask, B) MSCs retracted and floating on the trypsin solution, following 3min. of processing. Magnification x100.

3.3.6 Evaluation of MSCs colony sizes and population doubling time

The total area of colonies covering a 10cm diameter petri-dish, used for CFU-F assays, was calculated with Nikon NIS-Elements software, following scanning of the plates at 360dpi, using EPSON Perfection 2580 Photo scanner and imaging processing software ArcSoft Photostudio v.5. Briefly, the software following calibration, was able to measure the area of the plate covered by colonies and differentiate it from the empty space between them, calculating the total colony area. The single colony area was next calculated dividing the total area by the number of colonies.

Population doubling rates (PD) between passages, were calculated comparing the initial number of seeded cells (N1) and the final number of expanded cells (N2), and the results were divided by the number of days (D) in culture[178], using the formula $PD=Log_2(N2/N1)/D$.

3.4 CFU-F assays

CFU-F assays were performed, as previously described[179,180], in order to quantify MSCs from ICBM and VBBM using two isolation methods (LMP and AC). Following thawing and counting, $2x10^6$ cells were seeded (in duplicates) in 10cm diameter petri-dishes, with 8ml NH® MSCs expansion medium changed twice weekly and incubated at 37°C in 5% CO_2 for a 14 days period.

On the 14th day, culture was stopped, medium was removed and cells were washed twice with PBS and fixed with 7ml of 3.7% formalin (RT for 15min.). Formalin was next removed and colonies were stained with 5ml of 1% methylene blue, for 30min. at RT, then washed in tap water and air-dried.

Scoring was performed by counting the colonies under direct vision for each duplicate 10cm petri-dish. Afterwards the average number of colonies per dish was calculated and converted into number of colonies per million of cells plated and consequently into number of colonies per ml of BM.

3.5 Phenotypic characterisation of MSCs using flow cytometry

Reagents

Fluorescence-activated cell sorting (FACS) buffer;

Fixation/permeabilization buffer;

Permeabilization wash buffer;

5 ml round-bottom polystyrene FACS™ tubes;

Antibodies and fluorochromes, as listed below (**Tables 3,4,5**).

Procedure

Flow cytometry was performed with a two fold aim: first, to confirm the MSCs phenotype of cultured BM–derived plastic-adherent cells and secondly to investigate further the difference of osteogenic potential between IC and VB BM-MSCs.

Based on the minimal criteria for defining MSCs published from the International Society for Cellular Therapy (ISCT)[69], cells must meet three conditions: firstly, be capable of plastic-adherence when maintained in standard culture; secondly, be able to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*; and thirdly, express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Furthermore, SUSD2 surface marker for MSCs characterisation was also investigated in this study[181-183]. Additionally, in order to explore the different potential between IC and VB MSCs, especially in terms of osteogenic production, MSCA-1[182-186] (extracellular) and alkaline phosphatase[187,188] (ALP) (intracellular) markers, were also analysed (Tables 3,4). For technical reasons, flow cytometry experiment was performed in three groups (two for surface markers and one for the intracellular marker), as illustrated in Table 4.

The fluorochromes used in this particular set of experiments were designed to help characterize cells through surface and intracellular markers. Can be presented as a single dye (FITC, PE, APC, BV421) or as a tandem dye (PE CY-7). Both fluorochromes types following excitation by a laser beam produce a different wavelength energy, which is measured by separate detectors. Tandem technology, often used in combination with single dyes, in multicolour fluorescence studies, links two fluorochromes (a smaller and a bigger size) together, with the aim to increase the number of colours that can be produced by a single laser wavelength (Table 5).

In order to be able to compare the osteogenic potential between IC and VB BM-MSCs, same passage (P3) cultured MSCs, were used for flow cytometry and differentiation assays, utilising always the same four donors (AF28, AF29, AF39 AF50). The cells were thawed as described in paragraph 3.4.3, and subsequently spun for 10min. at 500xg at 4°C, the supernatant was discarded and the cells were resuspended in 1ml of ice cold PBS and counted. Following counting, cells were centrifuged again and resuspended in 150µl of ice cold FACS buffer. MSCs were divided into two groups for surface staining, and a third group for intracellular staining. Moreover, for the haematopoietic lineage, most of the negative markers (CD14, CD19, CD34, HLA-DR) were combined into a single tube, as per validated a published protocol of Leeds MSCs' laboratory[183]. 7AAD staining was also utilized, prior to acquisition, to exclude dead cells and debris[189,190]. Non-stained cells (for the first two groups of surface staining) and isotype control for intracellular staining (third group) were used as negative controls, respectively, to set gates on positive labelled cells and exclude non-specific binding. The events analysis and gate strategy used, will be illustrated in the results section 4.7.1.

The surface staining was performed via incubation of 50µl of cells suspension with the primary conjugated antibodies, for 30min. at RT in dark, using a 96-well rounded bottom plate. Following two washes in 200µl FACS buffer and centrifugation for 5min. at 500xg at 4°C, the supernatant was discarded and cells were resuspended in 200µl of FACS buffer and next moved into 5ml FACS tubes for analysis.

For intracellular staining, 50µl of cells suspension with 100µl fixation/permeabilization buffer were mixed, then added into a 96-well rounded bottom plate and incubated on ice for 20min. This step was used to permeabilize the plasma membrane allowing the antibody to penetrate intracellularly, maintaining the morphological characteristics of MSCs for phenotype characterization. Following a wash in 100µl of permeabilization wash-buffer and centrifugation for 6min. at 300xg at 4°C, the supernatant was discarded and ALT antibody was added, incubating for 30min. at RT in dark. Next, a further wash was performed with 100µl of permeabilization wash-buffer, as before, and the cells were resuspended in 200µl of FACS buffer and transferred into 5ml FACS tubes for analysis. Data were acquired using a LSR II 4 LASER flow cytometer (BD, Biosciences), and analysed using FlowJo v7.6.5 software, (Treestar, Oregon, USA).

Flow cytometry was performed with the valuable assistance of Dr. Jehan El-Jawhari, who contributed mainly to data acquisition and technical support with the flow cytometer.

Table 3: List of the MSCs markers used for flow cytometry and their features.

Marker	Function
Standard positive markers for MSCs	
CD105	Endoglin, is a type I integral transmembrane glycoprotein and is an accessory receptor for TGF-β complex. It is highly expressed on vascular endothelial cells, chondrocytes, syncytiotrophoblasts of term placenta, and it is also present on activated monocytes, MSCs and leukemic cells of lymphoid and myeloid lineages[191,192].
CD73	Ecto-5'-nucleotidase, commonly serves to convert AMP to adenosine, at neutral pH. Can be also related to stromal interaction and MSC migration. It is used as a marker of lymphocyte differentiation[191,194].
CD90	Originally discovered as a thymocyte antigen (Thy-1). Can facilitate cell-cell interactions and participate in adhesion of monocytes and leukocytes to endothelial cells and fibroblasts. It is used as a marker of MSCs and mature neurons[191].
Additional positive markers for MSCs	
SUSD2	Sushi Domain Containing 2, is novel and specific marker for BM-MSCs isolation and can be used on cryopreserved MSCs. Also it is expressed on vessel-surrounding smooth muscle cells, and a few neoplastic cell lines[181-183].
MSCA-1	W8-B2/MSCA-1 is expressed by BM MSCs, and is identical to tissue nonspecific alkaline phosphatase antigen (TNAP). Is also expressed at high levels in human liver, bone, kidney, embryonic stem cells and co-expressed on BM $CD271^+$ MSCs[182-186].
ALP	Used for detection of endogenous alkaline phosphatase activity from MSCs[187,188].
Standard negative markers for MSCs	
CD14	Acts as a co-receptor (along with the TLR 4 and MD-2 receptors) for the detection of bacterial lipopolysaccharides. It is mainly expressed on monocytes and macrophages[69,184].
CD19	Acts influencing the threshold for antigen receptor-dependent stimulation of B-Lymphocytes[69,184].
CD34	It is a cell surface glycoprotein expressed on hematopoietic progenitors and endothelial cells and functions as a cell-cell adhesion factor[69,184].
CD45	Protein tyrosine phosphatase, receptor type, C (PTPRC) is also known as leukocyte common antigen (LCA). It is a type I transmembrane protein present on differentiated hematopoietic cells, B- and T-Lymphocytes[69,184].
HLA-DR	Human leukocyte antigen complex-DR (HLA-DR) presenting cells. It is present on macrophages, B-Lymphocytes and dendritic cells[69,184].
Dye to exclude dead cells and debris	
7-AAD	7-Aminoactinomycin D, is a membrane impermeant dye that is generally excluded from viable cells. It binds to DNA by intercalating between base pairs in G-C-rich regions[183,193].

Table 4: List of antibodies and dyes used in flow cytometry to confirm MSCs' phenotype and their osteogenic potential.

Marker	Fluorochrome	µl/well	Isotype	Clone	Manufacturer / catalogue number
First group, surface markers					
CD105	FITC	10	IgG1, K	43A4E1	MACS Miltenyi Biotec 130098774
CD34	PE	10	IgG1, K	563	BD 550761
CD14	PE	10	IgG2a, K	M5E2	BD 555398
CD19	PE	10	IgG1, K	HIB19	BD 555413
HLA-DR	PE	10	IgG2a, K	G46-6	BD 555812
7ADD	Dye	10			BD 559925
Second group, surface markers					
CD73	BV421	5	IgG1, K	AD2	BD 562430
CD90	FITC	5	IgG1, K	F15-42-1	MCA90F ABD Serotec
CD45	PE Cy-7	5	IgG1, K	HI30	BD 557748
SUSD2	APC	5	IgG1, K	W5C5	Bio Legend 327408
MSCA-1	PE	10	IgG1, K	W8B2	MACS Miltenyi Biotec 130093587
7ADD	Dye	10			BD 559925
Third group, intracellular marker					
ALP	PE	15	IgG1, K	B4-78	R&D Systems FAB1448P

Table 5: List of fluorochromes used for flow cytometry and their spectrum range. Information illustrated in this table, has been provided by the manufacturer.

Fluorochromes	Absorption/Emission (max)
FITC: Fluorescein isothiocyanate, is sensitive to pH changes and to photo-induced degradation.	494/520nm
PE: Phycoerythrin, is an accessory photosynthetic pigment found in red algae.	496/578nm
PE CY-7: Phycoerythrin coupled to cyanine dye CY-7, is a combined fluorochrome, as bright as the PE. Particularly sensitive to photo-induced degradation, resulting in loss of fluorescence and changes in fluorescence spill-over.	496, 546/785nm
BV421: Brilliant violet 421, is a very bright polymer-based dye, excited by the violet laser.	407/421nm
APC: Allophycocyanine, is an accessory photosynthetic pigment found in blue-green algae. It has 6 phycocyanobilin chromophores per molecule, which makes it a very bright fluorochrome.	650/660nm

3.6 Tri-lineage differentiation assays

3.6.1 Osteogenic differentiation of MSCs (Calcium assay, Alizarin red staining, Alkaline phosphatase activity)

3.6.1a Spectrophotometry, calcium assay

Reagents and basic osteogenic medium preparation

Basal medium was prepared, under sterile conditions, dissolving 50ml of FBS (Gibco), for MSCs' nutritional supply and 5ml of penicillin and streptomycin antibiotics (Gibco), for infection prevention, in 445ml of DMEM (Gibco). Subsequently, dexamethasone, ascorbic-2-phosphate and beta-2-glycerophosphate, inducing osteoblastic and mitogenic effects in MSCs, were added, as previously described[195].

Dexamethasone (Sigma). 25mg of dexamethasone were dissolved in 1280μl of 100% ethanol, giving a 50mM stock solution. Next, it was aliquoted into 10μl volumes and frozen at -20°C. Subsequently, 1ml of DMEM was added to the 10μl volumes to give a solution of 500μM which was then aliquoted in 20μl and frozen at -20°C. The final solution was added to the osteogenic medium just before use, at 1:5000 dilution (20μl in 100ml medium) to give a final concentration of 10μM.

Ascorbic-2-phosphate (Sigma). 2.57g of ascorbic-2-phosphate were dissolved in 50ml dH_2O, giving a 200mM stock solution. Next, it was aliquoted into 50μl volumes and frozen at -20°C. The solution was next added to the osteogenic medium at 1:2000 dilution (50μl in 100ml medium) to give a final concentration of 0.1mM.

Beta-2-glycerophosphate (Sigma). 10g of beta-2-glycerophosphate were dissolved in 23ml dH_2O, giving a 2M stock solution. This it was next aliquoted into 500μl volumes and frozen at -20°C. The solution was next added to the osteogenic medium at 1:200 dilution (500μl in 100ml medium) to give a final concentration of 10mM.

Sentinel diagnostic calcium kit for spectrophotometric reading (Sigma). The kit contains 3 solutions: a) the calcium standard (10mg/dl or 100 μg/ml), which was used for the standard curves, b) reagent 1 (2-aminoethanol buffer, MEA) and c) reagent 2 (Cresolphtalein complex-one, CPC). Reagents 1 and 2 were mixed only before use at a rate of 5:2, and the working solution was added to all the wells containing standards and samples solution. Thereafter, the different levels of purple colour generated, were measured spectrophotometrically.

Procedure

For all differentiation assays, MSCs were expanded up to 3-4 passages (P3/P4), using 25-150cm^2 flasks, until reaching sufficient number of cells. At every passage trypsinisation and counting procedures were performed, as described in chapter 3.4. Osteogenic medium was made, supplementing 100ml of basal medium with 50μl of ascorbic-2-phosphate, 500μl of beta-2-glycerophosphate and adding before use, 20μl of dexamethasone.

MSCs osteogenic induction was performed in triplicates seeding 3 x 10^4 MSCs per well using a 6-well plate. In each well were added 5ml of osteogenic medium and half medium was changed twice a week, for a total of 14 days, in contrast to published osteogenic differentiation protocols which suggest 21 days of induction[200-202]. The reason for this decision was the vigorous growth of MSCs, risking to over-populate the plate and compromise the outcome of the assay.

At the end of the osteogenic induction period, the medium was discarded, the cultures were washed twice with PBS (Ca^{++} free) and 1ml of 0.5N HCl was added to the plate for 5min. at RT, to extract the Ca^{++} produced. Subsequently, with the help of a cell scraper, calcium deposits and fixed MSCs were lifted from the plates' surface and transferred to 1.5ml micro-centrifuge tubes. These, were next loaded on a rotator device and left overnight in continuous mixing at 4^{o}C, before storing at -20^{o}C freezer. When vials from all 4 donors were collected (a total of 48 vials) calcium assay was performed using spectrophotometry. For this purpose, vials were micro-centrifuged at 976g for 5min. and the supernatant was transferred to new micro-centrifuge tubes. Subsequently, a 96-well plate (flat bottom) was used for each donor, loaded with serial dilutions of the standard calcium solution, performed in triplicates, and 3 aliquots of the supernatant from each micro-centrifuge tube, as illustrated in Table 6. Each well was loaded first with 4μl of calcium solution (standards and samples) and next were added 196μl of the working solution (reagents 1 and 2), generating a different grades of purple colour, based on calcium concentration contained in each well. Calcium per well was then measured spectrophotometrically using a Mithras LB 940 reader, at 570nm wavelength. Optical density (OD) readings were then plotted using Microsoft Excel 2003 and analysed with GraphPad Prism 6, calculating the calcium concentrations per well, and therefore per ml of solution.

Table 6: Calcium assay, setup of a 96-well plate for spectrophotometry. Each 96-well plate was loaded using the same arrangement as illustrated below. The first 3 columns were for standard curves and the rest of the plate was divided in 4 areas, based on the same configuration in triplicates, used for the osteogenic differentiation assay (IC AC, IC LMP, VB AC, VB LMP), testing 3 aliquots from each micro-centrifuge tube. 1:2 and 1:5 dilutions were performed, when OD was above the standard curve range.

µg/ml	1	2	3	4	5	6	7	8	9	10	11	12
A	100µg	100µg	100µg	IC AC 1 (Triplicates)				V12 AC 1 (Triplicates)				
B	50µg	50µg	50µg	IC AC 2 (Triplicates)				V12 AC 2 (Triplicates)				
C	25µg	25µg	25µg	IC AC 3 (Triplicates)				V12 AC 3 (Triplicates)				
D	12.5µg	12.5µg	12.5µg	IC LMP 1 (Triplicates)				V12 LMP 1 (Triplicates)				
E	6.25µg	6.25µg	6.25µg	IC LMP 2 (Triplicates)				V12 LMP 2 (Triplicates)				
F	3.13µg	3.13µg	3.13µg	IC LMP 3 (Triplicates)				V12 LMP 3 (Triplicates)				
G	1.56µg	1.56µg	1.56µg									
H	0µg	0µg	0µg									

3.6.1b Matrix mineralisation, alizarin red staining

Reagents

Ammonium hydroxide 10% (Sigma), used for pH neutralisation. A 10% ammonium hydroxide solution was prepared with 30ml 28% ammonium hydroxide diluted in 60 ml of dH_2O.

Alizarin red solution (Sigma), used for staining of calcium-rich deposits as previously described[198]. A 40mM alizarin red solution was made by dissolving 2g of alizarin red in 100ml dH_2O. The solution was next made up to 4.1pH, by adding the necessary amount of 10% ammonium hydroxide.

Procedure

Osteogenic medium composition and culturing procedure were the same as described per calcium assay previously in section 3.7.1a.

Alizarin red staining was performed in duplicates, seeding 3 x 10^4 MSCs per well in a 6-well plate. At the 14^{th} day of osteogenic induction, the medium was discarded, cultures were washed twice with PBS (Ca^{++} free) and fixed adding 70% cold ethanol for 1 hour at RT. Subsequently, ethanol was removed, plates were rinsed 5 times

with dH_2O and calcium deposits were stained with alizarin red for 10min. at RT. Next, plates were rinsed again 5 times, as previously, and allowed to dry before scanning using EPSON Perfection 2580 Photo scanner and imaging processing software ArcSoft Photostudio v.5.

3.6.1c Alkaline phosphate assay, fast blue RR staining

Reagents

Citrate (Sigma), used as component of fixative solution. A 2% of citrate working solution was prepared by dissolving 2ml of citrate concentrate solution, into 98ml of dH_2O.

Fixative solution, used for MSCs fixation. 2 volumes of citrate working solution were mixed with 3 volumes of acetone.

Fast Blue solution (Sigma), used as hystochemical semi-quantitative demonstration of alkaline phosphatase activity in MSCs. A fast blue RR dye was prepared using a 50ml centrifuge tube shielded from light, by dissolving fast blue RR salt capsule (4-Benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi-zinc chloride) into 48ml of dH_2O, in a 37°C water-bath. Following, 2ml of Naphthol AS-MX phosphate alkaline solution (3- Hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate, Sigma) was added to fast blue solution.

Procedure

Osteogenic medium composition and culturing procedure were the same as described per calcium assay and alizarin red staining previously, sections 3.7.1a and 3.7.1b. Procedure was performed as per Sigma-Aldrich validated and published protocol (n85).

Alkaline Phosphatase assay was performed in duplicates, seeding 3×10^4 MSCs per well in a 6-well plate. At the 14^{th} day of osteogenic induction, the medium was discarded, cultures were rinsed twice with PBS (Ca^{++} free) and fixed using the fixative solution for 30sec and plates were rinsed twice with dH_2O. Next plates were incubated at RT with fast blue solution for 60min., protected from light. Upon the completion of staining, dye was discarded and plates rinsed twice with dH_2O allowing them to dry at RT, before scanning with an EPSON Perfection 2580 Photo scanner.

3.6.2 Adipogenic differentiation of MSCs (Oil red-O, Nile red/DAPI staining)

Reagents and basic medium preparation

Ready NH® AdipoDiff medium (Miltenyi Biotec), for MSCs adipogenic differentiation was used. Medium, was composed by: DMEM, L-Glutamine, FBS, phenol red and other not specified supplements, as per manufacturer description. No further information regarding the concentration of the single compounds of the solution was provided by the manufacturer.

Penicillin/Streptomycin (Gibco/Life Tech) was added to prevent culture infection. Antibiotic solution was aliquoted in 5ml and stored at –20ºC. This was added to adipogenic medium at a 1:100 dilution (1ml per 100ml of NH® AdipoDiff medium).

Procedure

MSCs were seeded in a 48 well plate at a density of 4 x 10^4 cells per well and cultured during 21 days in 1ml volume of adipogenic medium, performing half medium changes twice a week, as previously described[201,203,204]. Cell cultures were regularly checked under microscope, at every medium change, monitoring the cellular growth. A total of 3 wells were utilized per each condition (IC AC, IC LMP, VB AC, VB LMP), one well for Oil red-O staining and two for Nile red/ DAPI staining. At the 21st day, induction was stopped, culture medium was discarded, wells were rinsed with PBS, and stained accordingly.

3.6.2a Oil red-O staining

Reagents

Oil red (Sigma), used to stain the lipid droplets. A 0.5% of Oil red solution was made by dissolving 50mg of Oil red powder in 10ml of isopropanol in 37°C water-bath for 30min. Subsequently, 3 parts of Oil Red stock solution was diluted with 2 parts of dH_2O and next filtered through a 0.8μ pore filter followed by 0.2μ filter to remove particles.

Formalin 10% (Sigma), was used to fix cells before staining. A 10% formalin was made diluting 27ml of 37% formalin stock solution in 73 ml of dH_2O.

Procedure

At the 21st day of adipogenic induction, medium was discarded, wells rinsed twice with PBS and cells were fixed with 10% formalin for 10min. Next, plates were rinsed again twice in PBS and 1ml of filtered Oil red-O was added for 10min. Following incubation at RT, Oil red-O was removed and wells were rinsed again twice in PBS. The red-ruby coloured adipose vesicles, contained in MSCs, were observed and photographed using an inverted microscope (Olympus CKX41) and the Olympus digital camera attached.

3.6.2b Nile red/DAPI staining and spectrophotometric reading

Reagents

Saponin 0.2% (Sigma), used to increase membrane permeabilization and subsequently intracellular staining. A 0.2% of saponin solution was made by dissolving 0.2g of saponin powder in 100ml of PBS.

Nile red (Sigma), used to stain the intracellular lipid droplets. Nile red stock solution of 10mg/ml in methanol aliquot was made and stored at -20°C. A working solution was made adding 1μl of Nile red in 10ml of 0.2% saponin solution, (1:1000 dilution).

4,6-diamidino-2-phenylindole (DAPI) (Invitrogen), used to stain the nucleus. DAPI stock solution was made by adding 2μl of dH_2O to the entire contents of the DAPI vial to make a 14.3mM (5 mg/μl) and stored at –20°C. A working solution was made adding 100μl of DAPI in 10ml of 0.2% saponin solution.

Formalin, 3.7% (Sigma), used as fixative it was made by diluting 10ml of 37% formalin stock solution in 90ml of dH_2O.

Procedure

At the 21st day of adipogenic induction, medium was discarded, cultures were washed twice with PBS and next fixed with 3.7% formalin for 30min. Subsequently, formalin was removed and 200μl PBS were added to each well. Before Nile red/DAPI staining, the background fluorescence was read using excitation and emission filter sets for DAPI (355/460) and Nile red (485/535), as per manufacturer's instructions using a Mithras LB 940 reader. Wells were "spot read" in a 5x5 pattern, providing 25 readings per well. The PBS was then removed from the wells, and 200μl 0.2% saponin solution was added along with DAPI and Nile red (10ml of 0.2% saponin solution with 1μl/ml of Nile red and 1μl/ml of DAPI). The plate was then protected from light and incubated at RT for 15min. Following three washes with PBS, 200μl PBS were added, and plates were scanned again as before. The adipogenesis ratio was determined, calculating the different fluorescence emitted between Nile red and DAPI staining.

Subsequently, fluorescent microscopy was carried out using an inverted microscope (Olympus CKX41) combined with a light generator (Olympus U-RFLT50). Images were photographed using an attached Olympus digital camera.

3.6.3 Chondrogenic differentiation of MSCs
(Glycosaminoglycan assay, qualitative analysis of chondrogenic pellets)

Reagents and basic chondrogenic medium preparation

Chondrogenic basal differentiation medium, was prepared, under sterile conditions, supplementing DMEM-high glucose medium (4.5g/l) (Gibco), with the following ingredients, up to 500ml of total product, in order to promote chondroblastic and mitogenic effects[197,198] in MSCs.

Ascorbic-2-phosphate (Sigma), used to enhance chondrogenic differentiation and collagen matrix production of MSCs[197,198]. A 200mM stock solution was made by dissolving 2.895g in 50ml of distilled water. The stock solution was aliquoted into 0.5ml volumes and stored at -20°C and it was next added to basal chondrogenic medium at a 1:1000 dilution (0.5ml to 500ml of basal chondrogenic medium).

Sodium pyruvate (Sigma). A 100mM stock solution was made by dissolving 1.1g sodium pyruvate in 100ml of distilled water. The stock solution was aliquoted into 0.5ml volumes and stored at -20°C and it was next added to chondrogenic medium at a 1:100 dilution (5ml in 500ml of basal chondrogenic medium).

Proline (Sigma). Stored at RT, was added to basal chondrogenic medium at 20mg per 500ml.

Bovine serum albumin (BSA) (Sigma). Stored at 4ºC. It was added to chondrogenic medium, as MSCs' nutritional supply, at 1:5 dilution (50ml per 450ml of basal chondrogenic medium).

Penicillin/Streptomycin (Gibco/Life Tech). It was aliquoted in 5ml and stored at –20ºC. Next, it was added to chondrogenic medium at 1:100 dilution (5ml per 500ml of basal chondrogenic medium), to prevent culture infection.

Supplements added immediately before use, to complete the basal chondrogenic medium

Dexamethasone, TGFβ3 and ITS+ show a synergistic effect enhancing the expression of collagen genes and increasing the numbers of differentiated MSCs in culture, leading to a higher concentration of a functional cartilaginous tissue[197-199].

Dexamethasone (Sigma). A 25mg of dexamethasone were dissolved in 1280μl of 100% ethanol, giving a 50mM stock solution. This, was next aliquoted into 10μl volumes and frozen at -20°C. Subsequently, 1ml of DMEM was added to 10μl volumes to give a solution of 500μM which was aliquoted in 20μl and frozen at -20°C. The solution was added to the basal chondrogenic medium just before use, at a 1:5000 dilution (1μl in 5ml of medium).

Transforming growth factor (TGFβ3) (R&D Systems). For the TGFβ3 stock solution, the follow steps were performed: 1) 4mM HCl containing 1mg/mL BSA diluent solution was prepared (2.15mL HCl were added to 25ml of dH_2O, at 1:250 dilution, followed by 25mg of BSA). 2) 2μg TGFβ3 were dissolved in 2ml of the previously prepared diluent to give 1μg/ml TGFβ3 stock solution. It was aliquoted into 20μl and stored at –20ºC, and added to basal chondrogenic medium at 1:1000 dilution (50μl in 5ml of medium).

Insulin-Transferrin-Selenium (ITS+) (Invitrogen). ITS+, was added to chondrogenic medium at 1:1000 dilution (50μl in 5ml of basal chondrogenic medium).These important supplements are utilized by most mammalian cells. They promote glucose and amino acids uptake (insulin) and in combination with essential trace elements (transferrin and selenium) enhance MSCs proliferation and chondrogenic differentiation, in vitro[197-199].

Papain (Sigma) was used at a final stage in order to digest the chondrogenic pellet, for the GAG concentration measurement. For the final papain solution at pH 6.5, the following steps were performed: 1) a sodium phosphate buffer was made mixing 100mM Na_2HPO_4 (7.1g/500mL dH_2O) with 10mM EDTA (1.86g/500mL dH_2O) till reaching the desirable pH. 2) For the papain solution were mixed 125μg/ml papain, 10mM cysteine (0.035g/20mL) and 50U/mL hyaluronidase with the above buffer and filtered using a 0.2μ filter. Then the final product was aliquoted in 1ml vials and stored at –20ºC. It was added to chondrogenic pellets at 100μl per vial.

3.6.3a Glycosaminoglycan assay

Sulphated glycosaminoglycan (GAG) assay kit (Blyscan™), was used to stain the chondroitin 4-sulfate produced from chondrocytes. This kit contained 3 components: 1) dye reagent containing 1,9-dimethyl-methylene blue in an inorganic buffer, which also contains surfactants, 2) dissociation reagent containing the sodium salt of an anionic surfactant, formulated to dissociate the GAG-dye complex and enhance the spectrophotometric absorption of the dye, and 3) the reference standard solution made of bovine tracheal chondroitin 4-sulfate (100µg/ml).

Procedure

Chondrogenic basal medium was made, supplementing 500ml of DMEM (high glucose) with 0.5ml of ascorbic-2-phosphate, 5ml of sodium pyruvate, 20mg of proline, 500mg of BSA and 5ml of penicillin/streptomycin. To complete the medium the following it was added before use: 1µl of dexamethasone, 50µl of TGFβ3 and 50µl of ITS in 5ml of chondrogenic basal medium, as described in section 3.7.3.

Chondrogenic induction was performed in quintuplicates seeding 25 x 10^4 MSCs per micro-centrifuge tube. Three samples were dedicated to quantitative analysis of GAG content (GAG assay) and two for qualitative analysis cryo-sectioning and staining the chondrogenic pellets with toluidine blue dye, which stained purple the GAG molecules of the cartilage. To arrange the cells in a three-dimensional spherical model (pellet), MSCs were micro-centrifuged in centrifuge tubes at 2.439xg for 5min., in a provisional medium (0.5ml of DMEM with 2%FCS), which was replaced afterwards by equal amount of compete chondrogenic medium. The latter was changed 3 times a week (half medium each time), during the 21 days of chondrogenic induction, incubating at 37°C 5%CO_2, as per validated and published protocols[200,205] (Figure 11).

Chondrogenic induction was stopped after 3 weeks, the medium was discarded, the pellets were washed twice with PBS and to those intended to use for GAG assay, 100µl of papain was added in the micro-centrifuge tubes following overnight incubation in water-bath at 65°C. Next, centrifuge tubes were micro-centrifuged at 6.099xg for 5min. and stored at –20ºC. When vials from all 4 donors were collected (48 for GAG-assays and 19 for cryo-sectioning, 77 vials in total,) the assay was performed using spectrophotometry.

For this purpose, from each micro-centrifuge tube 30μl of the dissolved with papain chondrogenic pellet, were placed into a new micro-centrifuge tube (in duplicate), adjusting the contents to 100μl with dH_2O. Thereafter, serial dilutions of the standard GAG solution were made in duplicates making each standard up to 100μl with dH_2O. To each micro-centrifuge tube 100μl of the dye reagent were added, and loaded on rotator disc for 30min. in continuous mixing at RT. Following micro-centrifugation at 14.636xg for 10min., GAG-dye complex formed, precipitating as pellet, and the residual unbound dye was next discarded. At this stage, 0.5ml of the dissociation reagent was added in each tube and mixed using a vortex device followed by a second micro-centrifugation at 14.636xg for 5min. Subsequently, a 96-well plate (flat bottom) was loaded with serial dilutions of the standard GAG solution (in duplicates), and 3 aliquots of 100μl from each sample to test (in triplicates), as illustrated in Table 7.

As result of the chemical reaction between the GAG content of each sample and the 1,9-dimethyl-methylene blue dye, different levels of blue colour were generated. The amount of GAG per well, was determined spectrophotometrically using a Mithras LB 940 reader, at 620nm wavelength. OD readings were then plotted using Microsoft Excel 2003 and analyzed with GraphPad Prism 6, calculating the concentrations per well, and therefore per ml of solution.

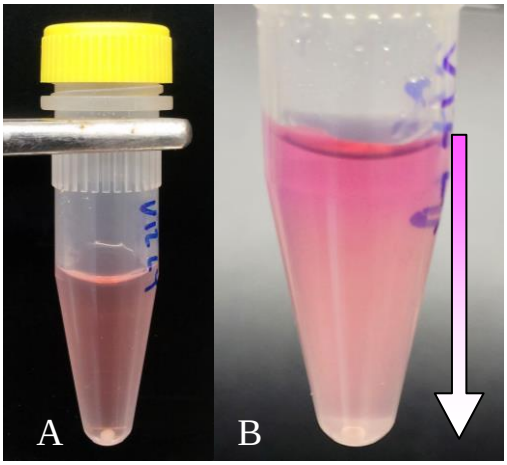


Figure 11: Chondrogenic pellets during induction period. A) The lid of the micro-centrifuge tube was left slightly loose to allow gas exchange. Typical spheroid pellet is visible at the bottom of the tube. B) Characteristic change of the chondrogenic medium colour around the pellet, due to metabolic activity of the developing chondrocytes, following two days from medium change. A total number of 77 chondrogenic cultures were performed for all four donors.

Table 7: Composition of the 96-well plate for spectrophotometry. The first 2 columns were dedicated to the standard curves and the rest of the plate was divided in groups of 4 areas per patient, based on the same configuration in triplicates, used for the previous steps of the GAG assay (IC AC, ICL MP, V12 AC, V12 LMP), testing 2 aliquots per eppendorf tube. 1:2 and 1:5 dilutions were performed, in case that samples' OD was above the standard curve range.

µg/ml	1	2	3	4	5	6	7	8	9	10	11	12
A	0µg	0µg	IC AC1 (Duplicate)		IC LMP1 (Duplicate)		V12 AC1 (Duplicate)		V12 LMP1 (Duplicate)			
B	1µg	1µg	IC AC2 (Duplicate)		IC LMP2 (Duplicate)		V12 AC2 (Duplicate)		V12 LMP2 (Duplicate)			
C	2µg	2µg	IC AC3 (Duplicate)		IC LMP3 (Duplicate)		V12 AC3 (Duplicate)		V12 LMP3 (Duplicate)			
D	3µg	3µg										
E	4µg	4µg										
F	5µg	5µg										
G												
H												

3.6.3b Reagents and materials for cryosection and toluidine blue staining for qualitative analysis of chondrogenic pellets

Toluidine blue (1% in 50% isopropanol) (Sigma), was prepared by mixing 50ml of 100% isopropanol with 50ml of dH_2O and subsequently 1g of toluidine blue powder was added to the solution, adjusting pH to 4. Next, the solution was filtered using a 0.45µ filter and kept in dark, to preserve the colour and the pH.

Optimisation staining of toluidine blue was performed prior to final staining of the pellet-slides. A combination of two different types of pH (2.5 and 4) and two different staining incubation times (10min. and 30min.) were tested, in order to determine the most appropriate combination for staining. The best result was obtained with a pH4 for 30min. incubation time (appendix 8.3). Chondrogenic differentiation was assessed by the purple stain of cartilaginous extracellular matrix (metachromasia), while undifferentiated or fibrous tissue was stained in blue.

Procedure for qualitative analysis of chondrogenic pellets

After 3 weeks of chondrogenic induction the culture was stopped, medium was discarded and pellets were embedded in OCT gel (optimal cutting temperature compound), an inert support made of polyethylene glycol and polyvinyl alcohol, avoiding bubbles formation, especially near the pellet[206]. OCT is characterized by a fluid nature at RT, which becomes solid, with a density similar to the pellet when frozen.

Next, OCT-pellets were snap-frozen in liquid nitrogen, to reduce ice-crystal formation, minimizing the morphologic damage and preventing cracks of the OCT-pellet block[207]. Samples were wrapped in labelled foil, and stored in -80°C.

When chondrogenic pellets from all 4 donors (4 condition per donor) were collected (n=16, in duplicates), a Leica CM1950 cryostat was used, to produce multiple sections of 4µm thickness. Sections were loaded on SuperFrost® yellow cut edge microslides, wrapped in labelled foil and stored in -80°C[208].

When ready to perform toluidine blue staining, the frozen sections on microslides were moved from –80°C freezer to RT and allowed to thaw gradually for 30min. In order to provide a better dye contrast, sectioned pellets were moisturised for 1min., by adding few drops of dH_2O directly onto the microslides. Subsequently, toluidine

blue staining was performed for 30min., adding the dye directly on the sections, at RT and protecting from light. Next dye excess was removed by gentle wash with dH_2O (3x immersions), the excess of water on microslides was wiped and sectioned pellets were dehydrated by xylene (3x immersions of 1min. each), under fume cupboard[206,208]. Finally, microslides were air dried for 5–10min., mounted in sub-x mounting medium (Leica) and covered by cover-glass applying gently a uniform pressure, to avoid air-bubbles[207]. Chondro-pellet images were captured at 64 and 160 magnification, using an Eclipse E1000 microscope.

Toluidine blue staining of chondro-pellets was performed with the valuable assistance of Dr. Filomena O.G. Esteves, providing mainly lab training and performing the quality control of the staining. Additionally, cryosection training and lab support has been provided by ex-Lab manager Karen Henshaw.

3.7 Enzyme-linked immuno sorbent assay (ELISA) analysis

Serum samples were thawed at RT and ensured to be thoroughly mixed on a roller. In order to avoid a long time exposure at RT of cytokines and repetitive freeze-thawing cycles, which can affect cytokines stability[209-211], serum was first aliquoted into a transient 96-well plates based on templates previously arranged, and cryopreserved at -80°C. Finally, serum was transferred to ELISA kits, at a later stage, for the measurement of pro-inflammatory and anti-inflammatory cytokines (IL-6, IL-18, PCT and IL-10) when ready to perform the assays.

Analysis was carried out with commercially available kits, from R&D Systems, MBL and Sigma-Aldrich (Table 8), employing the quantitative sandwich enzyme immunoassay technique. To minimize the variability within the different kits used ELISA kits with the same lot number were utilised per every molecule studied, (4 ELISA kits per molecule, for a total of 16 kits). Additionally, in order to be able to compare time-points between all four molecules, minimizing the variability, all samples from the same time-point of each patient were loaded on the same 96-well plate. ELISA kits codes and manufacturers for the protocols used, are located in appendix section 8.2.

Briefly, a monoclonal antibody specific for the molecule (IL-6, IL-18, PCT and IL-10) has been pre-coated on the micro plates, by the manufacturer. Duplicates of standards and serum samples, following appropriate dilutions, were added into the wells where the molecules of interest were bound to the corresponding antibodies. Following wash to remove any unbound substances, a further enzyme-linked polyclonal antibody (conjugate), specific for the molecule in question was subsequently added to the well. Following a further wash, to remove any unbound antibody/conjugate complexes, a colour reagent (substrate solution) was added and a different intensity of colour was developed in proportion to the test molecule concentration present in serum. The colour reaction was then stopped by adding sulphuric acid (stop solution) and the OD was measured within 30min. by a Mithras LB 940 spectrophotometer. The appropriate wavelength and the correction reading, when required (to neutralize plastic-ware, lamp and optical fluctuation effects), was set as per manufacturer instructions and every plate underwent a duplicate scanning. OD values were then transferred to Microsoft Excel software for analysis. The duplicate readings were averaged and subtracted to the averaged correction wavelength, obtaining the final absorbance. Standard curves were linearized using

log or 4-pl transformation based on manufacturer manual. Final concentrations were calculated considering the dilution factor used for each ELISA kit. Graphics were generated using GraphPad Prism for Windows. As every different molecule analysed with ELISAs, present variations in protocol, Table 8 summarises the differences for all the 4 molecules studied.

Table 8. Summary of ELISA protocols. The **i**nformation illustrated in this table, has been provided by the manufacturer.

	IL-6	IL-6 HS	IL-18	PCT	IL-10 HS
Assay/Sample dilution	1:2 dilution	1:2 dilution	1:5 dilution	No dilution	1:1.2 dilution
Addition of standard /sample per well	100µl	100µl	50µl	100µl	200µl
1st incubation at RT	2 hours	2 hours	1 hour	2.5 hours	2 hours
Wash after 1st incubation	4 times	6 times	3 times	4 times	6 times
Addition of conjugate solution per well	200µl	200µl	100µl	100µl	200µl
2nd incubation at RT	2 hours	2 hours	1 hour	1 hour	2 hours
Wash after 2nd incubation	4 times	6 times	3 times	4 times	6 times
Addition of substrate solution per well	200µl	50µl	100µl	100µl	50µl
3rd incubation at RT	20min.	60min.	30min.	45min.	1 hour
Addition of amplifier solution per well	No	50µl	No	100µl	50µl
4th incubation at RT	N/A	30 min.	N/A	30min.	30min.
Stop solution per well	50µl	50µl	100µl	50µl	50µl
OD reading OD correction	450nm 540nm	490nm 650nm	450nm 620nm	450nm ----	490nm 650nm
Standard Range	3.13-300 pg/ml	0.156-10 pg/ml	36.1-257.8 pg/ml	27.43-2x10^4 pg/ml	0.78-50 pg/ml
Manufacturer	R&D Systems	R&D Systems	MBL	Sigma-Aldrich	R&D Systems

3.8 Statistics

Statistical analysis and graphing were performed using Windows Excel 2003 and Graph Pad Prism v6 for Windows (San Diego California, USA). Median, mean values, along with interquartile ranges were calculated throughout all the experiments. All statistical analysis were based on mean or median values, depending on the Gaussian distribution of the data analyzed. Gaussian distribution was determined using Shapiro-Wilk and D'Agostino & Pearson omnibus normality tests. For paired values presenting Gaussian distribution, paired t test was performed, otherwise Wilcoxon test was used. For unpaired values showing Gaussian distribution unpaired t test was performed, applying additionally Welch's correction in case of different standard deviation between the compared groups. For unpaired values without Gaussian distribution Mann-Whitney U test was used. The cut-off value for significance was $p<0.05$.

Variability of the results, including intra-assay and inter-assay variability, was measured analyzing the coefficient of variation (CV) by dividing the standard deviation (SD) by the mean and multiplying by 100, CV = (SD/mean) x 100). Furthermore, to determine the relationship between two variables, Spearman's rank correlation coefficient test (R values) with a two tailed p-value was calculated using the Graph Pad Prism v6 software.

For the immuno-inflammatory component of the thesis, due to the limitations of GraphPad Prism v6 to support two-way nonparametric analysis of variance (ANOVA), was used the IBM SPSS v23 software. Nevertheless, in order to present the data with consistency throughout the thesis, the final analysis and graphics were performed using GraphPad Prism v6 and presented as mean ± SEM.

Moreover, given the lack of peripheral blood sampling (especially at T5, T6) for part of the cohort due to the early hospital discharges or their challenging clinical management, the missing data were treated by mean regression imputation generating unbiased estimates of means, using IBM SPSS v23 software. Additionally, prior to the final analysis, a pilot analysis was conducted to examine the assumptions of the statistical tests. Subsequently, polynomial regression coefficients were calculated and the missing values were substituted by the predicted mean for each time point (Appendix 8.1).

Given the GraphPad Prism v6 limitations to support a two-way ANOVA calculations, nonparametric analysis were performed at each time point using Friedman test (nonparametric equivalent of a repeated measure), Kruskal-Wallis test (nonparametric equivalent of one way ANOVA) and Mann-Whitney U test (nonparametric equivalent of independent samples t-test) separately.

Friedman test was used to examine differences in outcomes over one-week period in each approach, gender and duration of surgery on all four molecules examined (IL-6, IL-18, PCT and IL-10). Moreover, Kruskal-Wallis test was conducted to test for differences among the three surgical approaches (Posterior, Posterior-Costoplasty and Antero-Posterior) at each time point, while Mann-Whitney U test was conducted to test for differences of outcomes between genders and different durations of surgery (fast and slow groups) at each time point.

The one-way ANOVA section of the GraphPad Prism v6 in a first place determined whether there is an overall significance for Kruskal-Wallis and Friedman methods, without specifying the exact point. In case of statistically significant results, via the Dunn's multiple comparisons test, the exact points were located. However, in few occasions due to cohort limitations, even though an overall significant difference was present, multiple comparison could not determine where was the significance.

4. Results

4.1 Patients' recruitment

Research with minors presents some unique challenges, compared to adults, related mainly to their difficulty, to express their needs or defend their interests[212]. Moreover, the recruitment and consenting process involves minimum two individuals and often the entire family, increasing the complexity of the interactions and patient's management. It is also fundamental to gain their confidence and of their parents, who are not always familiar with a research process. Despite the additional difficulties, medical research involving children is essential for advancing child health and wellbeing, because often it is not scientifically or ethically appropriate to apply adult research findings into children necessities[212].

To all children involved in this research, it was given the possibility to express themselves unbiased. It was asked to family members or legal representatives to have a neutral role and understand that they should not attempt to influence or interpret the responses of participants, as it is also described in the article 12 of the United Nations Convention on the Rights of the Child (UNCRC) "*all children and young people who are capable of forming their own views, have a right to express those views freely in all matters affecting them, with the views of the child being given due weight in accordance with their age and maturity*"[213].

For children under 16 years old, consent was obtained from parents or legal representatives and also assent was required from the child involved. For adolescents between 16-17 years old, consent was obtained from them directly, however parents or legal representatives were present.

During all stages of recruitment, data collection and blood sampling, type of language and approaches utilized were adapted accordingly, to generate a more productive researcher-child rapport[213-215]. Also, almost always it was asked to parents or legal representatives to stay during data collection or peripheral blood sampling in order to guarantee a more comfortable environment for the child[214].

MRC Ethics Guide[212],Research with Children and Young People[213] and Good Clinical Practise (paediatrics) guidelines, were followed for the whole duration of the project.

4.2 Overview of patient's demographics

Over a 20 months period (09/2012-06/2014), 55 patients satisfying the inclusion/exclusion criteria were approached and 50 of them were successfully recruited. The remaining five patients did not agree to participate in this study, merely for personal reasons. Out of the 50 patients recruited, it was not possible to harvest BM and peripheral blood (PB) from 13 patients, due to unexpected circumstances or hospital organization issues, (5 patients decided to postpone the surgery until a later date and 8 patients were lost due to last minute changes of the theatre operation lists).

From the remaining 37 patients, PB was collected in different time points, isolating both plasma and serum. ICBM and VBBM were also harvested from 19 patients (14F/5M). Additionally, in seven patients BM was harvested from a second vertebra, usually the thoracic 11^{th} (V11), or the first lumbar (L1), to investigate whether the cell concentration, is similar from adjacent vertebrae, and if it can be affected by differences in vertebral size. An overview of the PB and BM harvested and related assays performed (enumeration, differentiations, flow cytometry and ELISA), is shown in Table 9. Patients' availability, for PB sampling, was often limited for the 5^{th} and 6^{th} time points, due the early hospital discharge.

Twenty-six patents were operated with posterior approach (21F/5M), 5 patients with posterior approach and costoplasty (5F), and 6 patients (3F/3M) with antero-posterior approach. For the purpose of the immuno-inflammatory component of this study a total of 23 patients have been selected. Serum from 12 patients (9F/3M) having posterior approach was analysed and compared to the remaining two groups (posterior approach and costoplasty (5F), and antero-posterior approach (3F/3M).

The overall age and sex distribution of the recruited patients is shown in Table10. A female predominance (29F/8M, F/M-ratio:3.6) was observed, as expected[1]. The age range was 13-17.8 years (median 15.4 years).

Table 9: Patient's overview. Blood sampling (time points), BM enumeration, proliferation and differentiation assays, phenotypic characterization and ELISA assays are illustrated.

N.	Patient Code	P P+Costoplasty AP	Time 0a	Time 0	Time 1	Time 2	Time Turn	Time 3	Time 4	Time 5	Time 6	Bone Marrow Harvesting	CFU-F assay	Differenti-ation assay	Flow-cytometry	ELISA assay
1	AF 01	P		√	√	√		√	√	√	√					√
2	AF 02	P	√	√	√	√		√	√							√
3	AF 03	P	√	√	√			√	√							√
4	AF 05	AP		√	√	√		√	√	√						√
5	AF 06	P		√	√	√		√	√		√					√
6	AF 07	P		√	√	√		√	√							√
7	AF 08	P	√	√	√	√		√	√							√
8	AF 09	P		√	√	√		√								
9	AF 10	P		√	√	√		√	√							
10	AF 11	P		√	√	√		√								
11	AF 12	P+Costoplasty		√	√	√		√		√						√
12	AF 14	P+Costoplasty		√	√	√		√								√
13	AF 16	P		√	√	√		√	√							
14	AF 17	P+Costoplasty		√	√	√		√	√							√
15	AF 18	P		√	√	√		√		√						√
16	AF 19	AP		√	√	√	√	√	√	√	√	√	√			√
17	AF 20	P		√	√	√		√	√			√	√			
18	AF 21	P		√	√	√		√	√							
19	AF 22	P+Costoplasty		√	√	√		√	√	√	√					√
20	AF 23	P		√	√	√		√	√	√		√	√			√
21	AF 24	P		√	√	√		√	√			√	√			
22	AF 26	P		√	√	√		√	√			√	√			√
23	AF 27	P		√	√	√		√	√	√	√	√	√			√
24	AF 28	AP		√	√	√	√	√	√	√		√	√	√	√	√
25	AF 29	P		√	√	√		√				√	√	√	√	√
26	AF 31	P		√	√	√		√	√			√	√			
27	AF 32	P		√	√				√			√	√			
28	AF 33	P		√	√	√		√	√	√		√	√			√
29	AF 34	P	√	√	√	√		√	√			√	√			
30	AF 35	P		√	√	√			√	√		√	√			√
31	AF 39	P+Costoplasty		√	√	√		√	√	√		√	√	√	√	√
32	AF 40	P		√	√	√		√	√	√	√	√	√			√
33	AF 43	P		√	√	√		√	√			√	√			√
34	AF 47	P		√	√	√		√	√							
35	AF 48	AP		√	√	√	√	√	√	√		√	√			√
36	AF 49	AP		√	√	√	√	√	√	√	√	√	√			√
37	AF 50	AP		√	√	√	√	√	√	√		√	√	√	√	√

The blank boxes indicate assays, peripheral blood and BM sampling, that were not performed.

Table 10. Patients' demographics, at the time of recruitment.

N.	Patient Code	Gender	Age (At recruitment)
1	AF 01	Female	13.19
2	AF 02	Female	17.07
3	AF 03	Female	15.27
4	AF 05	Male	17.21
5	AF 06	Female	14.99
6	AF 07	Female	15.71
7	AF 08	Male	16.37
8	AF 09	Female	14.70
9	AF 10	Female	15.81
10	AF 11	Female	14.98
11	AF 12	Female	17.03
12	AF 14	Female	15.91
13	AF 16	Female	15.43
14	AF 17	Female	16.43
15	AF 18	Female	15.56
16	AF 19	Female	13.15
17	AF 20	Female	15.89
18	AF 21	Female	14.56
19	AF 22	Female	15.32
20	AF 23	Female	13.78
21	AF 24	Female	14.66
22	AF 26	Male	14.73
23	AF 27	Male	16.98
24	AF 28	Male	14.25
25	AF 29	Male	15.60
26	AF 31	Female	15.73
27	AF 32	Male	16.66
28	AF 33	Female	16.04
29	AF 34	Female	14.36
30	AF 35	Female	15.39
31	AF 39	Female	15.33
32	AF 40	Female	16.83
33	AF 43	Female	16.29
34	AF 47	Female	14.27
35	AF 48	Female	13.29
36	AF 49	Female	15.20
37	AF 50	Male	17.60

4.3 Power and Sample Size Calculation

For power analysis and sample size of the MSCs component of the study, normality was assumed, based on the Gaussian distribution of the CFU-F assay data, tested with D'Agostino and Pearson omnibus normality test. Null hypothesis mean (μ_0=1.5), type I error rate, (α=5%), and power (1-β=0.80), have been setup accordingly; also true mean value (μ) and standard deviation, (σ) were determined based on the anatomic site and the cells-isolation method used. Consequently, sample size (n=19) satisfied the statistic criteria. Moreover, sample size and study power, were considered to represent an appropriate balance between a statistically significant difference and a scientific/clinical meaningful difference.

4.4 Total cells/ml from IC BM and VB BM: Comparison of AC and LMP

it was evident, since the first stages of BM processing (from both anatomic sites) using AC and LMP, the remarkable difference in terms of cell pellet volume, when AC was used (Figure 12). The different outcome between both techniques, it was expected for the ICBM[54-57,216,217], however to best of our knowledge, it was never tested before on VBBM.

AC following red cells lysis, concentrates the leukocyte fraction (WBCs) of the BM, resulting in a bigger cell pellet, compared to LMP which concentrates only the MNC fraction, having density 1.077 ± 0.001 g/ml, without the voluminous part of the granulocytes.

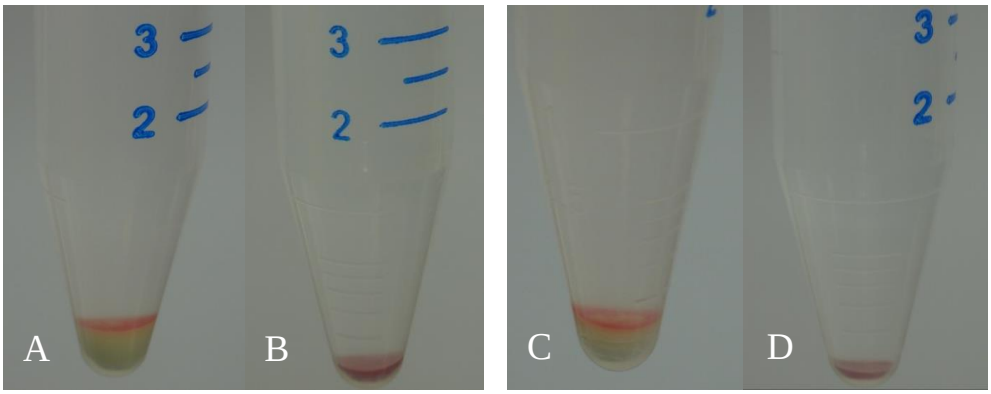


Figure 12: Falcon tubes containing cell pellets. (A,B) Harvested from ICBM and (C,D) from VBBM of the same donor (AF 48), using AC and LMP, respectively.

These differences were also confirmed quantitatively by counting total cells per ml of BM aspirate. From ICBM, using AC, a significantly higher number of cells was recovered (median $20x10^6$/ml total cells) compared to LMP ($7.23x10^6$/ml total cells). This 2.8-fold difference was statistically significant, p=0.0080, (Table 11, Figure 13). Similarly, from VBBM, using AC, a significantly higher number of cells was recovered (median $15x10^6$/ml total cells) compared to LMP ($5x10^6$/ml total cells). In this case also the 3-fold difference was statistically significant, p=0.0002, (Table 11, Figure 13). Despite the higher numbers of total cells yielded using AC, compared to LMP, no statistical significance was observed between VB and IC.

Table 11: Total cells/ml isolated from ICBM and VBBM using AC and LMP.

Patient Code	# Cells / ml of IC AC (10^6)	# Cells / ml of IC LMP (10^6)	# Cells / ml of VB AC (10^6)	# Cells / ml of VB LMP (10^6)
AF 28	12.36	2.43	9.25	2.67
AF 29	12.88	8.05	17.6	8.9
AF 31	32.89	24.47	8.33	3.83
AF 32	23.1	7.83	18.67	12.25
AF 35	18.2	5.89	17.9	10.2
AF 40	18.18	4.91	7.56	2.67
AF 43	27.08	8.27	20.89	12.22
AF 24	16	4	10.4	4.4
AF 23	25.26	16.8	33.87	20.82
AF 19	(n/a)	3.7	(n/a)	5
AF 20	18	3	20	3.1
AF 26	20	10	15.6	5
AF 27	15.3	7	15	3.4
AF 33	23.9	3.67	14	2.75
AF 34	7.63	7.46	7,5	4.2
AF 39	22.91	7.55	16	7.45
AF 49	24.50	12.19	12.84	4.84
AF48	30.11	5.26	14.74	5.26
AF50	28	44	38.38	15.79
MEAN	**20.79**	**9.94**	**16.62**	**7.09**
SD	**6.87**	**10.09**	**8.52**	**5.06**
MEDIAN	**20**	**7.23**	**15**	**5**

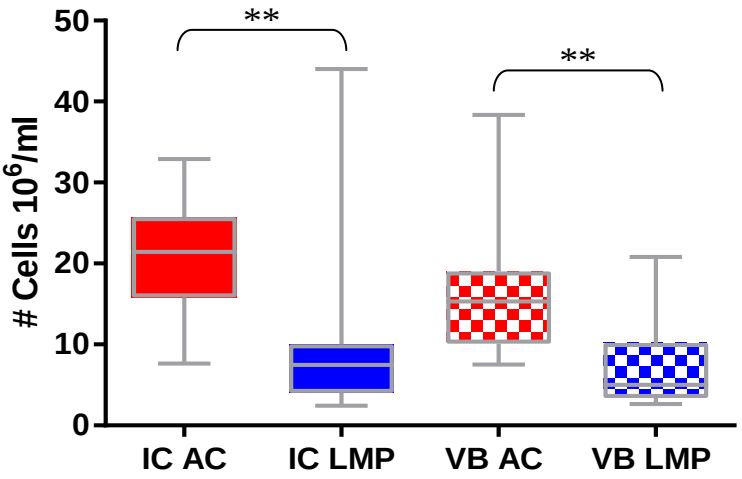


Figure 13: Comparison of total number of cells, recovered from 1ml of BM, using AC and LMP. IC AC (n=18, p=0.0080) and VB AC (n=18, p=0.0002), showed significantly higher concentrations, compared to IC LMP and VB LMP. Box & whiskers with min to max range and Wilcoxon matched-pairs test were used.

4.5 Cells recovery following thawing

CFU-F, proliferation, differentiation and phenotypic assays were performed using fresh-frozen total cells and expanded MSCs. Therefore cells recovery following thawing for both locations ICBM and VBBM, were compared for both isolation techniques used (AC and LMP). During cryopreservation, approximately $20x10^6$ cells were placed in each cryovial; this was considered as a reference value (100%) and the number of recovered cells, following thawing, was calculated as percentage. Comparisons based on 19 patients' samples, were made in order to assess whether the isolation technique used, can influence the thawing outcome (Figure 14, Table 12).

These findings show a significative loss of cells as a result of the cryoinjury, as expected[218]. Moreover, no statistically significant differences were found between anatomic sites (ICBM and VBBM) or cell-isolation methods (AC and LMP), suggesting that any numeric differences of MSCs observed, following thawing, are unlikely to be related to the cell-isolation method used.

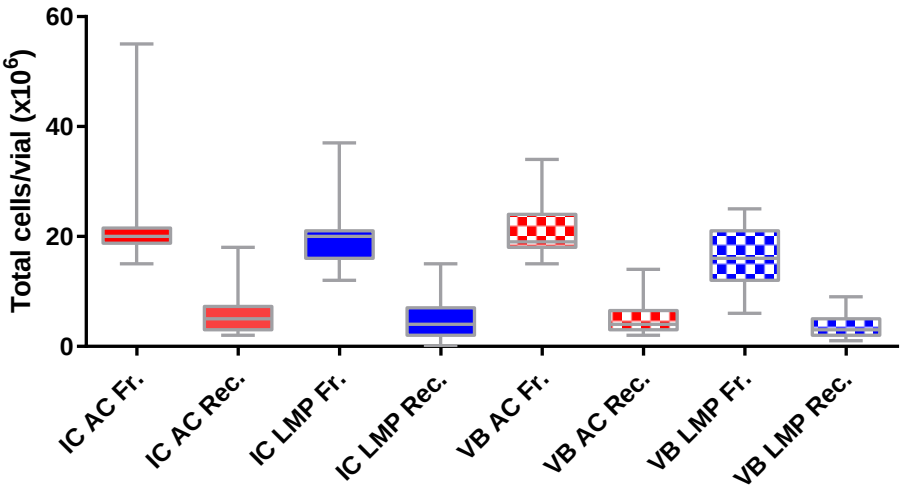


Figure14: Cells recovery, following cryopreservation. ICBM, presents a 23% and 17.5% of cells recovery, from AC and LMP, respectively, whilst VBBM, presents a 17.14% and 19.33% of cells recovery, from AC and LMP, respectively. Box & whiskers with min to max range and Wilcoxon matched-pairs test were used.

Table 12: Total cells/vial cryopreserved and recovered, from ICBM and VBBM using AC and LMP.

Patient Code	IC AC Frozen cells/vial (10^6)	IC AC Recovered cells/vial (10^6)	% of Recovery	IC LMP Frozen cells/vial (10^6)	IC LMP Recovered cells/vial (10^6)	% of Recovery	VB AC Frozen cells/vial (10^6)	VB AC Recovered cells/vial (10^6)	% of Recovery	VB LMP Frozen cells/vial (10^6)	VB LMP Recovered cells/vial (10^6)	% of Recovery
AF 28	22.6	7	30.88	17	5.6	32.94	18.5	9	48.65	16	5.5	34.38
AF 29	20.6	15	72.82	21.5	6.5	30.28	22	8.2	37.27	22.25	4.3	19.33
AF 31	21.14	5.32	25.16	21	6.7	31.76	16.7	4	24.00	12.5	1	8.00
AF 32	18.48	3.8	20.56	15.6	2.4	15.32	18.7	3.2	17.14	18.38	1.8	9.80
AF 35	18.2	6	32.97	20.6	9.4	45.63	17.9	12	67.04	20.4	4	19.61
AF 40	20	17.6	88.00	13	8.9	68.46	17	14	82.35	6	5	83.33
AF 43	20.1	2	9.94	14.33	2.4	16.74	18.8	2.4	12.77	18.33	1.8	9.82
AF 24	16	4.4	27.63	20	3.5	17.50	20.8	4.18	20.10	16.5	5.52	33.45
AF 23	20	2.7	13.50	20	2.5	12.50	21.8	3.55	16.31	23.35	3.2	13.70
AF 19	(n/a)	(n/a)	(n/a)	37	14.6	39.46	(n/a)	(n/a)	(n/a)	25	9	36.00
AF 20	23.63	2.8	12.02	15.8	8.5	53.97	32	5.3	16.56	12.4	6.4	51.61
AF 26	46.66	10.3	21.99	35	2.8	7.86	33	5	16.27	16.25	4.2	25.85
AF 27	54.83	6.2	11.22	22.8	3.4	15.12	32	3.4	10.46	11.05	2	18.10
AF 33	19.12	3.2	16.47	12	7	0.58	17.5	3	17.14	8.25	1.5	18.18
AF 34	15.25	5.6	36.72	18.7	3.8	20.38	15	2.8	18.67	12.6	3.2	25.40
AF 39	21	5.2	24.76	20.8	5	24.10	17.6	3.6	20.45	20.5	4.4	21.46
AF 49	21.43	7.5	35.00	21.33	2.4	11.25	20.3	3.2	15.76	11.5	1.5	13.04
AF48	20.43	2.57	12.58	12.5	2.1	16.80	17.5	2.42	13.83	12.5	2.1	16.80
AF50	20	2.8	14.00	36.6	1.39	3.80	30.36	2	6.59	25	0.75	3.00
Mean	**23.31**	**6.11**	**28.12**	**20.81**	**4.84**	**24.44**	**21.59**	**5.1**	**25.63**	**16.25**	**3.54**	**24.26**
SD	**10.28**	**4.287**	**21.03**	**7.60**	**3.55**	**17.61**	**6.1**	**3.45**	**20.44**	**5.57**	**2.15**	**18.44**
Median	**20.27**	**5.26**	**23.37**	**20**	**3.5**	**17.50**	**18.73**	**3.58**	**17.14**	**16.25**	**3.2**	**19.33**
CV %	**44**	**70**		**37**	**73**		**28**	**68**		**34**	**61**	

4.6 Colony forming unit fibroblast (CFU-F) assays

As mentioned previously, one of the characteristics of MSCs is the ability to adhere on plastic surface and proliferate in standard culture conditions optimized for MSCs. These features have been exploited in the CFU-F assay. In this study, BM cells were separated by differential gradient centrifugation (LMP) and also red cells lysis techniques (AC), followed by centrifugation, isolating MNCs and WBCs, respectively. Each colony has been shown to originate from a single colony-forming cell or CFU-F[219] and traditionally, MSCs are enumerated as CFU-Fs based on this assay[219,220]. MSCs' colonies from ICBM and VBBM were found in all samples tested (Figure 15) and manually counted (Figure 16).

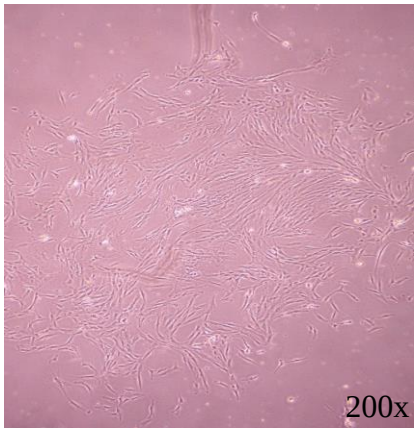


Figure 15: Microscopic images showing the characteristic aspect of a BM-MSCs colony, after 10 days in culture. Magnification x200.

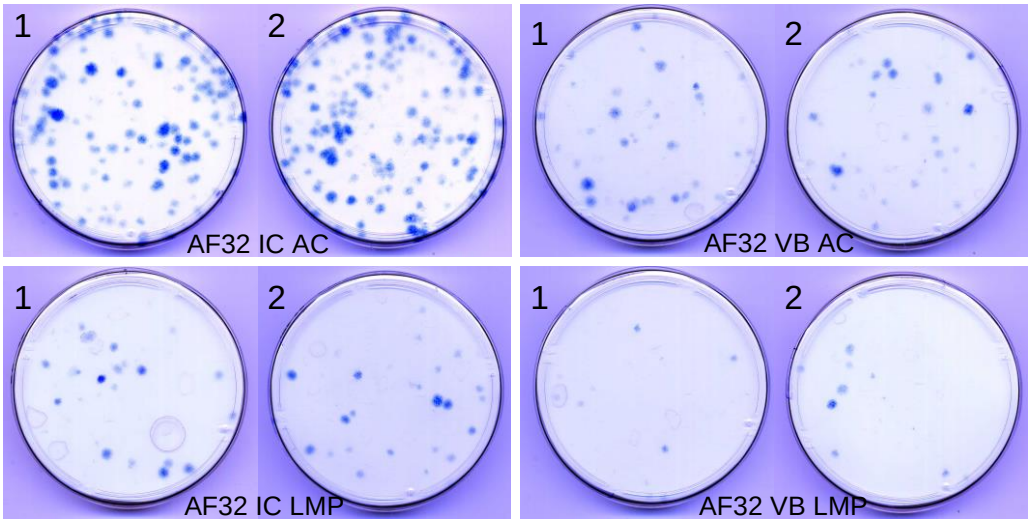


Figure 16: Example of CFU-F assay. MSCs colonies stained with methylene blue in 10cm petri-dishes. All experiments were performed, in duplicates.

4.6.1 MSCs/10^6 from MNCs / WBCs: Comparison of ICBM and VBBM

A comparison of CFU-F colonies from ICBM and VBBM samples was performed, based on the isolation and plating of $2x10^6$ mononuclear cells (MNCs) and leucocytes (WBCs), respectively, normalising subsequently the data to a million of cells seeded, (Tables 13, 14). Despite the young age of the subjects and the consistency of techniques and conditions used for all patients, some of them presented relatively low numbers of MSCs.

From both anatomic regions, LMP-harvested cells gave more colonies per million of seeded cells, with 1.3-fold and 1.2-fold, respectively, nearly missing statistical significance (Tables 4, 5, Figure 17).

Table 13. MSCs /10^6 of cells in ICBM.

IC LMP (n=19)				**IC AC (n=18)**		
Patients Code	CFU-F/ $2x10^6$ 1st petri-dish	CFU-F/$2x10^6$ 2nd petri-dish	CFU-F/10^6 of MNCs	CFU-F /$2x10^6$ 1st petri-dish	CFU-F/$2x10^6$ 2nd petri-dish	CFU-F/10^6 of WBCs
AF28	131	229	90	62	57	29.75
AF29	14	6	5	31	33	16
AF31	7	2	2.25	7	6	3.25
AF32	29	26	22.92	133	138	71.32
AF35	273	265	134.50	158	202	90
AF40	6	6	3	4	4	2
AF43	18	13	12.92	12	15	13.50
AF34	98	108	54.21	11	9	5
AF48	65	74	66.19	53	46	38.52
AF24	325	400	207.14	195	170	91.25
AF23	6	7	5.20	12	13	9.26
AF33	1	1	2.86	4	10	4.44
AF27	29	28	16.57	89	72	40.25
AF26	57	54	40.36	223	201	106
AF39	193	206	99.75	200	228	107
AF19	232	230	115.50	(n/a)	(n/a)	(n/a)
AF20	186	181	91.75	82	81	57.39
AF49	97	104	83.75	115	104	54.75
AF50	16	28	31.43	20	11	11.7
Mean			**55.29**			**41.71**
SD			**56.18**			**37.47**
Median			**40.36**			**34.14**

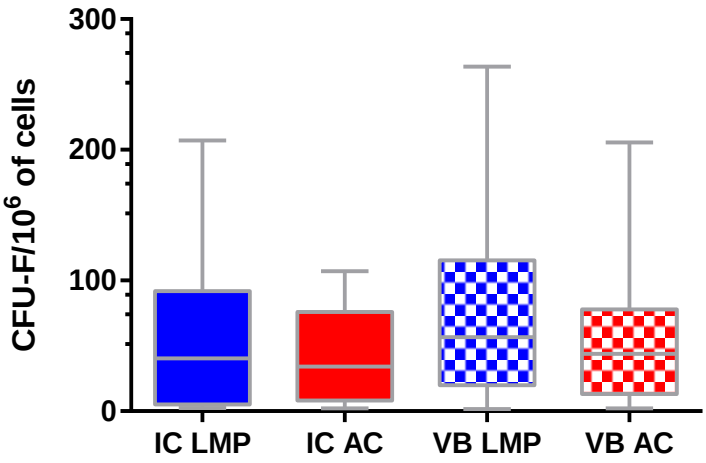


Figure 17: CFU-F/10^6 of cells harvested from IC and VB using both AC and LMP. VBBM presented a higher number of colonies per million of cells when LMP was used, compare d to VB AC, however without statistical significance. Box & whiskers with min to max range and Wilcoxon matched-pairs test was used.

Table 14. MSCs/10^6 of cells in VBBM.

	VB LMP (n=19)			**VB AC (n=18)**		
Patients Code	CFU-F/ 2x10^6 1st petri-dish	CFU-F/2x10^6 2nd petri-dish	CFU-F/10^6 of MNCs	CFU-F/ 2x10^6 1st petri-dish	CFU-F/2x10^6 2nd petri-dish	CFU-F/10^6 of WBCs
AF28	134	117	62.75	20	23	10.75
AF29	92	94	46.50	33	31	16
AF31	19	20	19.50	31	25	14
AF32	11	14	13.89	45	45	28.13
AF35	102	175	69.25	53	60	28.25
AF40	4	2	1.50	3	5	2
AF43	4	2	3.33	3	6	3.75
AF34	81	86	52.19	79	61	50
AF48	100	68	80	44	47	37.60
AF24	550	504	263.50	363	459	205.50
AF23	83	59	44.38	117	97	60.28
AF33	107	66	115.33	170	173	114.33
AF27	257	259	258	130	114	71.76
AF26	46	33	19.75	193	191	96
AF39	246	262	127	201	195	110
AF19	289	280	142.25	(n/a)	(n/a)	(n/a)
AF20	121	131	63	104	123	56.75
AF49	39	46	56.67	9	11	6.25
AF50	8	19	27	60	77	68.5
Mean			**77.15**			**54.44**
SD			**75.81**			**52.08**
Median			**56.67**			**43.80**

4.6.2 MSCs/ml from ICBM and VBBM: Comparison of AC and LMP

The total number of MSCs (CFU-F) per ml of BM harvested from both IC and VB, using LMP and AC, was calculated by the following equation: CFU-F/ml of BM = CFU-F/10^6 x Cells/ml (Tables 15, 16). Both IC and VB showed a significatively higher number of MSCs when AC was utilized (IC AC p=0.0009, VB AC p=0.0038), by 2.6-fold and 1.9-fold, respectively (Figure 18). These results suggested a loss of MSCs following LMP processing. This finding was consistent with previous studies on IC BM, showing loss of LMP-harvested MSCs (compared to AC-harvested MSCs), in the residual cell pellet mixed with erythrocytes and granulocytes[54,55,57].

Concerning MSCs' gender variability, given the demographic characteristics of scoliosis, this cohort presents an uneven female/male distribution (15F/4M) which did not allow a robust statistical comparison. However, the only possible trend noticed within the male cohort, was a higher concentration of MSCs, for both VB AC (2.6-fold) and IC AC (4.4-fold), compared to females. Nevertheless, a bigger evenly distributed cohort is needed for a definitive answer.

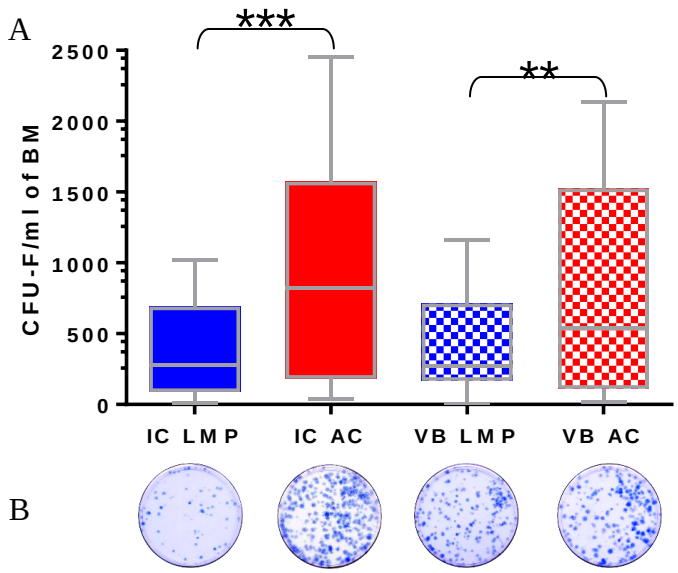


Figure 18: CFU-F/ml of BM harvested from IC and VB using both AC and LMP. A) AC-harvested MSCs presented a statistically higher number of colonies from both ICBM (p=0.0009) and VBBM (p=0.0038). Column bar-graph with range and mean values was used. Gaussian distribution was assumed. Paired t-test was performed. B) Representative example of CFU-F assays showing MSCs colonies per anatomic site and cells-isolation method used.

Table 15. MSCs/ml from ICBM, comparing LMP and AC.

IC LMP (n=19)				IC AC (n=18)		
Patients Code	CFU-F/10^6 of MNC	MNC of BM (10^6/ml)	MSCs/ml of BM	CFU-F/10^6 of WBC	WBC of BM (10^6/ml)	MSCs/ml of BM
AF28	90	2.43	218.70	29.75	12.36	367.71
AF29	5	8.05	40.25	16	12.88	206.08
AF31	2.25	24.47	55.06	3.25	32.89	106.89
AF32	22.92	7.83	179.44	71.32	23.1	1647.39
AF35	134.50	5.89	792.21	90	18.2	1638
AF40	3	4.90	14.70	2	18.81	37.62
AF43	12.92	8.27	106.82	13.50	27.08	365.58
AF34	54.21	7.46	404.41	5	7.63	38.15
AF48	66.19	5.26	348.16	38.52	30.11	1159.88
AF24	207.14	4	828.57	91.25	16	1460
AF23	5.20	16.84	87.57	9.26	25.26	233.89
AF33	2.86	3.69	10.54	4.44	23.9	106.22
AF27	16.57	7	115.99	40.25	15.3	615.83
AF26	40.36	10	403.64	106	20	2120
AF39	99.75	7.55	753.11	107	22.91	2451.37
AF19	115.50	3.70	427.35	(n/a)	(n/a)	(n/a)
AF20	91.75	3	275.25	57.39	18	1033.10
AF49	83.75	12.20	1021.75	54.75	24.5	1341.38
AF50	31.43	22	691.43	11.07	140	1550
Mean			**356.58**			**915.50**
SD			**317.21**			**779.55**
Median			**275.25**			**824.46**

Table 16. MSCs/ml from VBBM, comparing LMP and AC.

VB LMP (n=19)				VB AC (n=18)		
Patients Code	CFU-F/10^6 of MNC	MNC of BM (10^6/ml)	MSCs/ml of BM	CFU-F /10^6 of WBC	WBC of BM (10^6/ml)	MSCs/ml of BM
AF28	62.75	2.67	167.54	10.75	9.25	99.44
AF29	46.50	8.9	413.85	16	17.6	281.60
AF31	19.50	3.83	74.69	14	8.33	116.62
AF32	13.89	12.25	170.14	28.13	18.67	525.09
AF35	69.25	10.2	706.35	28.25	17.9	505.68
AF40	1.50	2.67	4.01	2	7.56	15.12
AF43	3.33	12.22	40.73	3.75	20.89	78.34
AF34	52.19	4.2	219.19	50	7.5	375
AF48	80.00	5.26	420.80	37.60	14.74	554.27
AF24	263.50	4.4	1159.40	205.50	10.4	2137.20
AF23	44.38	20.82	923.89	60.28	33.87	2041.74
AF33	115.33	2.75	317.17	114.33	14	1600.67
AF27	258.00	3.4	877.20	71.76	15	1076.47
AF26	19.75	5	98.75	96	15.6	1497.60
AF39	127.00	7.45	946.15	110	16	1760
AF19	142.25	5	711.25	(n/a)	(n/a)	(n/a)
AF20	63	3.1	195.30	56.75	20	1135
AF49	56.67	4.84	274.27	6.25	12.84	80.25
AF50	27	7.5	202.50	68.50	18.2	1246.70
Mean			**417.01**			**840.38**
SD			**356.57**			**728.53**
Median			**274.27**			**539.68**

4.6.3 CFU-F: Colony size of ICBM and VBBM, using AC and LMP

In line with other authors[171,219,221], the CFU-F colony sizes varies between different donors or different anatomic regions of the same donor, but could also vary within the same culture (petri-dish). Hence, larger colony sizes, may represent a higher proliferative potential or alternatively a bigger size of MSCs in culture. In order to study the colonies' morphology further, dishes were scanned using an Epson 2580 digital scanner and digital images were analyzed using NIS elements BR 2.20 imaging software (Nikon, Tokyo, Japan). The dish area ($A=70.88cm^2$) was determined by using the formula $A=\pi \times r^2$ ($\pi=3.14159$, r(radius)=4.75). Furthermore, total colony area per dish was calculated, allowing next to determine the average

surface area per single colony, using the formula: Single colony area (cm^2)=Total area of colonies (cm^2)/number of colonies (Table 17).

VB AC-harvested MSC presented a significantly smaller colony area, compared to IC AC, (p=0.0039). Moreover, similarly to other authors[171], it was noticed a trend from VBBM to produce smaller colonies with a faster doubling rate, even though it was not statistically significant (Figures 19, 20).

Table 17. MSCs' total colonies area.

Patients Code	IC AC area (%)	IC AC area (cm^2)	IC LMP area (%)	IC LMP area (cm^2)	VB AC area (%)	VB AC area (cm^2)	VB LMP area (%)	VB LMP area (cm^2)
AF 28	10.31	7.31	31.62	22.41	7.59	5.38	23.61	16.73
AF 29	8.19	5.80	9.56	6.78	4.22	2.99	24.47	17.34
AF 31	3.38	2.39	3.15	2.23	3.24	2.29	5.09	3.60
AF 32	23.06	16.34	10.78	7.64	9.97	7.06	6.59	4.67
AF 35	15.30	10.84	30.61	21.69	8.13	5.76	26.10	18.50
AF 40	3.55	2.52	4.28	3.03	4.52	3.20	3.51	2.49
AF 43	3.98	2.82	4.17	2.95	3.57	2.53	1.35	0.96
AF 34	5.06	3.58	21.98	15.58	14.76	10.46	11.00	7.80
AF 48	10	7.08	12.52	8.87	9.14	6.48	13.49	9.56
AF 24	14.44	10.24	58.75	41.64	27.09	19.20	61.10	43.30
AF 23	5.15	3.65	4.75	3.36	15.12	10.72	15.13	10.72
AF 33	5.27	3.73	1.70	1.20	11.22	7.95	11.48	8.14
AF 27	8.56	6.06	4.84	3.43	19.52	13.84	64.61	45.79
AF 26	54.74	38.80	10.14	7.19	16.86	11.95	4.35	3.08
AF 39	24.06	17.05	45.10	31.97	18.50	13.11	37.26	26.41
AF 19	(n/a)	(n/a)	30.93	21.92	(n/a)	(n/a)	11.48	8.14
AF 20	12.30	8.71	17.07	12.10	19.00	13.47	17.24	12.22
AF 49	13.46	9.54	10.49	7.44	6.39	4.53	7.10	5.03
AF50	7.34	5.20	6.90	4.89	11.90	8.43	5.80	4.11
Mean	**12.67**	**8.98**	**16.81**	**11.91**	**11.71**	**8.30**	**18.46**	**13.08**
SD	**12.15**	**8.61**	**15.82**	**11.21**	**6.68**	**4.73**	**18.16**	**12.87**
Median	**9.28**	**6.57**	**10.49**	**7.44**	**10.59**	**7.51**	**11.48**	**8.14**

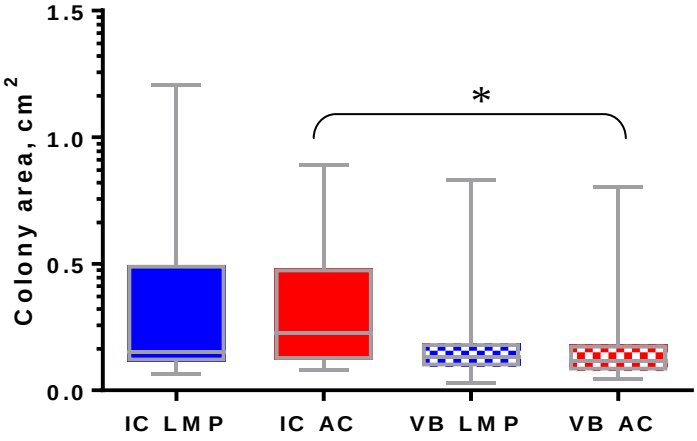


Figure 19: MSCs colony area, following CFU-F assay. IC AC presented a significatively bigger colony area, compared with VB AC (p=0.039). Additionally, a trend was noticed for VBBM-harvested MSCs to generate smaller colonies, with median values of ICBM: LMP 0.15 cm^2, AC 0.23 cm^2, VBBM: LMP 0.13 cm^2, AC 0.12 cm^2. Box & whiskers with min to max range and Wilcoxon matched-pairs test were used.

4.6.4 MSCs' population doubling for ICBM and VBBM, using AC and LMP

MSCs were passaged at approximately 80% of confluence and then counted, as described previously, on section 3.4.1. Population doubling (PD) rates were calculated from eight donors, using the formula $PD=Log_2(N2/N1)$ (N2 # of cells at P1, N1 # of cells seeded), and the results were divided by the number of days (D) in culture. No statistically significant differences were observed within the 4 groups (Figure 20).

During the proliferation assay, a trend was noticed for VBBM-MSCs to grow slightly faster compared to ICBM, as also described by other authors[62,171], however this trend did not reach a statistical significance, at least with the number of donors examined. Moreover, LMP-harvested MSCs, tended to grow slightly faster, compared to AC, however no statistical significance was observed, (median values of ICBM: AC 1.94 D, LMP 1.89 D, VBBM: AC 1.89 D, LMP 1.82 D).

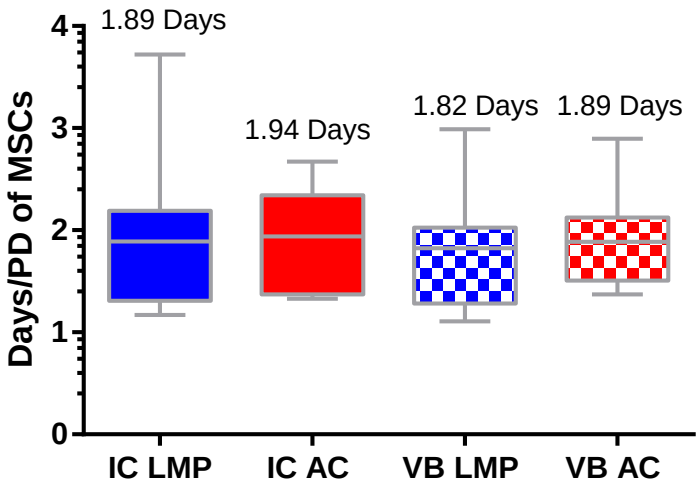


Figure 20: MSCs DP rates, following tissue culturing. A trend was observed for VBBM-MSCs and LMP-harvested MSCs to proliferate faster, from both sites, however without a statistical significance. Box & whiskers with min to max range and Wilcoxon matched-pairs test were used.

4.7 Flow cytometry

4.7.1 Phenotypic characterisation of MSCs

Flow cytometry was performed with scope to confirm MSCs nature, based on the ISCT criteria[69,222], in addition to CFU-F and differentiation assays. Furthermore, the aim of the flow cytometry was to investigate whether ICBM-MSCs differ from VBBM-MSCs, with regards to standard (CD105, CD73, CD90) and novel (SUSD2, MSCA1 ALP) MSCs' markers, considering also that different isolation methods were employed. Moreover, although CFU-F assay, represent the most common method for MSCs analysis, is a plastic adherence–based cultivation method, which may lead to the loss of some poorly adherent, but highly potent MSCs[223]. Contrarily flow cytometry is based on superficial and intracellular markers detection, hence is not affected by tissue culturing techniques allowing the same cells to be studied, from a different perspective.

For MSCs phenotypic characterization, were analysed the same donors, used also for differentiation assays, (AF28, AF29, AF39, AF50). All MSCs were culture-expanded at P3 and their phenotype was investigated as explained in paragraph 3.6. Cultures were expanded and cryopreserved as described in section 3.4. In order to avoid variation of the fluorescent intensity, which is sensitive to experimental conditions, (e.g. laser fluctuations) all cultures (4 conditions/donor, 16 cultures in total) were thawed simultaneously[189,190]. Moreover, to exclude dead cells, debris and aggregates, 7-AAD dye[193] was used. Gating strategy is illustrated below, in Figure 21.

The flow cytometric analysis is illustrated using histograms with the marker recorded on the x-axis, and the total count on the y-axis (appendix 8.4). The latter was gated between 10^2-10^5 of positive staining cells. Grouped-bars graphics, present an overview of median florescent intensity (MFI), positive events detected and percentage of positively stained cells. Results, showed positive expression of the common MSCs' markers (CD105, CD73, CD90) and negligible levels of hematopoietic cell markers (CD34, CD45, CD14, CD19 and HLA-DR) for all donors examined (Figures 22-25).

CD271 represent an ideal marker for the identification of fresh-harvested MSCs, as showed in previous publications[191,193,223,224].However, in this study culture-expanded MSCs were used, which tend to adapt physiologically their function, based on the culturing conditions *in vitro*, decreasing the concentration of the CD271 marker[183,193,223,225]. Hence, in this study, the novel MSCs' surface markers SUSD2[181-183], MSCA1[182-186] and also intracellular marker ALP[188,226] were used, in order to investigate possible differences between IC- and VB-harvested MSCs, isolated utilising two different techniques (AC, LMP)[54,57].

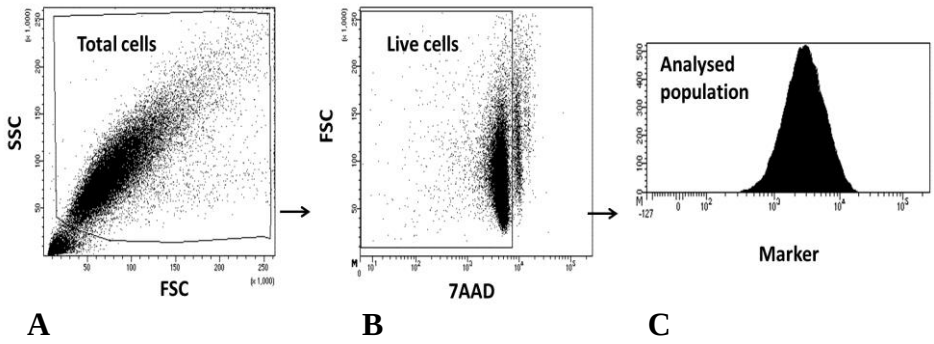


Figure 21: Flow cytometric analysis dot plot, gating strategy. A) Cell debris were excluded based on their forward and side scatter, B) 7-AAD dye was also used to exclude dead cells, C) Filtered population of MSCs was then examined for surface and intracellular markers. Each dot represents a cell, (an 'event') recorded from the laser detectors.

4.7.2 Individual analysis of each donor

First donor, AF 28, (14.3y, M).

The intracellular marker ALP presented a higher MFI with LMP-harvested MSC, from both IC and VB by 1.4-fold and 2.1-fold, respectively. With regards to the anatomic location, ALP presented a higher MFI from VB for both AC and LMP-harvested MSCs, by 1.2-fold and 1.8-fold difference, respectively (Figure 22, Table 18).

Conversely to ALP, the extracellular marker MSCA1, presented a higher MFI with AC-harvested MSCs, from both IC and VB by 3.5-fold and 1.8-fold, respectively. Concerning the anatomic site, MSCA1 presented an overall better outcome from VB, for both AC- and LMP-harvested MSCs, by 1.2-fold and 2.3-fold, respectively (Figure 22, Table 18).

Marker SUSD2, showed a higher MFI for AC-harvested MSCs from IC by 1.5-fold, whereas LMP-harvested MSCs from VB by 1.2-fold difference. With regards to the anatomic site, the overall expression appeared to be similar for both IC and VB, however IC presented a higher MFI from AC-harvested MSCs, by 1.5-fold difference (Figure 22, Table 18).

The CD90 presented the highest MFI, compared to CD105 and CD73 standard MSC characterization markers. For both IC and VB, AC-harvested MSCs showed a higher MFI, in both cases by 2.1-fold. In terms of the anatomic site, the overall expression appeared to be similar for both IC and VB (Figure 22, Table 18).

Marker CD105, presented a higher MFI from VB LMP-harvested MSCs, by 1.5-fold, whilst IC gave similar results for both isolation methods. In terms of the anatomic site, VB presented a higher outcome for both AC and LMP-harvested MSCs, by 1.8-fold and 1.2-fold, respectively (Figure 22, Table 18).

CD73 presented a similar to CD90 trend. For both IC and VB, AC-harvested MSCs showed a slightly higher MFI, by 1.2-fold and 1.1-fold, respectively. In terms of the anatomic site, VB presented a slightly better outcome by 1.2-fold, from both LMP and AC-harvested MSCs (Figure 22, Table 18).

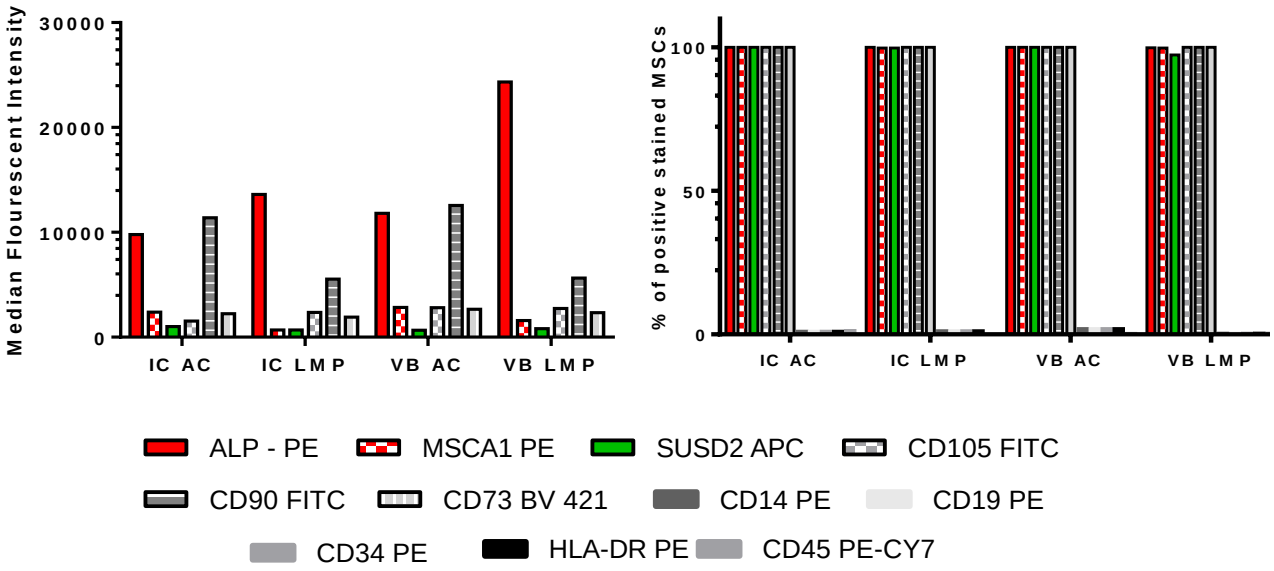


Figure 22: Graphics of median fluorescent intensity, and percentage of positively stained MSCs for donor AF 28. MFI results illustrated, are limited only to the positive markers characterising MSCs, due to the negligible values of the haematopoietic markers (CD14, CD19, CD34, HLA-DR and CD45). The percentage of positive MSCs was determined based on the negative controls, applying the isotype control concept for all the fluorochromes used. Data are illustrated as grouped bars, median values are calculated. The legend for both graphics is showed at bottom figure.

Table 18. MFI values of donor AF 28.

AF 28	ALP	MSCA1	SUSD2	CD105	CD90	CD73
IC AC	9790	2392	1020	1544	11396	2231
IC LMP	13623	693	689	2350	5543	1918
VB AC	11807	2839	667	2824	12558	2667
VB LMP	24337	1597	822	2743	5638	2333

Second donor, AF 29, (15.6y, M).

The intracellular marker ALP, presented a higher MFI from LMP-harvested MSCs, from both IC and VB by 6- and 3.5-fold difference, respectively. With regards to the anatomic location, ALP presented a higher MFI from IC, for both AC and LMP-harvested MSCs, by 4.4-fold and 7.5-fold difference, respectively (Figure 23,Table 19).

Extracellular marker for ALP, MSCA1, concerning the isolation method used, presented a higher MFI from IC LMP-harvested MSCs by 1.5-fold and from VB AC-harvested MSCs by 3-fold difference. Concerning the anatomic site, MSCA1 presented an overall better outcome from IC, by 1.6-fold, using AC-harvested MSCs and 7.3-fold, using LMP-harvested MSCs (Figure 23,Table 19).

Marker SUSD2, showed a higher MFI for LMP-harvested MSCs from VBBM by 1.4-fold, whereas ICBM presented a similar outcome from both MSCs-isolation methods. Concerning the anatomic site, the overall expression was similar for both ICBM and VBBM (Figure 23,Table 19).

Also in this case, CD90 presented the highest MFI, compared to CD105, and CD73 standard MSC characterization markers. Both IC and VB, presented similar results from both AC and LMP-harvested MSCs. In terms of the anatomic site, IC showed the highest outcome, by 1.5-fold and 1.6-fold difference, from AC and LMP-harvested MSCs, respectively (Figure 23,Table 19).

CD105, presented a higher MFI for LMP-harvested MSCs, from IC by 1.5-fold, whereas VB presented a similar outcome for both MSCs-isolation methods. In terms of the anatomic site, VB presented the highest outcome, by 1.7-fold and 1.2-fold difference for both AC and LMP-harvested MSCs, respectively (Figure 23,Table 19).

CD73 presented a similar trend to CD90. LMP-harvested MSCs presented a higher MFI, by 1.2-fold from IC and 1.3-fold for AC-harvested MSCs from VB, respectively. In terms of the anatomic site, IC presented the highest outcome, by 1.3-fold and 2.2-fold, from both AC and LMP-harvested MSCs, respectively (Figure 23,Table 19).

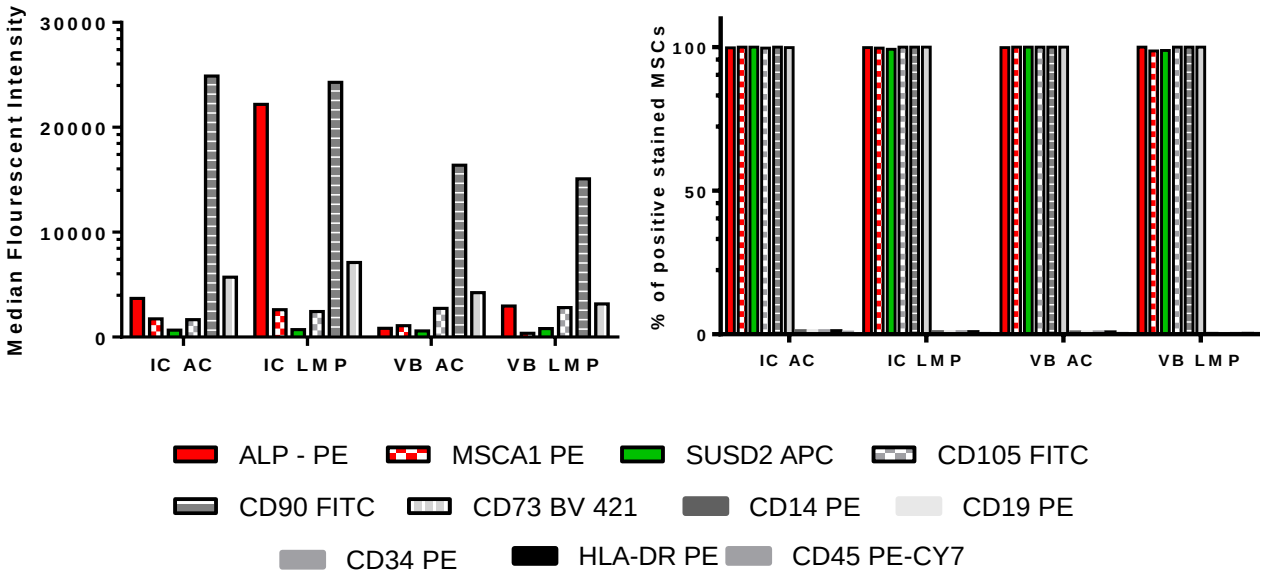


Figure 23: Graphics of median fluorescent intensity and percentage of positively stained MSCs for donor AF 29. MFI results illustrated, are limited only to the positive markers characterising MSCs, due to the negligible values of the haematopoietic markers (CD14, CD19, CD34, HLA-DR and CD45). The percentage of positive MSCs was determined based on the negative controls, applying the isotype control concept for all the fluorochromes used. Data are illustrated as grouped bars, median values are calculated. The legend for both graphics is showed at bottom figure.

Table 19. MFI values of donor AF 29.

AF 29	ALP	MSCA1	SUSD2	CD105	CD90	CD73
IC AC	3688	1743	656	1650	24878	5715
IC LMP	22194	2624	715	2445	24282	7103
VB AC	841	1090	591	2730	16380	4247
VB LMP	2964	360	814	2818	15094	3165

Third donor, AF 39, (15.3y, F).

The intracellular marker ALP, presented a higher MFI from IC LMP-harvested MSCs, by 1.3-fold and from VB AC-harvested MSCs by 1.7-fold, respectively. Regarding the anatomic site, ALP presented a higher MFI from IC, for both AC and LMP-harvested MSCs, by 1.4-fold and 3.2-fold difference, respectively (Figure 24,Table 20).

The extracellular marker for ALP, MSCA1, presented a higher MFI from AC-harvested MSCs, from both IC and VB by 1.3-fold. Regarding the anatomic site, MSCA1 showed a better outcome from IC, for both harvesting methods used by 1.6-fold (AC) and 1.7-fold (LMP) (Figure 24,Table 20).

Marker SUSD2, presented a higher MFI for LMP-harvested MSCs from ICBM by 1.3-fold, whereas VBBM presented a similar outcome from both MSCs-isolation methods. Regarding the anatomic site, the overall expression was similar for both ICBM and VBBM (Figure 24,Table 20).

CD90 presented, also in this case, the highest MFI, compared to the other standard MSCs characterization markers. AC-harvested MSCs, from both IC and VB presented a higher MFI, by 1.9- and 1.2-fold difference, respectively. Regarding the anatomic site, IC presented a higher outcome, by 1.6-fold from AC-harvested MSCs, whilst VB showed a better outcome, by 1.2-fold from LMP-harvested MSCs (Figure 24,Table 20).

CD105, presented a higher MFI for AC-harvested MSCs from IC by 1.7-fold, whereas VB presented a better outcome from LMP-harvested MSCs, by 1.6-fold. Concerning the anatomic site, VB showed the highest outcome from LMP-harvested MSCs, by 3-fold, while AC-harvested MSCs gave similar results from both IC and VB (Figure 24,Table 20).

CD73 presented, also in this case, a similar trend to CD90. AC-harvested MSCs presented a higher MFI, by 1.8-fold from IC and 1.2-fold for LMP-harvested MSCs from VB. In terms of the anatomic region, IC presented a similar outcome for both MSCs-harvesting methods, while VB presented a better outcome form LMP-harvested MSCs, by 1.9-fold difference (Figure 24,Table 20).

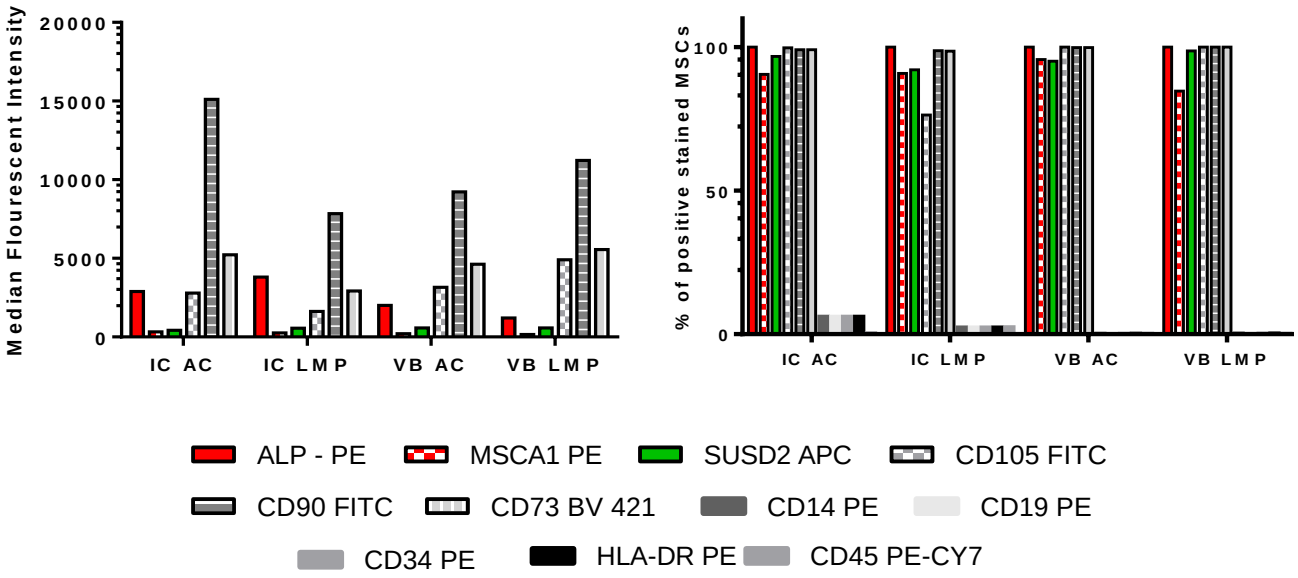


Figure 24: Graphics of median fluorescent intensity and percentage of positively stained MSCs for donor AF 39. MFI results illustrated, are limited only to the positive markers characterising MSCs, due to the negligible values of the haematopoietic markers (CD14, CD19, CD34, HLA-DR and CD45). The percentage of positive MSCs was determined based on the negative controls, applying the isotype control concept for all the fluorochromes used. Data are illustrated as grouped bars, median values are calculated. The legend for both graphics is showed at bottom figure.

Table 20. MFI values of donor AF 39.

AF 39	ALP	MSCA1	SUSD2	CD105	CD90	CD73
IC AC	2893	322	421	2790	15115	5231
IC LMP	3805	252	565	1625	7843	2918
VB AC	2012	204	582	3157	9225	4633
VB LMP	1201	152	570	4901	11231	5550

Forth donor, AF 50, (17y, M).

Intracellular marker ALP, presented a better MFI from both IC and VB LMP-harvested MSC, by 4.7-fold and 6.8-fold, respectively. With regards to the anatomic site, ALP presented the same MFI, from both IC and VB, when AC-harvested MSC was used, however, VB presented a higher MFI with LMP-harvested MSC, by 1.5-fold difference (Figure 25,Table 21).

The extracellular marker for ALP, MSCA1, presented a better MFI with AC-harvested MSC, from both IC and VB by 5.2-fold and 2.3-fold, respectively. Concerning the anatomic site, MSCA1 presented an overall better outcome from VB, by 1.9-fold, using AC-harvested MSCs and 4.3-fold, using LMP-harvested MSCs (Figure 25,Table 21).

Marker SUSD2, presented a higher MFI for AC-harvested MSCs from IC by 1.9-fold, whereas LMP-harvested MSCs from VB showed better results by 1.5-fold difference. From the anatomic point of view, the overall expression was similar from both ICBM and VBBM (Figure 25,Table 21).

Similarly to all the previous donors, CD90 presented the highest MFI, compared to CD105, and CD73 standard MSC characterization markers. Both IC and VB, AC-harvested MSCs presented a higher MFI, by 1.7-fold and 1.3-fold, respectively, compared to LMP. Concerning the anatomic site, VB presented the highest outcome for CD90, by 3.5- and 2.1-fold from LMP and AC-harvested MSCs, respectively (Figure 25,Table 21).

CD105, presented a higher MFI from LMP-harvested MSCs, for both IC and VB, by 1.7-fold and 1.6-fold, respectively. With regards to the anatomic site, IC presented the highest outcome, by 1.2-fold for both LMP and AC-harvested MSCs (Figure 25,Table 21).

In line with all the above donors, CD73 presented a similar to CD90 trend. Both IC and VB, AC-harvested MSCs showed a higher MFI, by 1.2-fold and 1.4-fold, respectively. Regarding the anatomic region, VBBM shown the higher outcome, by 1.9-fold and 1.6-fold, from both AC and LMP-harvested MSCs, respectively (Figure 25,Table 21).

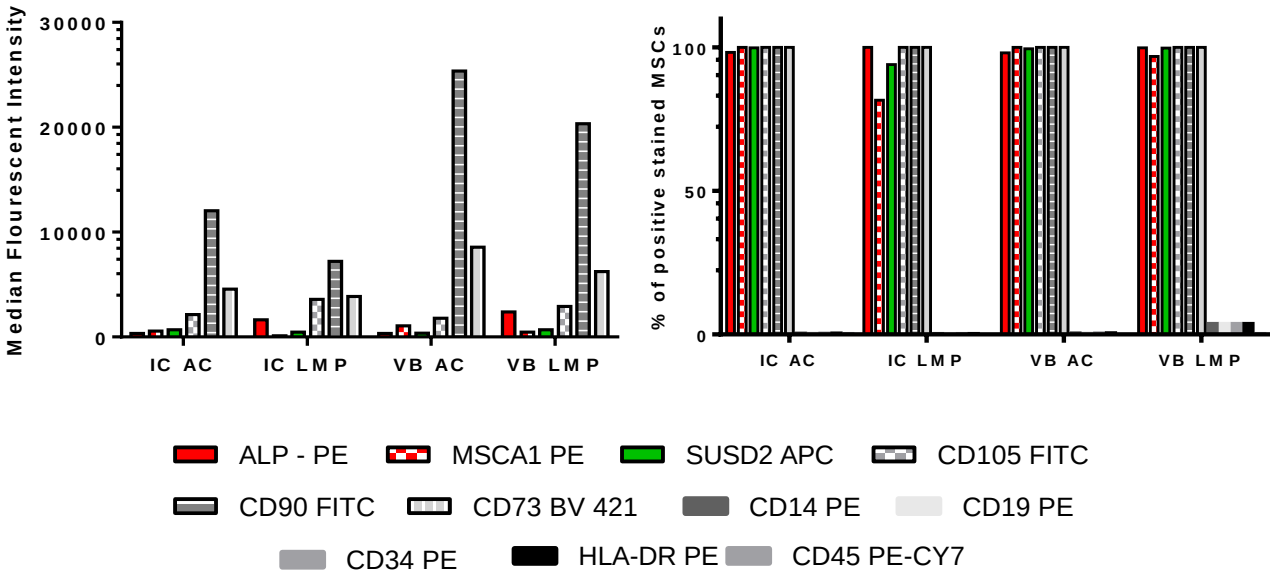


Figure 25: Graphics of median fluorescent intensity and percentage of positively stained MSCs for donor AF 50. MFI results illustrated, are limited only to the positive markers characterising MSCs, due to the negligible values of the haematopoietic markers (CD14, CD19, CD34, HLA-DR and CD45). The percentage of positive MSCs was determined based on the negative controls, applying the isotype control concept for all the fluorochromes used. Data are illustrated as grouped bars, median values are calculated. The legend for both graphics is showed at bottom figure.

Table 21. MFI values of donor AF 50.

AF 50	ALP	MSCA1	SUSD2	CD105	CD90	CD73
IC AC	348	569	689	2126	12041	4555
IC LMP	1634	110	450	3596	7204	3859
V12 AC	349	1064	369	1784	25354	8556
V12 LMP	2379	469	689	2900	20340	6243

4.7.3 Overall comparison (anatomic sites and isolation methods)

The ALP intracellular marker presented a higher MFI from LMP-harvested MSCs from all donors, compared to AC, for both anatomic sites (IC 2.6-fold, VB 1.9-fold). APL presented the highest MFI from IC LMP (8714), followed by IC AC (3290.5), VB LMP (2671.5), and VB AC (1426.5). Despite the trend of IC to show a higher MFI, compared to VB, for both AC and LMP by 2.3-fold 3.3-fold, respectively, no statistical significance was observed (Figures 26-28, Table 22).

Conversely, the extracellular marker for ALP, MSCA1, presented a higher MFI from AC-harvested MSCs, compared to LMP, from the majority of donors (AF28, AF29, AF50), for both anatomic sites (IC 2.4-fold, VB 2.6-fold). MSCA1 presented the highest MFI from IC AC (1156), followed by VB AC (1077), IC LMP (472.5), and VB LMP (414.5). Concerning the anatomic location, the overall expression was similar from both IC and VB (Figures 26-28, Table 22).

SUSD2 did not show any substantial trend, regarding the harvesting method used or the anatomic site. VB LMP-harvested MSCs presented a slightly higher MFI (751.5), compared to VB AC (586.5) by 1.3-fold difference, followed by IC AC (672.5), and IC LMP (627) (Figures 26-28, Table 22).

In relation to MSCs standard characterization markers, CD90 and CD73 presented a similar pattern throughout all donors. In particular, IC AC-harvested MSCs, presented a higher MFI for both CD90 (1.8-fold) and CD73 (1.4-fold), compared to IC LMP. Moreover, both presented a similar MFI from VB regardless the MSCs-harvesting method used. Additionally, CD90 was the only marker to present statistical significance ($p=0.048$) from IC AC-harvested MSCs, compared to IC LMP. Also CD90 presented always the highest MFI independently from the harvesting method used or the anatomic site. CD105 presented the higher MFI from VB LMP-harvested MSCs (2859), followed by VB LMP (2777), IC LMP (2397.5) and IC AC (1888). IC LMP presented a higher MFI by 1.3-fold difference, compared to IC AC. (Figures 26-28, Table 22).

The overall expression of all three markers CD105, CD90 and CD73, concerning the anatomic site, was similar for both IC and VB. Moreover, VB did not present any substantial difference between harvesting methods used, for CD105, CD90 and CD73, (Figures 26-28, Table 22).

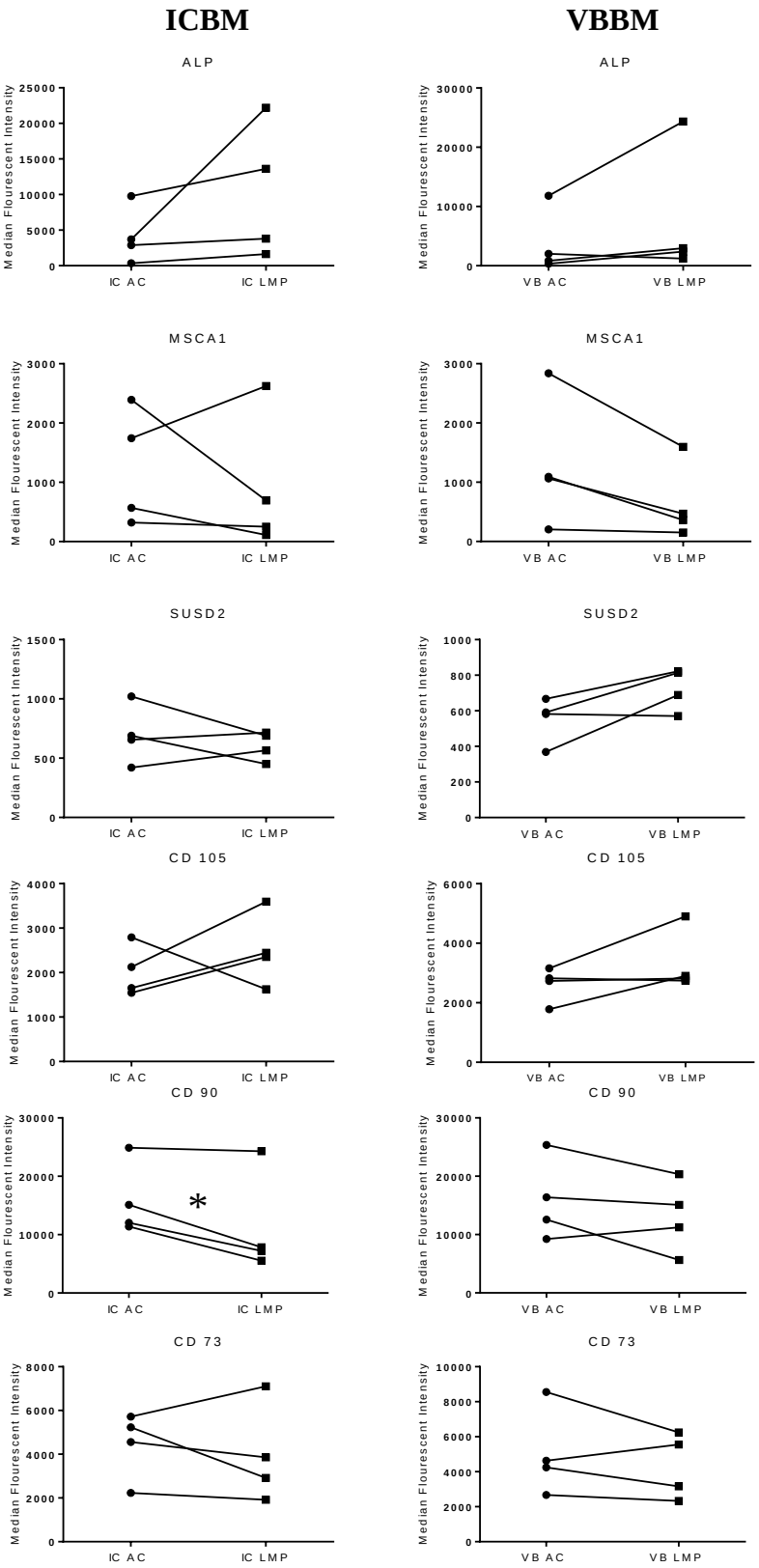


Figure 26: Comparison of MFI values between AC- and LMP-harvested MSCs. Data are illustrated as before/after-type of graphics. No statistical significance was found, except from IC AC harvested-MSCs CD90 marker (p=0.048).

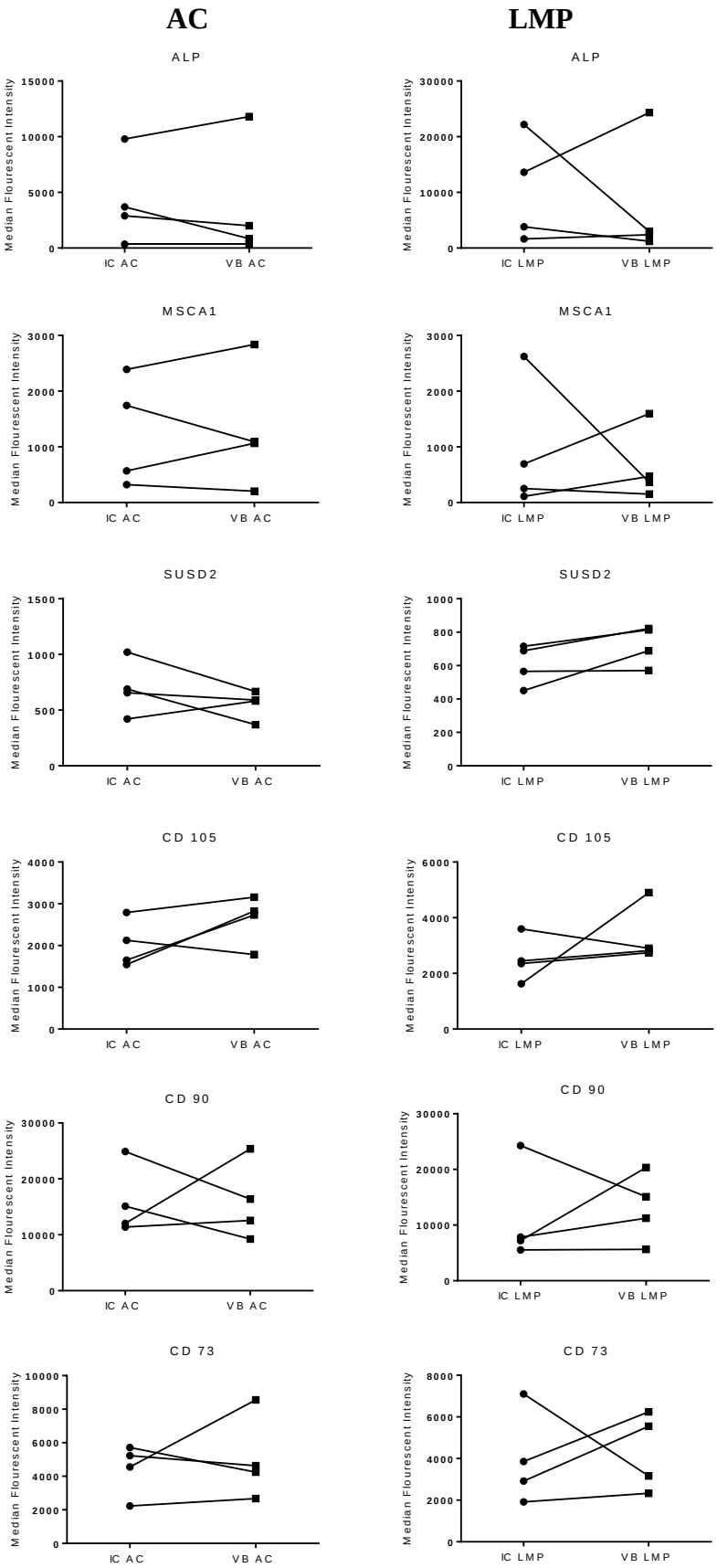


Figure 27: Comparison of MFI values between anatomic sites VB and IC MSCs. Data are illustrated as before/after-type of graphic. No statistical significance was found.

Table 22. Overview of MFI values, for all the donors tested with flow cytometry.

Due to a non Gaussian distribution, median values were used for analysis.

Donors	ALP PE	CD105 FITC	CD90 FITC	CD73 BV 421	SUSD2 APC	MSCA1 PE
AF28 IC AC	9790	1544	11396	2231	1020	2392
AF29 IC AC	3688	1650	24878	5715	656	1743
AF39 IC AC	2893	2790	15115	5231	421	322
AF50 IC AC	348	2126	12041	4555	689	569
Median	**3290.5**	**1888.0**	**13578.0**	**4893.0**	**672.5**	**1156.0**
SD	4002.3	567.9	6228.7	1543.2	246.5	978.4
Mean	4179.75	2027.5	15857.5	4433	696.5	1256.5
AF28 IC LMP	13623	2350	5543	1918	689	693
AF29 IC LMP	22194	2445	24282	7103	715	2624
AF39 IC LMP	3805	1625	7843	2918	565	252
AF50 IC LMP	1634	3596	7204	3859	450	110
Median	**8714.0**	**2397.5**	**7523.5**	**3388.5**	**627.0**	**472.5**
SD	9483.2	814.9	8763.1	2246.8	122.2	1163.0
Mean	10314	2504	11218	3949.5	604.75	919.75
AF28 VB AC	11807	2824	12558	2667	667	2839
AF29 VB AC	841	2730	16380	4247	591	1090
AF39 VB AC	2012	3157	9225	4633	582	204
AF50 VB AC	349	1784	25354	8556	369	1064
Median	**1426.5**	**2777.0**	**14469.0**	**4440.0**	**586.5**	**1077.0**
SD	5414.9	589.0	6960.2	2502.5	128.0	1106.0
Mean	3752.25	2623.75	15879.25	5025.75	552.25	1299.25
AF28 VB LMP	24337	2743	5638	2333	822	1597
AF29 VB LMP	2964	2818	15094	3165	814	360
AF39 VB LMP	1201	4901	11231	5550	570	152
AF50 VB LMP	2379	2900	20340	6243	689	469
Median	**2671.5**	**2859.0**	**13162.5**	**4357.5**	**751.5**	**414.5**
SD	11102.1	1042.3	6206.6	1870.2	119.2	648.5
Mean	7720.25	3340.5	13075.75	4322.75	723.75	644.5

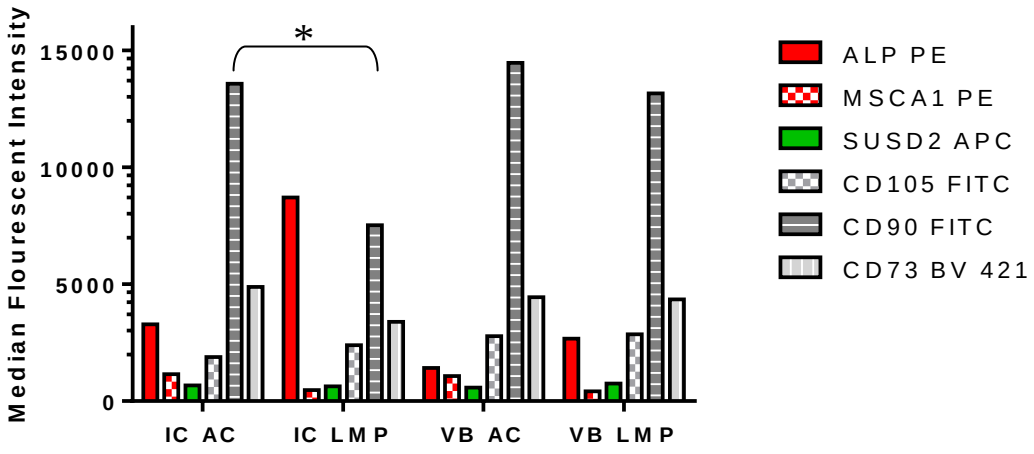


Figure 28: Overall comparison of median fluorescent intensity for all four donors. Median fluorescent intensity was performed only on markers characterising MSCs. CD90 from IC AC-harvested MSCs, was the only marker to present statistic significance (p=0.048). Data are illustrated as grouped bars, median values were calculated and Wilcoxon matched-pairs test were used.

4.8 Tri-lineage differentiation: Osteogenesis

4.8.1 Osteogenesis, introduction.

Following 14 days of osteogenic induction, osteogenesis was compared between the different groups; quantitatively (in triplicates), with spectrophotometric measurement of calcium production and qualitatively (in duplicates) by detection of alkaline phosphatase (ALP) activity with fast blue RR staining and matrix mineralization assay using alizarin red (AR) dye (Figures 29,30). All MSCs cultured under osteogenic conditions stained positively for all donors, with the only exception of a single contaminated well.

ALP activity was more pronounced from VB with no significant deference between AC and LMP. Contrarily, within the IC group the colour was less marked, meaning a slightly lower ALP activity. In particular, IC LMP presented a slightly more intense activity compared to IC AC. Concluding, VB AC presented remarkably higher levels of ALP activity compared to IC AC, in all the donors studied. Whilst IC LMP gave better results from donors AF 39, AF 50 and only donor AF 29 expressed more ALP activity from VB LMP. Donor AF 28 presented similar activities for both harvesting methods (Figure 29).

Similarly, matrix mineralization was higher from VB and in particular from VB AC-harvested MSCs (donors: AF 29, AF 50), compared to VB LMP. Similarly to Figure 34, the colour from IC was less marked, meaning a lower calcium deposition. In particular, IC LMP presented a slightly more intense activity compared to IC AC, especially for donors AF 39, AF 50. Concluding, VB AC presented the highest levels of matrix mineralization compared to VB LMP, IC AC and IC LMP, which present comparable levels between them (Figure 30).

Despite the thorough tissue culture techniques, the staining presented an heterogeneous distribution within the same well. VB AC showed a more uniform colour distribution for both ALP and matrix mineralization (Figures 29,30). As it was expected, donor-to-donor variability was observed. This is a constant characteristic among similar studies and it is vastly documented in the literature[51].

All osteogenic cultures were stopped prematurely at the 14th day (instead of the 21st as per standard protocols), due to their high proliferation capacity, having almost

reached confluency of the well-plate at the end of the second week. Further extension of the culture, could have led to MSCs detachment from the plastic surface prejudging the outcome of the assay (Figure 31).

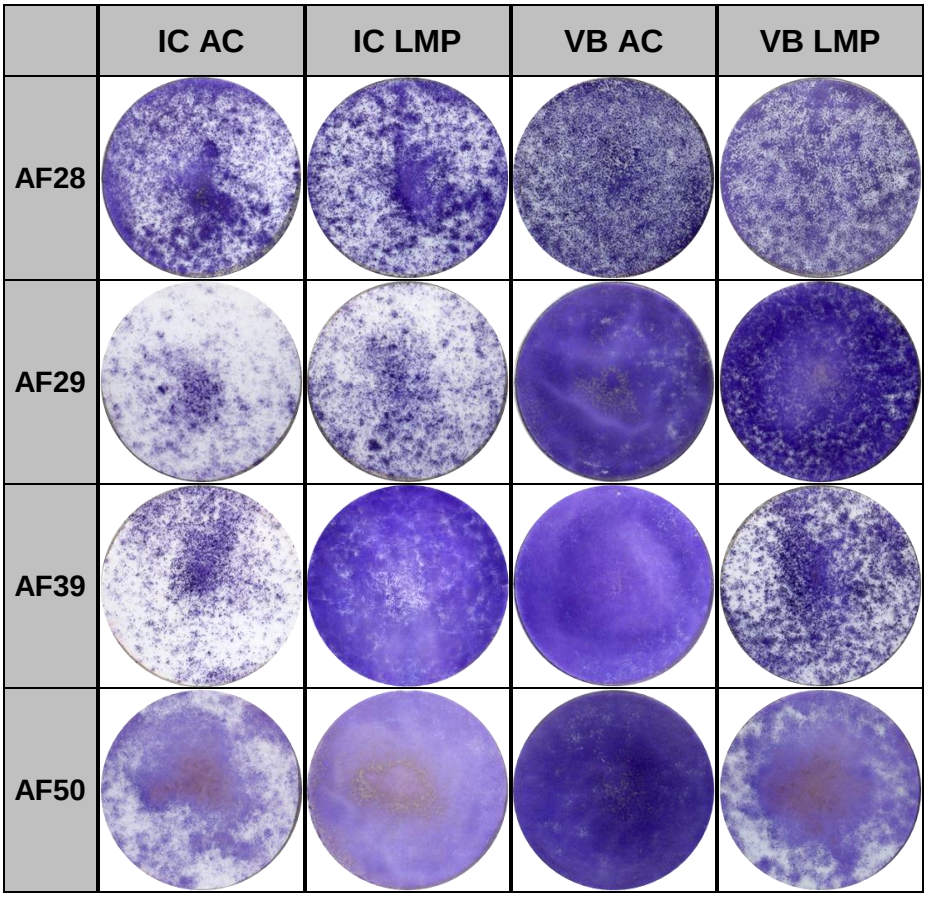


Figure 29: ALP colourimetric assay at 14 days post-induction. Overall comparison of the entire surface of the well plate (3.5 cm diameter) showing ALP activity, stained with Fast blue RR salt dye.

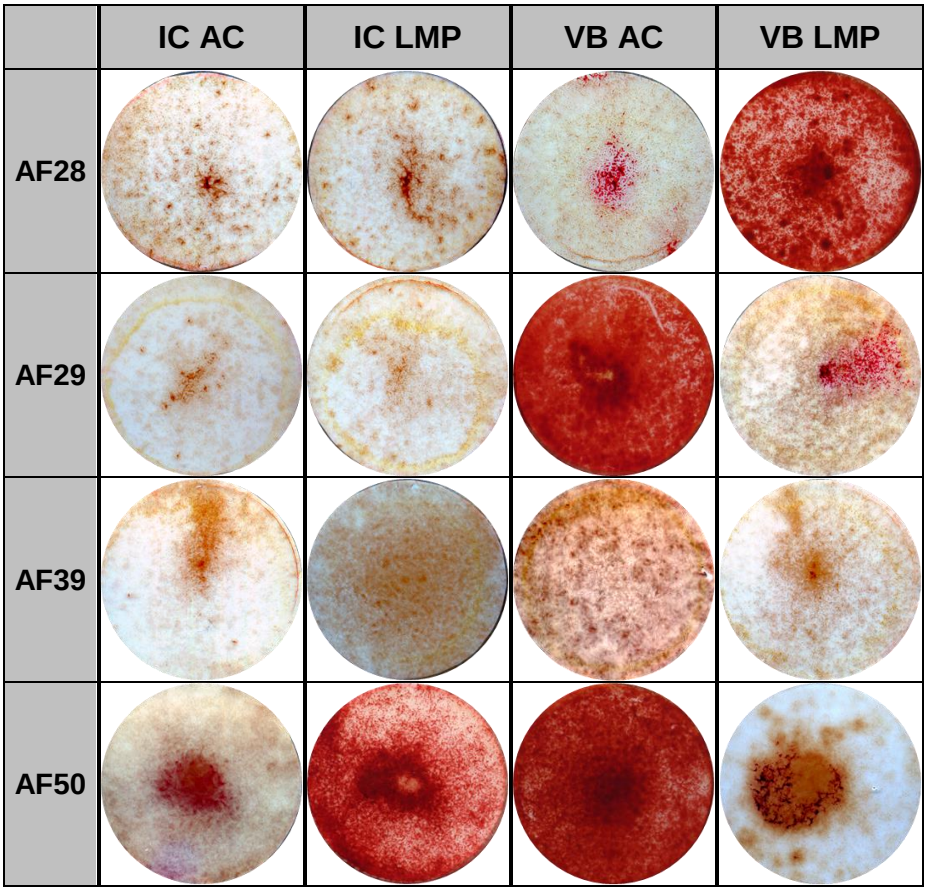


Figure 30: AR colourimetric assay at 14 days post-induction. Overall comparison of the entire surface of the well plate (3.5 cm diameter) showing mineral deposition, stained with AR dye.

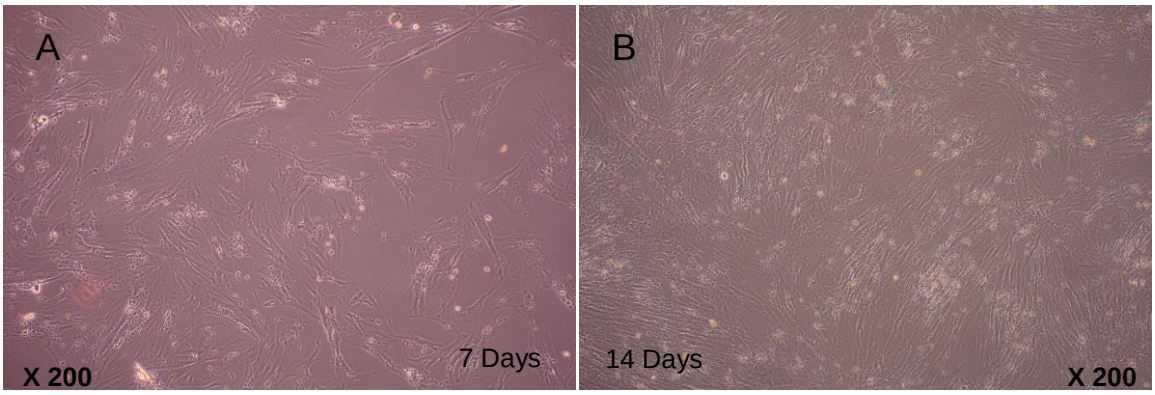


Figure 31: Bone marrow MSCs in osteogenic environment at passage 3. At this stage of MSCs' differentiation, the typical growth at radial pattern is less pronounced, however MSCs are still characterized by a high doubling rate, due to the young donors' age. A) Proliferation at the end of the 1st week. B) Reached confluency by the end of the 2nd week. Images at x200 magnification.

Validation of calcium assay performance

Variability between calcium assay triplicates was assessed to establish its accuracy. The CV was calculated at two different stages: 1) intra-variability within the triplicates, used for the spectrophotometric analysis (cut-off point ≤ 10%) and 2) inter-variability between the three different dishes used for the MSCs osteogenic induction (cut-off point ≤ 30%).

Moreover, calcium concentrations were compared with the staining outcomes of colourimetric assays (ALP activity and AR), adding an additional level of validation to the results.

Calcium assay performance (spectrophotometry)

Standard curves have been produced using standard calcium values (Sigma kit) showing satisfactory linearity and consistent results across the different experiments performed (Figure 32). Following 144 readings, the optical density was analyzed and the overall CV was 2.63% (range 0%-10%), showing satisfactory intra-experimental performance with minimal pipetting error.

Calcium assay performance (osteogenesis)

Total calcium concentration was calculated in μg/ml, from each group of triplicates for all the different test cultures. Following 47 osteogenic assays, the average CV was 16.1 (range 8.1%-24.7%) remaining, for all the cases, below the cut-off level of 30% for this type of assay.

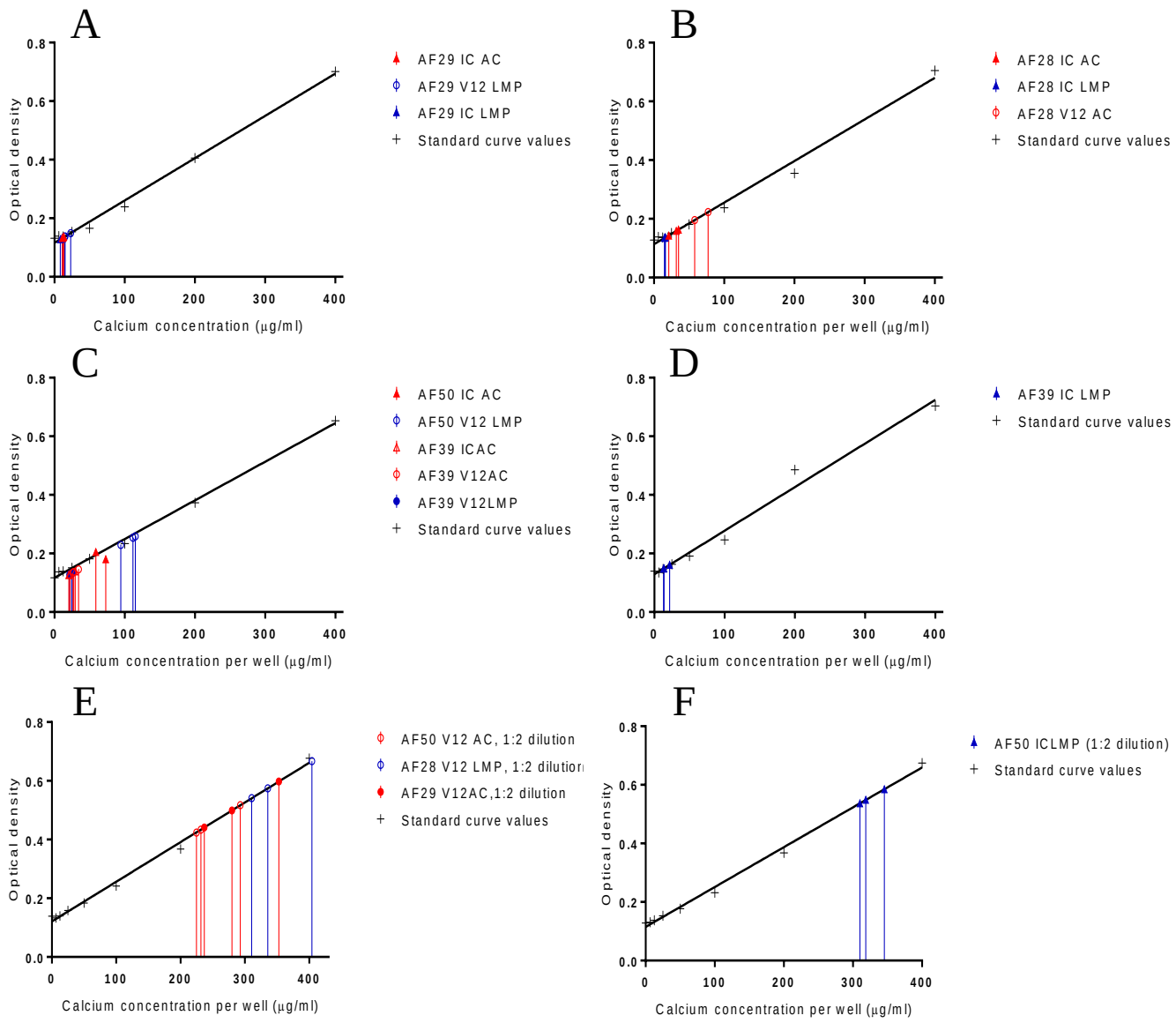


Figure 32: Spectrophotometric readings of calcium assays. A-D) Standard curves and calcium concentrations in each set of triplicates, using 96-wells plate. E, F) Repetition of the assays performing 1:2 dilution of the samples, due to calcium concentrations, above the standard curves.

4.8.2 Individual analysis of each donor

First donor, AF 28, (14.3y, M).

CVs of the triplicates, following spectrophotometry (OD) and total calcium concentration (μg/ml), were 2.2% and 15.9%, far below the cut-off points of 10% and 30% respectively, showing satisfactory intra- and inter-assay accuracy (Tables 23, 24).

The first donor exhibited higher calcium concentrations from VBBM (Figure 33a) and both AC and LMP harvesting methods performed better from VBBM. However, calcium concentrations from VB LMP-harvested MSCs, were higher compared to VB AC, IC LMP, and IC AC, as illustrated in Table 24. Additionally, considering the 15.9% of the overall CV, the differences between the values of VB AC, IC LMP and IC AC were within the intra-experimental variability, therefore were not significant (Table 24, Figure 33).

Matrix mineralization assay outcome, was consistent with the above spectrophotometric results confirming in such way the accuracy of both tests (Figure 33b). Similarly, ALP activity assay, showed also a trend to be slightly more pronounced within the VBBM group, but without a marked difference between AC and LMP (Figure 33c).

Table 23: First donor, variability between triplicates of Calcium assay. OD readings, in triplicates. Mean, SD and CV were performed per single group.

AF 28		Optical density in 96well-plate (x3)					
		1	2	3	MEAN	SD	CV (%)
IC AC	1 dish	0.159	0.159	0.158	0.159	0.00	0.36
	2 dish	0.162	0.166	0.161	0.163	0.00	1.62
	3 dish	0.146	0.141	0.143	0.143	0.00	1.76
						CV Mean	1.25
IC LMP	1 dish	0.135	0.133	0.136	0.135	0.00	1.13
	2 dish	0.137	0.135	0.138	0.137	0.00	1.12
	3 dish	0.136	0.134	0.138	0.136	0.00	1.47
						CV Mean	1.24
VB AC	1 dish	0.225	0.222	0.221	0.223	0.00	0.93
	2 dish	0.194	0.194	0.199	0.196	0.00	1.48
	3 dish	contaminated				CV Mean	1.21
VB LMP (1:2 dilution)	1 dish	0.497	0.548	0.577	0.541	0.04	7.49
	2 dish	0.674	0.638	0.689	0.667	0.03	3.93
	3 dish	0.595	0.573	0.555	0.574	0.02	3.49
						CV Mean	4.97
					Total CV Mean		**2.17**

Table 24: First donor, total calcium concentrations and experimental variability. A) Calcium concentration in triplicates, SD and intra-donor CV within the groups, B) Intra-donor comparisons of calcium concentrations, in fold-difference.

A	Total Calcium (µgr/ml)			
	IC AC	IC LMP	VB AC	VB LMP
1 dish	7.947	3.709	19.249	155.271
2 dish	8.713	4.062	14.481	202.019
3 dish	5.24	3.944	16.865	167.729
MEAN	**7.3**	**3.905**	**16.865**	**175.007**
SD	1.825	0.18	3.372	24.209
CV (%)	24.998	4.605	19.991	13.833
Mean of Overall CV 15.86				

B	Intra-donor comparison based on total calcium	Fold-difference
	IC AC - IC LMP	1.9
	VB AC - VB LMP	10.4
	IC AC - VB AC	2.3
	IC LMP - VB LMP	23.97

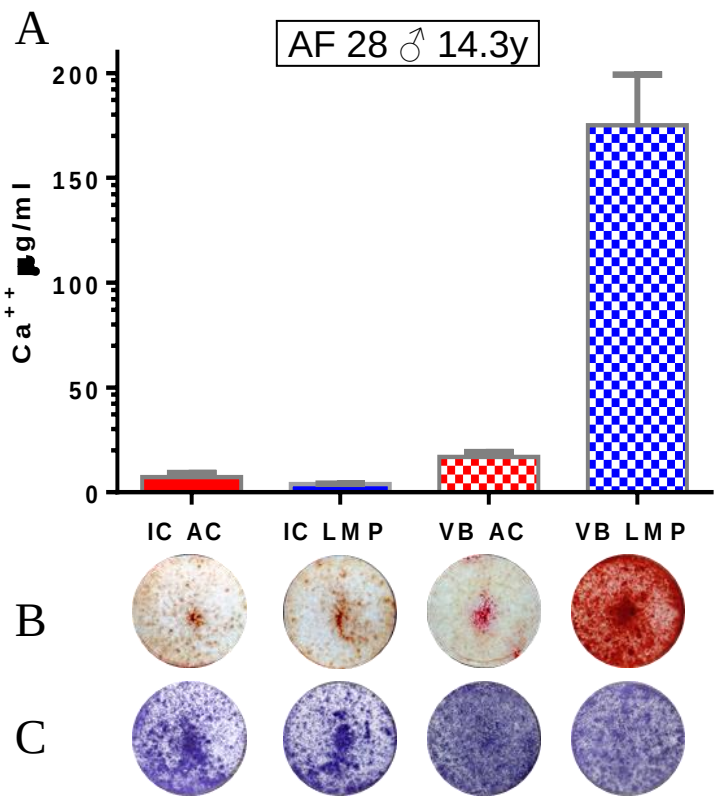


Figure 33: First donor, comparison between calcium concentration, AR and ALP results. A) Calcium concentration was higher in VB, compared to IC. VB LMP showed remarkably higher results, among all groups. B) Calcium deposits stained with AR, confirmed the spectrophotometric readings. C) ALP activity was similar in all groups. The text box indicates donor's details. B, C) Illustration of the entire surface of the wells. Graphic presented as bars with mean and SD.

Second donor, AF 29, (15.6y, M).

In this case also, CVs of the triplicates, following spectrophotometry (OD) and total calcium concentration (μg/ml), were 2.3% and 18%, far below the cut-off points of 10% and 30% respectively showing satisfactory intra- and inter-assay accuracy (Tables 25, 26).

The second donor also exhibited higher concentrations of calcium from VBBM (Figure 34a) and both AC and LMP harvesting methods performed better from VBBM. Nevertheless, calcium concentration from VB AC-harvested MSCs, was higher compared to VB LMP, IC LMP, and IC AC as illustrated in Table 26. Additionally, considering the 18% of the overall CV, the differences between the values of VB AC, IC LMP and IC AC were within the intra-experimental variability, therefore were not significant (Table 26, Figure 34).

Matrix mineralization assay outcome, was consistent with the above spectrophotometric results, confirming in this manner the accuracy of both tests (Figure 34b). Similarly, ALP activity assay, was also remarkably more pronounced within the VBBM group, but without difference between AC and LMP (Figure 34c).

Table 25: Second donor, variability between triplicates of Calcium assay. OD readings, in triplicates. Mean, SD and CV were performed per single group.

AF 29		Optical density in 96well-plate (x3)					
		1	2	3	**MEAN**	**SD**	**CV (%)**
IC AC	1 dish	0.132	0.133	0.132	0.132	0.00	0.44
	2 dish	0.137	0.135	0.139	0.137	0.00	1.46
	3 dish	0.133	0.136	0.134	0.134	0.00	1.14
						CV Mean	1.01
IC LMP	1 dish	0.133	0.132	0.131	0.132	0.00	0.76
	2 dish	0.131	0.132	0.133	0.132	0.00	0.76
	3 dish	0.129	0.127	0.128	0.128	0.00	0.78
						CV Mean	0.77
VB AC (1:2 dilution)	1 dish	0.451	0.457	0.415	0.441	0.02	5.15
	2 dish	0.520	0.462	0.516	0.499	0.03	6.49
	3 dish	0.557	0.588	0.648	0.598	0.05	7.74
						CV Mean	6.46
VB LMP	1 dish	0.149	0.147	0.152	0.149	0.00	1.69
	2 dish	0.138	0.137	0.135	0.137	0.00	1.12
	3 dish	0.138	0.138	0.138	0.138	0.00	0.00
						CV Mean	0.93
					Total CV Mean		**2.29**

Table 26: Second donor, total calcium concentrations and experimental variability. A) Calcium concentration in triplicates, SD and intra-donor CV within the groups, B) Intra-donor comparisons of calcium concentrations, in fold-difference.

A	**Total Calcium (µgr/ml)**			
	IC AC	**IC LMP**	**VB AC**	**VB LMP**
1 dish	2.816	2.758	118.391	5.758
2 dish	3.335	2.758	139.976	3.566
3 dish	3.162	2.066	176.363	3.797
MEAN	**3.104**	**2.527**	**144.91**	**4.374**
SD	0.264	0.4	29.3	1.205
CV (%)	8.517	15.815	20.219	27.545
Mean of Overall CV 18.02				

B	**Intra-donor comparison based on total calcium**	**Fold-difference**
	IC AC - IC LMP	1.2
	VB AC - VB LMP	33.1
	IC AC - VB AC	46.7
	IC LMP - VB LMP	1.7

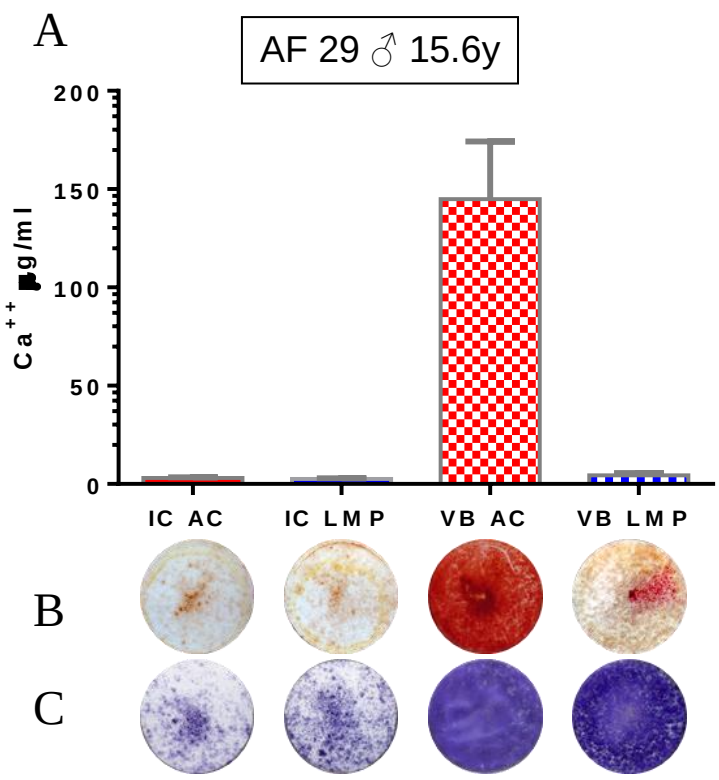


Figure 34: Second donor, comparison between calcium concentration, AR and ALP results. A) Calcium concentration was higher in VB, compared to IC. VB AC gave the highest results, among all groups. B) Calcium deposits stained with AR, confirmed the spectrophotometric results. C) ALP activity was higher in VB group. The text box indicates donor's details. B, C) Illustration of the entire surface of the wells. Graphic presented as bars with mean and SD.

Third donor, AF 39, (15.3y, F).

CVs of the triplicates, following spectrophotometry (OD) and total calcium concentration (µg/ml), were 1.7% and 17.9%, below the cut-off points of 10% and 30% respectively, showing satisfactory intra- and inter-assay accuracy (Table 27, 28).

The third donor, in contrast with the previous cases, did not show any significant difference between the compared groups and both harvesting methods, AC and LMP, performed slightly better from VBBM (Figure 35a). In particular, calcium concentration from VB AC-harvested MSCs, was higher compare to VB LMP, IC LMP, and IC AC as illustrated in Table 28. Additionally, considering the 17.9% of the overall CV, the differences between VB LMP, IC AC and IC LMP were within the intra-experimental variability (Table 28, Figure 35).

Comparison between matrix mineralization assay and ALP activity, showed similar colourimetric results (Figure 35b,c). This donor did not present a strong relation, between spectrophotometric and colourimetric assays, however it is still possible to identify a trend, which correlates between the tests (Figure 35a,b,c). This difference from the rest of the donors, maybe is related to the different gender of this donor. From the anatomic point of view, both sites presented a similar outcome.

Table 27: Third donor, variability between triplicates of Calcium assay. OD readings, in triplicates. Mean, SD and CV were performed per single group.

AF 39		Optical density in 96well-plate (x3)					
		1	2	3	**MEAN**	**SD**	**CV (%)**
IC AC	1 dish	0.139	0.142	0.136	0.139	0.00	2.16
	2 dish	0.127	0.129	0.130	0.129	0.00	1.19
	3 dish	0.124	0.127	0.128	0.126	0.00	1.65
						CV Mean	1.66
IC LMP	1 dish	0.148	0.150	0.150	0.149	0.00	0.77
	2 dish	0.148	0.149	0.148	0.148	0.00	0.39
	3 dish	0.162	0.161	0.160	0.161	0.00	0.62
						CV Mean	0.60
VB AC	1 dish	0.140	0.135	0.134	0.136	0.00	2.36
	2 dish	0.132	0.139	0.134	0.135	0.00	2.67
	3 dish	0.144	0.143	0.150	0.146	0.00	2.60
						CV Mean	2.54
VB LMP	1 dish	0.135	0.135	0.129	0.133	0.00	2.60
	2 dish	0.132	0.130	0.134	0.132	0.00	1.52
	3 dish	0.127	0.127	0.130	0.128	0.00	1.35
						CV Mean	1.82
					Total CV Mean		**1.66**

Table 28: Third donor, total calcium concentrations and experimental variability. A) Calcium concentration in triplicates, SD and intra-donor CV within the groups, B) Intra-donor comparisons of calcium concentrations, in fold-difference.

A	**Total Calcium (µgr/ml)**			
	IC AC	**IC LMP**	**VB AC**	**VB LMP**
1 dish	7.355	3.463	6.876	6.277
2 dish	5.498	3.295	6.636	6.097
3 dish	5.079	5.424	8.552	5.378
MEAN	**5.977**	**4.060**	**7.355**	**5.917**
SD	1.211	1.184	1.044	0.475
CV (%)	20.263	29.150	14.198	8.033
Mean of Overall CV 17.91				

B	**Intra-donor comparison based on total calcium**	**Fold-difference**
	IC AC - IC LMP	1.5
	VB AC - VB LMP	1.2
	IC AC - VB AC	1.2
	IC LMP - VB LMP	1.4

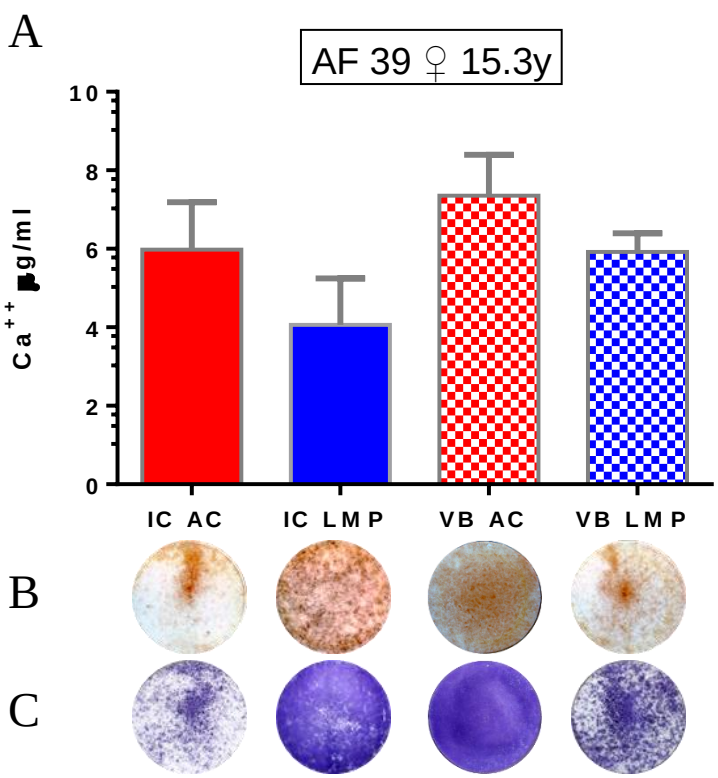


Figure 35: Third donor, comparison between calcium concentration, AR and ALP results. A) Calcium concentration was slightly higher in VB, compared to IC, but without any substantial difference. B) Calcium deposits stained with AR, did not present a strong correlation with the spectrophotometric results. C) ALP activity was similar in both anatomic regions. The text box indicates donor's details. B, C) Illustration of the entire surface of the wells. Graphic presented as bars with mean and SD.

Forth donor, AF 50, (17y, M).

In the fourth donor, CVs of the triplicates, following spectrophotometry (OD) and total calcium concentration (μg/ml), were 4.4% and 11.2%, far below the cut-off points of 10% and 30% respectively, showing satisfactory intra- and inter-assay accuracy (Tables 29, 30).

The forth donor, exhibited higher concentrations of calcium from IC LMP-harvested MSCs followed by VB AC. AC-harvesting method performed better from VBBM, but contrary to the rest of the cases, LMP performed better from ICBM (Figure 36a). In particular, calcium concentration from IC LMP-harvested MSCs, was higher compared to VB AC, VB LMP, and IC AC as illustrated in Table 30. Additionally, considering the 11.2% of the overall CV, the difference between IC LMP and VB AC was above the intra-experimental variability, and the difference between VB LMP and IC AC was within the intra-experimental variability (Table 30, Figure 36).

Matrix mineralization and ALP activity assays showed consistent colourimetric outcome, coinciding with the above spectrophotometric results, confirming the accuracy of both tests (Figure 36a,b,c).

Table 29: Forth donor, variability between triplicates of Calcium assay. OD readings, in triplicates. Mean, SD and CV were performed per single group.

AF 50		Optical density in 96well-plate (x3)					
		1	2	3	MEAN	SD	CV (%)
IC AC	1 dish	0.195	0.211	0.208	0.205	0.01	4.15
	2 dish	0.172	0.185	0.183	0.180	0.01	3.89
	3 dish	0.191	0.207	0.201	0.200	0.01	4.05
						CV Mean	4.03
IC LMP (1:2 dilution)	1 dish	0.562	0.567	0.626	0.585	0.04	6.08
	2 dish	0.514	0.574	0.558	0.549	0.03	5.66
	3 dish	0.524	0.526	0.561	0.537	0.02	3.87
						CV Mean	5.21
VB AC (1:2 dilution)	1 dish	0.424	0.424	0.424	0.424	0.00	0.00
	2 dish	0.477	0.517	0.556	0.517	0.04	7.65
	3 dish	0.439	0.417	0.446	0.434	0.02	3.49
						CV Mean	3.71
VB LMP	1 dish	0.254	0.251	0.270	0.258	0.01	3.95
	2 dish	0.219	0.229	0.240	0.229	0.01	4.58
	3 dish	0.248	0.243	0.270	0.254	0.01	5.66
						CV Mean	4.73
					Total CV Mean		**4.42**

Table 30: Forth donor, total calcium concentrations and experimental variability. A) Calcium concentration in triplicates, SD and intra-donor CV within the groups, B) Intra-donor comparisons of calcium concentrations, in fold-difference.

A	Total Calcium (µgr/ml)			
	IC AC	IC LMP	VB AC	VB LMP
1 dish	19.153	172.676	112.368	28.795
2 dish	14.721	159.324	146.390	23.584
3 dish	18.254	155.036	115.800	27.956
MEAN	**17.376**	**162.345**	**124.853**	**26.779**
SD	2.343	9.200	18.731	2.798
CV (%)	13.483	5.667	15.002	10.448
Mean of Overall CV 11.15				

B	Intra-donor comparison based on total calcium	Fold-difference
	IC AC - IC LMP	9.3
	VB AC - VB LMP	4.7
	IC AC - VB AC	7.2
	IC LMP - VB LMP	6.1

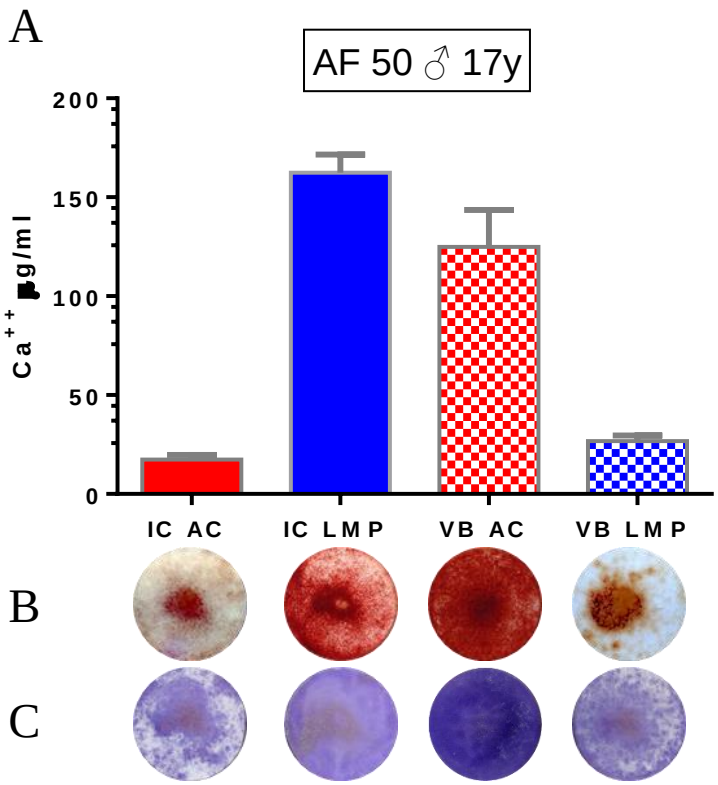


Figure 36: Forth donor, comparison between calcium concentration, AR and ALP results. A) Calcium concentration presented similar results between both anatomic regions, however, IC LMP yielded the highest outcome compared to VB AC, VB LMP and IC AC, respectively. B, C) Calcium deposits stained with AR and ALP activity was in line with the spectrophotometric results. The text box indicates donor's details. B, C) Illustration of the entire surface of the wells. Graphic presented as bars with mean and SD.

4.8.3 Osteogenesis, overall comparison

The overall analysis of 16 different cultures from a cohort of 4 donors (3 males and 1 female) showed higher calcium concentrations from VBBM MSCs, compared to ICBM, with both harvesting methods (VBBM: AC 73.5µg/ml, LMP 53µg/ml, ICBM: AC 8.4µg/ml, LMP 43.21µg/ml, mean values). In particular, VB AC-harvested MSCs showed significantly higher calcium concentration, compared to IC AC-harvested MSCs by 8.7-fold difference, p=0.001 (Figure 37). Contrarily, comparing the calcium production between VB LMP and IC LMP, the difference was only 1.2-fold and without any statistical significance.

Matrix mineralization assay, showed a higher calcium deposition from VBBM, especially from the VB AC-harvested MSCs' group (Figure 30), which was consistent with the spectrophotometric results in all the above experiments.

ALP activity was also higher from the VBBM and especially from VB AC-harvested MSCs' group, however it was not always matching the spectrophotometric outcomes with the same accuracy as the AR staining (Figure 29). This is because, although spectrophotometry and matrix mineralization assays use different techniques, both measure calcium deposition following its staining. Contrarily ALP assay, represents a qualitative assessment of the ALP expression, detecting its enzymatic activity. Therefore, they show different aspects of the same process and for this reason they present a similar pattern, but is not exactly identical.

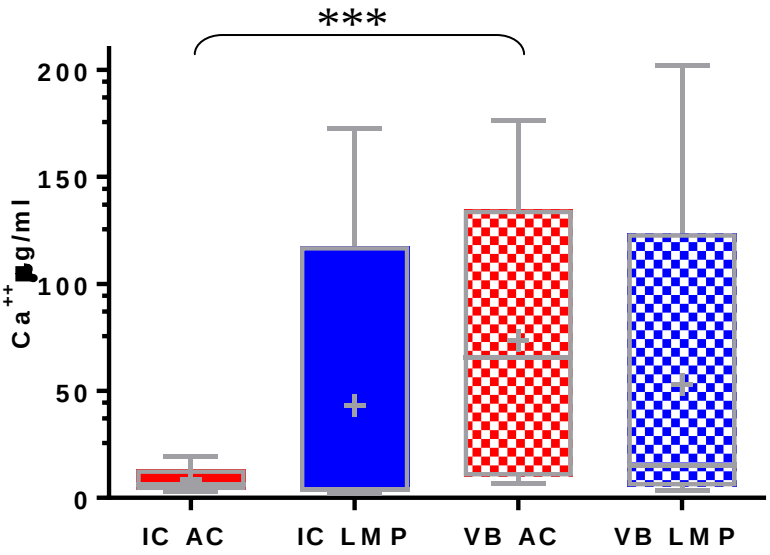


Figure 37: Summarized data from all 4 donors. Average values of total calcium production (μg/ml), showing a statistically significant difference between VB AC- and IC AC-harvested MSCs (***, p=0.001). Values processed with Wilcoxon T test and are represented as Box & Whiskers graphic. Mean and median are illustrated as "+" and "bar" symbols, respectively.

4.9 Tri-lineage differentiation: Chondrogenesis

4.9.1 Chondrogenesis, introduction

Following 21 days of MSCs chondrogenic induction, cultures stained positively for all donors. Chondrogenesis was compared between the different groups; quantitatively, with spectrophotometric measurement of glycosaminoglycan (GAG) production and qualitatively by toluidine blue staining of sectioned chondrogenic pellets at 10°C temperature using an oscillo-cryotome (Figure 38).

However, despite the thorough tissue culture technique used, MSCs-chondrogenic induction, presented a more complex management compared to osteogenesis and adipogenesis, resulting in three contaminated eppendorf tubes, subsequently excluded from the assay. This higher rate of infection, compared to other assays, could be possibly related to a more complex medium composition and manipulation of the numerous eppendorf tubes used (5 eppendorf tubes per condition, 77 in total) to reproduce the 3D culture model.

Similarly to osteogenesis, donor-to-donor variability was also noticed, however, similar patterns between donors were observed, and will be discussed in the following paragraphs.

Comparing chondrogenesis from a quantitative perspective, VBBM showed a significantly higher production of GAG from all patients, regardless the harvesting technique used. Contrarily, within the ICBM group, were statistically significant differences between AC and LMP-harvested MSCs, resulting in a higher production of GAG from IC LMP-harvested MSCs (Figure 44).

In particular, VB LMP presented slightly higher concentration of GAG from donors AF29 and AF50 (Figures 41, 43), while donors AF28 and AF39 (Figures 40, 42) gave higher concentration of GAG from VB AC. Therefore, no statistical significance was found between VBBM harvesting method. Regarding ICBM MSCs, a consistent performance was observed across all donors, presenting always a higher concentration of GAG from LMP-harvested MSCs, which was statistically significant (Figure 40-44).

Validation of GAG assay performance

Variability between the different groups of GAG assay was assessed to establish its accuracy. The CV was calculated at two different stages: 1) between the two samples tested from each eppendorf tube, between each group of triplicates (cut-off point of OD, ≤ 10%) and 2) between the total GAG concentrations (cut-off point, ≤ 30%).

Moreover, the quantitative outcomes of GAG concentrations were compared with the qualitative analysis of the staining patterns of chondrogenic pellets (Figure 38), performed in duplicates, adding an extra level of validation of the results.

GAG assay performance (spectrophotometry)

Standard curves were produced, in duplicates, using standard GAG values (Blyscan™ s-GAG assay kit) showing satisfactory linearity and consistent results across the different experiments performed (Figure 39). Following spectrophotometry, OD was analyzed and the overall CV was 3.03% (range 1.29%-4.24%), showing satisfactory intra-experimental performance.

GAG assay performance (chondrogenesis)

Total GAG concentration was calculated in μg/ml, from each group of triplicates. The average CV was 17.78% (range 13.24%-21.08%) remaining below the cut-off level of 30% for this type of assay.

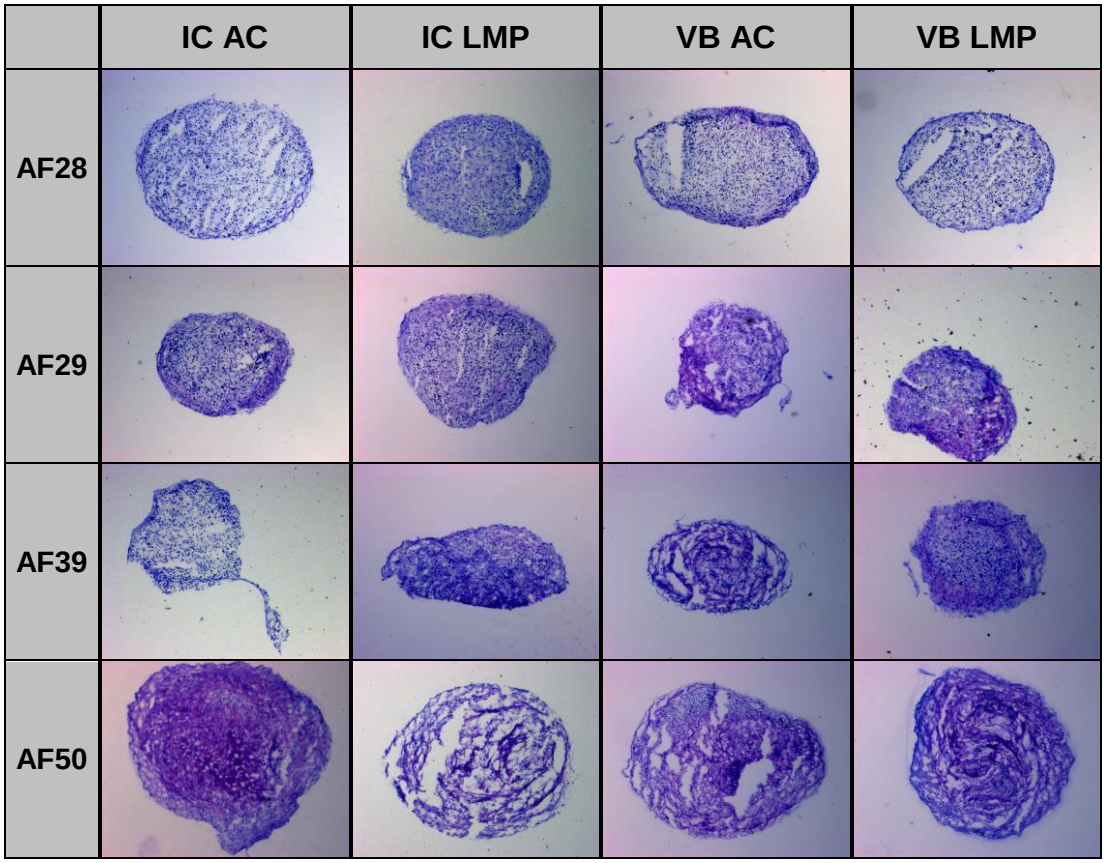


Figure 38: Qualitative analysis of chondrogenic pellets. Overall comparison of the pellets sectioned into 0.04µm in OCT and stained with toluidine blue dye, following 21 days post-induction. GAG is stained in purple and fibrous tissue is stained in blue. Microphotography was performed under consistent photographic parameters to allowed comparison of the results. Magnification x64.

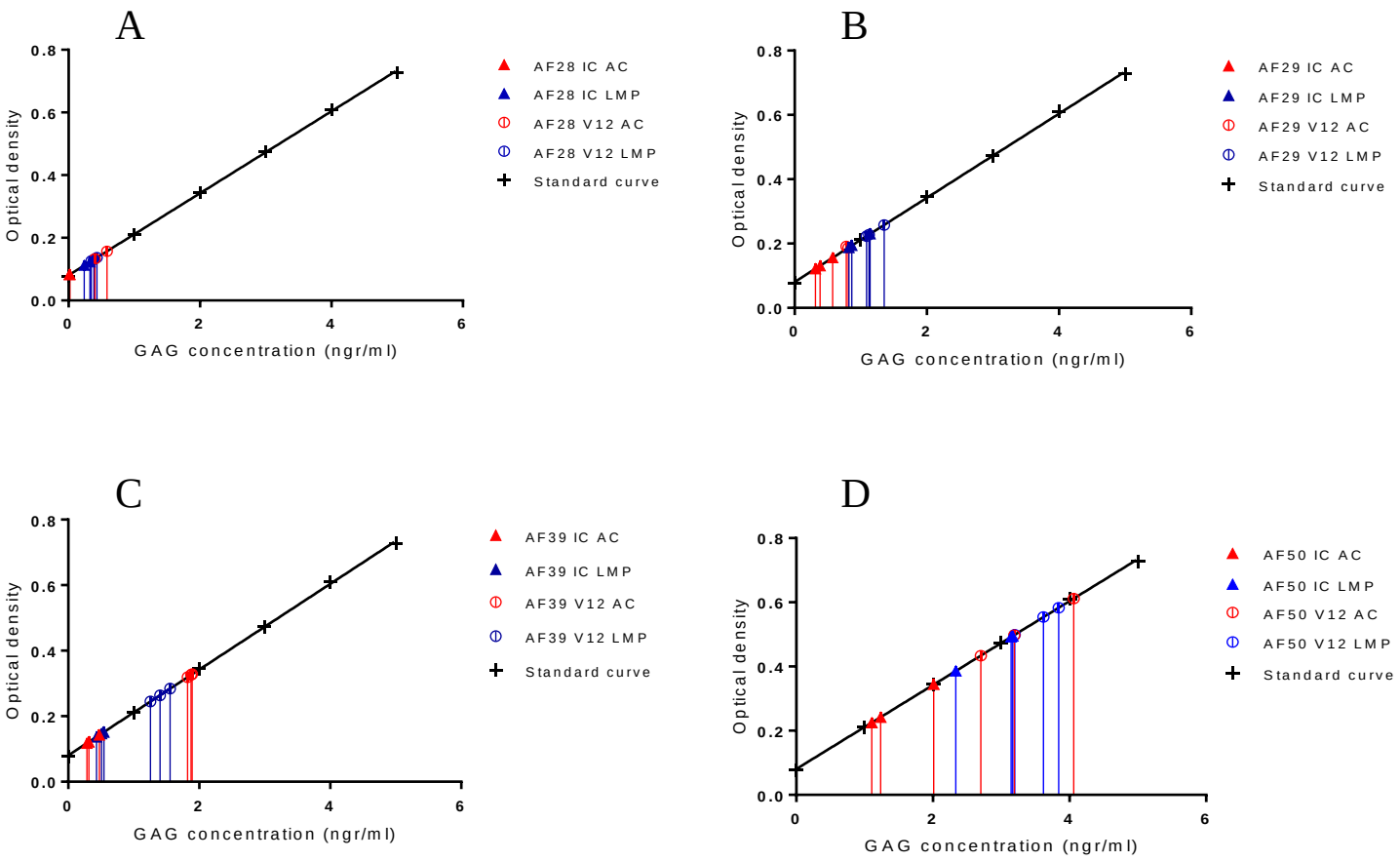


Figure 39: Spectrophotometric readings of GAG assays. A-D) Standard curves and GAG concentrations performed in duplicates, per each condition examined, using 96-flat bottom well plates.

4.9.2 Individual analysis of each donor.

First donor, AF 28, (14.3y, M).

Following spectrophotometry (OD) and calculation of total GAG concentrations (μg/ml), the CVs were 3.99% and 21.81%, far below the cut-off points of 10% and 30% respectively, showing satisfactory intra- and inter-assay accuracy (Tables 31, 32).

The first donor produced overall the lowest amount of GAG, however, presented a similar pattern compared to the rest of the donors, showing higher GAG concentrations from VBBM either using AC or LMP (Figure 40a). More specifically, VB AC-harvested MSCs produced the highest amount of GAG followed by VB LMP, IC LMP and IC AC with 1.3-fold, 1.7-fold and 32.2-fold difference, respectively (Table 32). Additionally, considering the 21.8% of the overall CV, the differences between GAG values of IC LMP and IC AC, were above the intra-experimental variability, contrarily the difference between IC LMP and VB LMP, was within the intra-experimental variability (Table 32, Figure 40).

Moreover, this donor did not present a strong relation, between spectrophotometric and colourimetric assays (Figure 40b).

Table 31: First donor, variability between duplicates of GAG assay. OD readings, with Mean, SD and CV were calculated per single group.

AF 28		Optical density in 96well-plate (x2)				
		1	2	**MEAN**	**SD**	**CV (%)**
IC AC	1 eppendorf	0.081	0.083	**0.082**	0.001	1.72
	2 eppendorf	0.084	0.081	**0.083**	0.002	2.57
	3 eppendorf	0.084	0.082	**0.083**	0.001	1.70
					CV Mean	2.00
IC LMP	1 eppendorf	0.125	0.121	**0.123**	0.003	2.30
	2 eppendorf	0.117	0.107	**0.112**	0.007	6.31
	3 eppendorf	contaminated			CV Mean	4.31
VB AC	1 eppendorf	0.155	0.159	**0.157**	0.003	1.80
	2 eppendorf	0.129	0.137	**0.133**	0.006	4.25
	3 eppendorf	contaminated			CV Mean	3.03
VB LMP	1 eppendorf	0.119	0.133	**0.126**	0.010	7.86
	2 eppendorf	0.129	0.133	**0.131**	0.003	2.16
	3 eppendorf	0.127	0.146	**0.137**	0.013	9.84
					CV Mean	6.62
				Total CV mean		**3.99**

Table 32: First donor, total GAG concentrations. GAG production in triplicates (µg/ml), SD and intra-donor CV within groups were calculated.

Total GAG (µg/ml)				
	IC AC	**IC LMP**	**VB AC**	**VB LMP**
1 eppendorf	0.340	10.788	19.453	11.553
2 eppendorf	0.595	7.985	13.337	12.827
3 eppendorf	0.595	contaminated	contaminated	14.356
MEAN	**0.510**	**9.387**	**16.395**	**12.912**
SD	0.147	1.982	4.325	1.404
CV (%)	28.867	21.117	26.379	10.870
Mean of Overall CV 21.81				

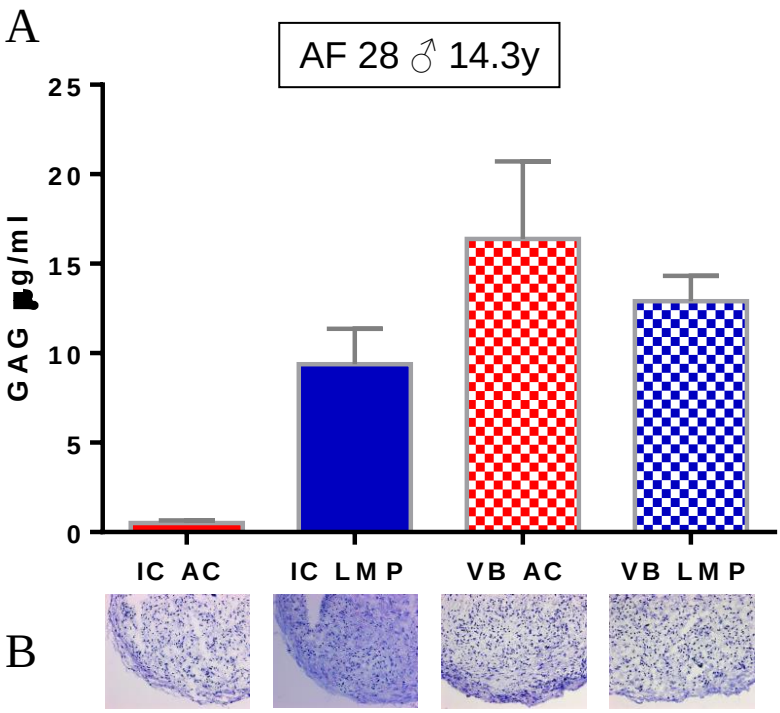


Figure 40: First donor, comparison between GAG concentrations A) GAG concentration was higher in VB, compared to IC, showing slightly higher results from VB AC, among all groups. B) It was not possible to identify a strong correlation, between GAG deposits stained with toluidine blue and the spectrophotometric results. The text box indicates donor's details. A) Graphic presented as bars with mean and SD, B) Microphotography of chondrogenic pellet slides. Magnification x160.

Second donor, AF 29, (15.6y, M).

In this case also, following spectrophotometry (OD) and calculation of total GAG concentrations (μg/ml), the CVs were 4.24% and 16.05%, far below the cut-off points of 10% and 30%, respectively, showing satisfactory intra- and inter-assay accuracy (Tables 33, 34).

The second donor also exhibited higher concentration of GAG from VBBM, especially from VB LMP-harvested MSCs (Figure 41a). In particular, VB LMP-harvested MSCs produced the highest amount of GAG followed by IC LMP, VB AC and IC AC with 1.3-fold, 1.4-fold and 2.8-fold difference, respectively. In addition, considering the 16.05% of the overall CV, the differences between the GAG values of VB AC, IC LMP and IC AC, were all above the intra-experimental variability (Table 33, Figure 41).

GAG staining outcome was similar with the above mentioned spectrophotometric results, showing satisfactory correlation between colourimetric and spectrophotometric assays (Figure 41b).

Table 33: Second donor, variability between duplicates of GAG assay. OD readings, with Mean, SD and CV were calculated per single group.

AF 29		**Optical density in 96well-plate (x2)**				
		1	2	**MEAN**	**SD**	**CV (%)**
IC AC	1 eppendorf	0.158	0.153	**0.156**	0.004	2.27
	2 eppendorf	0.123	0.138	**0.131**	0.011	8.13
	3 eppendorf	0.114	0.13	**0.122**	0.011	9.27
					CV Mean	6.56
IC LMP	1 eppendorf	0.236	0.223	**0.230**	0.009	4.01
	2 eppendorf	0.193	0.183	**0.188**	0.007	3.76
	3 eppendorf	0.193	0.195	**0.194**	0.001	0.73
					CV Mean	2.83
VB AC	1 eppendorf	0.19	0.192	**0.191**	0.001	0.74
	2 eppendorf	0.192	0.181	**0.187**	0.008	4.17
	3 eppendorf	contaminated			CV Mean	2.46
VB LMP	1 eppendorf	0.23	0.216	**0.223**	0.010	4.44
	2 eppendorf	0.241	0.274	**0.258**	0.023	9.06
	3 eppendorf	0.225	0.231	**0.228**	0.004	1.86
					CV Mean	5.12
				Total CV mean		**4.24**

Table 34: Second donor, total GAG concentrations. GAG production in triplicates (µg/ml), SD and intra-donor CV within groups were calculated.

Total GAG (µg/ml)				
	IC AC	**IC LMP**	**VB AC**	**VB LMP**
1 eppendorf	19.198	38.056	27.863	36.273
2 eppendorf	12.827	27.353	27.098	45.192
3 eppendorf	10.533	28.882	contaminated	37.547
MEAN	**14.186**	**31.431**	**27.480**	**39.670**
SD	4.489	5.789	0.541	4.824
CV (%)	31.646	18.418	1.967	12.160
Mean of Overall CV 16.05				

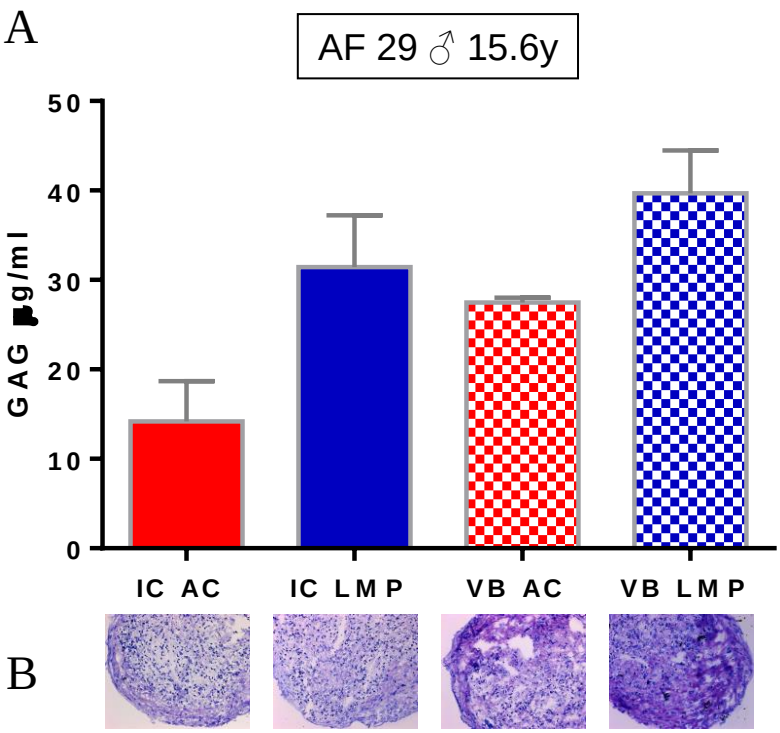


Figure 41: Second donor, comparison between GAG concentrations. A) GAG concentration was higher in VB, compared to IC; showing higher results from VB LMP, among all groups. B) GAG deposits stained with toluidine blue were in line with the spectrophotometric results. The text box indicates donor's details. A) Graphic presented as bars with mean and SD, B) Microphotography of chondrogenic pellet slides. Magnification x160.

Third donor, AF 39, (15.3y, F).

Following spectrophotometry (OD) and calculation of total GAG concentrations (µg/ml), the CVs were 2.26% and 13.24%, far below the cut-off points of 10% and 30% respectively, showing satisfactory intra and inter-assay accuracy (Tables 35, 36).

Likewise, AF 39 also exhibited a higher concentration of GAG from VBBM (Figure 42a). In particular, V12 AC-harvested MSCs produced the highest amount of GAG, followed by VB LMP, IC LMP, and IC AC with 1.3-fold, 3.8-fold and 5.2-fold, difference, respectively. In addition, considering the 13.24% of the overall CV, the differences between the GAG values of VB LMP, IC LMP and IC AC, were all above the intra-experimental variability. However, the difference between IC LMP and IC AC, was within the intra-experimental variability (Table 36, Figure 42).

Similarly to osteogenic assay, this donor did not present a strong relationship, between spectrophotometric and colourimetric assays, however is still possible to identify a trend (Figure 42b). This difference from the rest of the donors, possibly could be related to the different gender of this donor.

Table 35: Third donor, variability between duplicates of GAG assay. OD readings, with Mean, SD and CV were calculated per single group.

AF 39		**Optical density in 96well-plate (x2)**				
		1	2	**MEAN**	**SD**	**CV (%)**
IC AC	1 eppendorf	0.117	0.119	**0.118**	0.001	1.20
	2 eppendorf	0.123	0.121	**0.122**	0.001	1.16
	3 eppendorf	0.142	0.143	**0.143**	0.001	0.50
					CV Mean	**0.95**
IC LMP	1 eppendorf	0.153	0.151	**0.152**	0.001	0.93
	2 eppendorf	0.138	0.136	**0.137**	0.001	1.03
	3 eppendorf	0.147	0.146	**0.147**	0.001	0.48
					CV Mean	**0.82**
VB AC	1 eppendorf	0.31	0.345	**0.328**	0.025	7.56
	2 eppendorf	0.315	0.322	**0.319**	0.005	1.55
	3 eppendorf	0.311	0.341	**0.326**	0.021	6.51
					CV Mean	**5.21**
VB LMP	1 eppendorf	0.251	0.238	**0.245**	0.009	3.76
	2 eppendorf	0.291	0.277	**0.284**	0.010	3.49
	3 eppendorf	0.258	0.27	**0.264**	0.008	3.21
					CV Mean	**3.49**
				Total CV mean		**2.61**

Table 36: Third donor, total GAG concentrations. GAG production in triplicates (µg/ml), SD and intra-donor CV within groups were calculated.

Total GAG (µg/ml)				
	IC AC	**IC LMP**	**VB AC**	**VB LMP**
1 eppendorf	9.514	18.179	63.031	41.879
2 eppendorf	10.533	14.356	60.737	51.818
3 eppendorf	15.885	16.905	62.521	46.721
MEAN	**11.978**	**16.480**	**62.097**	**46.806**
SD	3.422	1.946	1.204	4.970
CV (%)	28.572	11.811	1.939	10.618
Mean of Overall CV 13.24				

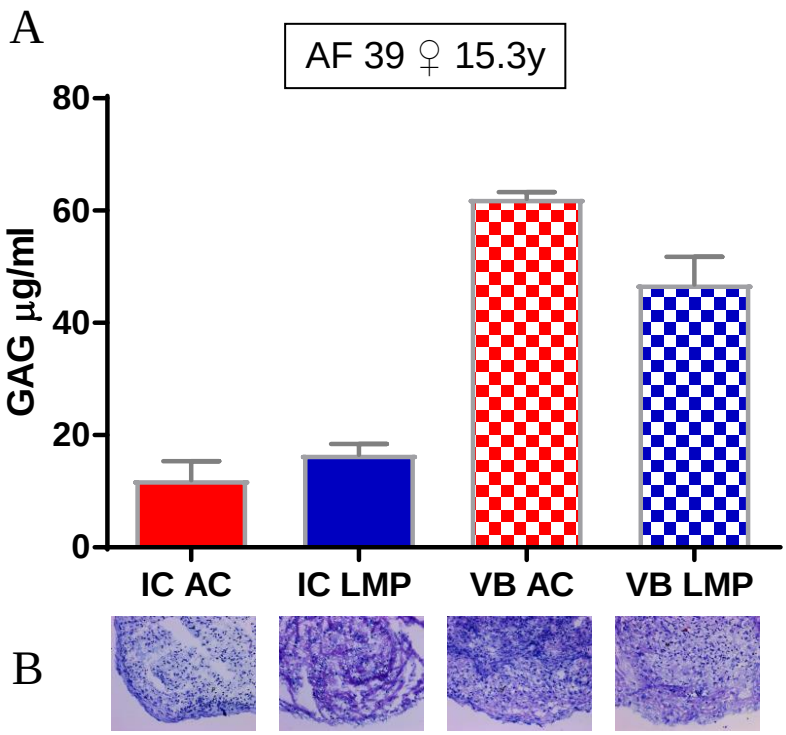


Figure 42: Third donor, comparison between GAG concentrations. A) GAG concentration was higher in VB, compared to IC, showing higher results from VB AC, among all groups. B) It was not possible to identify a strong correlation for all the groups, between GAG deposits stained with toluidine blue and the spectrophotometric results. The text box indicates donor's details. A) Graphic presented as bars with mean and SD, B) Microphotography of chondrogenic pellet slides. Magnification x160.

Forth donor, AF 50, (17y, M).

The forth donor, following spectrophotometry (OD) and calculation of total GAG concentrations (μg/ml), the CVs were 1.29% and 20.09%, below the cut-off points of 10% and 30% respectively, showing satisfactory intra- and inter-assay accuracy (Tables 37, 38).

The fourth donor, produced the highest amount of GAG among all donors, maintaining in the meantime a similar pattern like the previous donors. Also in this case, the highest concentration of GAG was from VBBM (Figure 43a). In particular, VB LMP-harvested MSCs produced the highest amount of GAG, followed by VB AC, IC LMP, and IC AC with 1.1-fold, 1.2-fold and 2.4-fold, difference, respectively. Additionally, considering the 20.09% of the overall CV, the difference between the GAG value of VB AC, VB LMP and IC LMP were within the intra-experimental variability, with exception of IC AC which was above it (Table 38, Figure 43).

GAG staining outcome was similar with the above mentioned spectrophotometric results, showing satisfactory correlation between colourimetric and spectrophotometric assays (Figure 43b).

Table 37: Fourth donor, variability between duplicates of GAG assay. OD readings, with Mean, SD and CV were calculated per single group.

AF 50		Optical density in 96well-plate (x2)				
		1	2	**MEAN**	**SD**	**CV (%)**
IC AC	1 eppendorf	0.24	0.244	**0.242**	0.003	1.17
	2 eppendorf	0.34	0.347	**0.344**	0.005	1.44
	3 eppendorf	0.227	0.223	**0.225**	0.003	1.26
					CV Mean	1.29
IC LMP	1 eppendorf	0.493	0.491	**0.492**	0.001	0.29
	2 eppendorf	0.493	0.496	**0.495**	0.002	0.43
	3 eppendorf	0.382	0.389	**0.386**	0.005	1.28
					CV Mean	0.67
VB AC	1 eppendorf	0.591	0.632	**0.612**	0.029	4.74
	2 eppendorf	0.429	0.439	**0.434**	0.007	1.63
	3 eppendorf	0.497	0.499	**0.498**	0.001	0.28
					CV Mean	2.22
VB LMP	1 eppendorf	0.581	0.584	**0.583**	0.002	0.36
	2 eppendorf	0.505	0.492	**0.499**	0.009	1.84
	3 eppendorf	0.551	0.557	**0.554**	0.004	0.77
					CV Mean	0.99
				Total CV mean		**1.29**

Table 38: Fourth donor, total GAG concentrations. GAG production in triplicates (µg/ml), SD and intra-donor CV within groups were calculated.

Total GAG (µg/ml)				
	IC AC	**IC LMP**	**VB AC**	**VB LMP**
1 eppendorf	41.115	104.825	135.406	128.016
2 eppendorf	67.108	105.590	90.044	106.609
3 eppendorf	36.782	77.812	106.354	120.625
MEAN	**48.335**	**96.075**	**110.601**	**118.417**
SD	16.402	15.821	22.977	10.873
CV (%)	33.934	16.468	20.775	9.182
Mean of Overall CV 20.09 %				

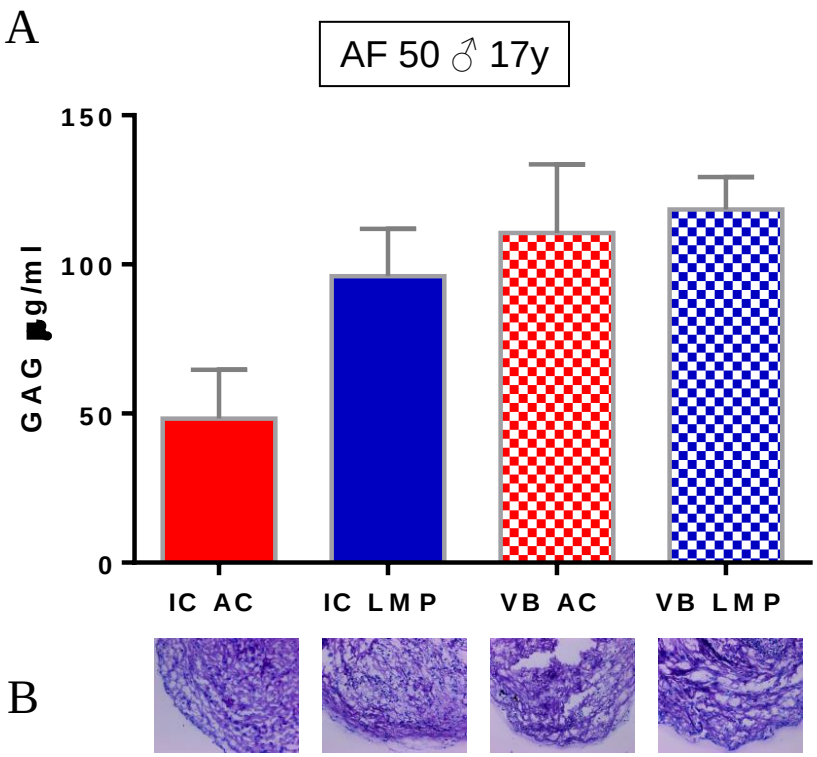


Figure 43: Four donor, comparison between GAG concentrations. A) GAG concentration was higher in VB, compared to IC, showing higher results from VB LMP, among all groups. B) GAG deposits stained with toluidine blue were similar with the spectrophotometric results. The text box indicates donor's details. A) Graphic presented as bars with mean and SD, B) Microphotography of chondrogenic pellet slides. Magnification x160.

4.9.3 Chondrogenesis, overall comparison

The overall analysis of 16 different groups of cultures from a cohort of 4 donors (3 males and 1 female), showed consistently higher GAG concentrations from VBBM-MSCs, compared to ICBM-MSCs (VBBM: AC 44.3μg/ml, LMP 43.5μg/ml, ICBM: AC 11.7μg/ml, LMP 22.8μg/ml) (Figure 44). In particular, GAG production from VB AC- and LMP-harvested MSCs was statistically higher, compared to IC AC- and LMP-harvested MSCs, by 3.8-fold (p=0.0005) and 1.9-fold (p=0.0030) difference, respectively.

Regarding ICBM, all four donors presented a consistently higher production of GAG from LMP-harvested MSCs by 1.9-fold difference (p=0.0005), compared to AC-harvested MSCs. Contrarily, VBBM donors showed an equal GAG production between AC and LMP-harvested MSCs, without any statistical difference.

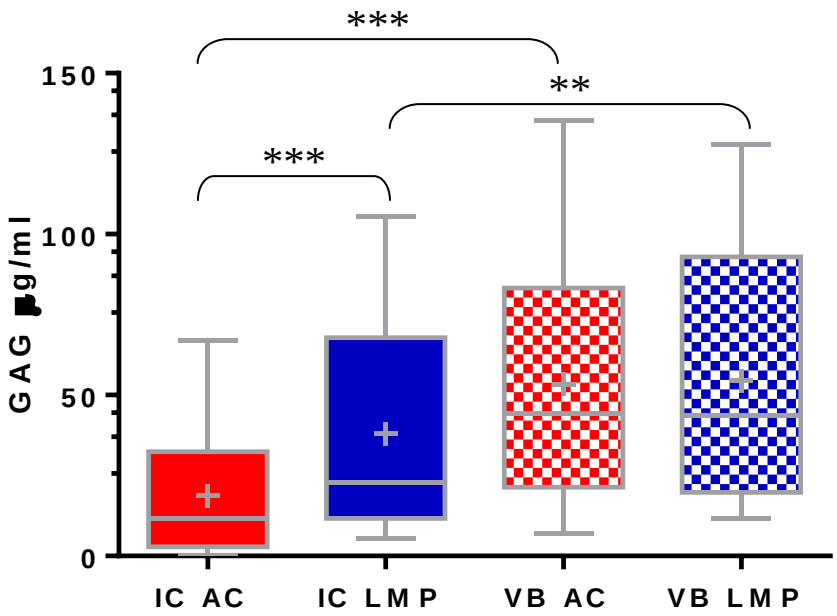


Figure 44: Summarized data from all 4 donors. Average values of total GAG production (μg/ml), showing statistical significant differences between IC LMP - IC AC (***, p=0.0005), IC LMP - VB LMP (**, p=0.0030), and IC AC - VB AC (***, p=0.0005). Values processed with Wilcoxon T test and are represented as Box & Whiskers graphic. Mean and median are illustrated as "+" and "bar" symbols, respectively.

4.10 Tri-lineage differentiation: Adipogenesis

4.10.1 Adipogenesis, introduction

Following 21 days of adipogenic induction (Figure 45), adipogenesis was compared between the different groups, using Oil red-O and Nile red/DAPI staining.
Oil red-O dye binds lipids present in cells and represents one of the most commonly used staining method for adipogenically-differentiated MSCs[227,228] (Figure 46). Additionally, this method can be used to assess the differentiation of MSCs through a semi-quantitative grading system[227] (Figure 47).

Moreover, Nile red and DAPI staining, combined together in the same well, provide a series of advantages for the adipogenesis study. Firstly, it allows the spectrophotometric measurement of fat content (Nile red). Secondly, DAPI staining of the nuclei, provides information on the cells' proliferation rate during the induction period. Moreover, the combination of two dyes allows to determine the relationship between proliferation and differentiation rate, calculated by dividing DAPI by Nile red fluorescence.

Finally, the spectrophotometric results were compared to fluorescent images of the same cultures. This offered a visual verification of the fat content (Figure 48). In this manner, further information could be gathered from the analysis of the fat droplets distribution per cell and their levels of fluorescence. For example, it allowed to distinguish between cultures presenting equal fluorescence, but with a different pattern of fat distribution.

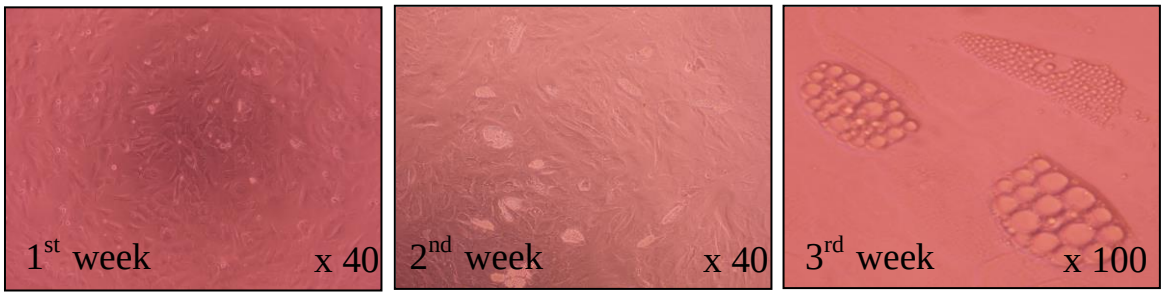


Figure 45: Progress of adipogenic differentiation of MSCs over a 21 days period. Fat droplets begin to be visible from the second week onwards. In the third week, fat content tends to cover a larger area of the cytoplasm. For the same time-course of adipogenic induction, were noticed cells presenting different size of fat droplets (3rd week). Images illustrated are from donor AF28 IC LMP before staining.

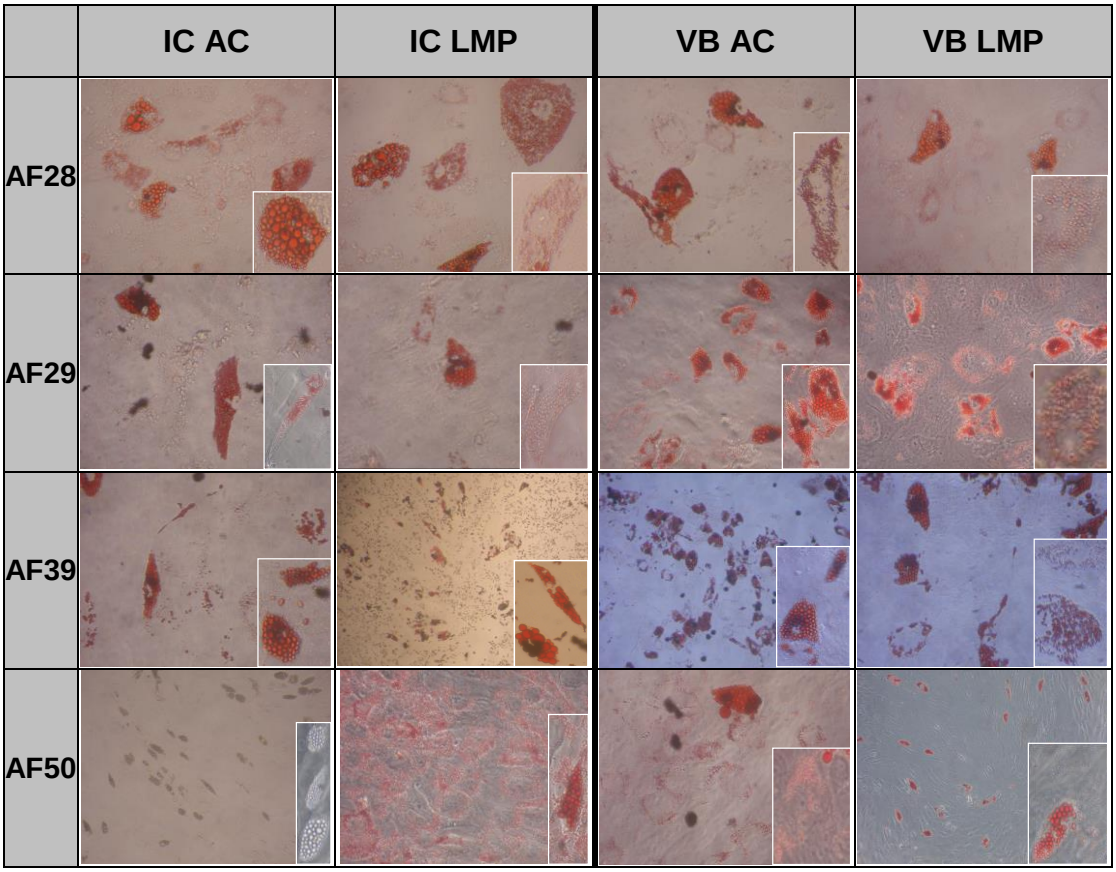


Figure 46: Oil red-O staining of MSCs. Representative images of MSCs from all four donors following 21 days of adipogenic induction. Intracellular fat droplets stained red for all cultures, except AF50 IC AC where dye didn't bind the fat content of the droplets, which however it is still visible. The bigger and more uniform size of fat droplets is evident in IC AC compared to IC LMP which appear to be slightly smaller. MSCs from VB AC presented a bigger variety of fat droplet sizes containing prevalently medium size droplets, while VB LMP presented mainly smaller size fat droplets. Images were taken using an Olympus inverted microscope in x40 and x200 (inserts) magnifications.

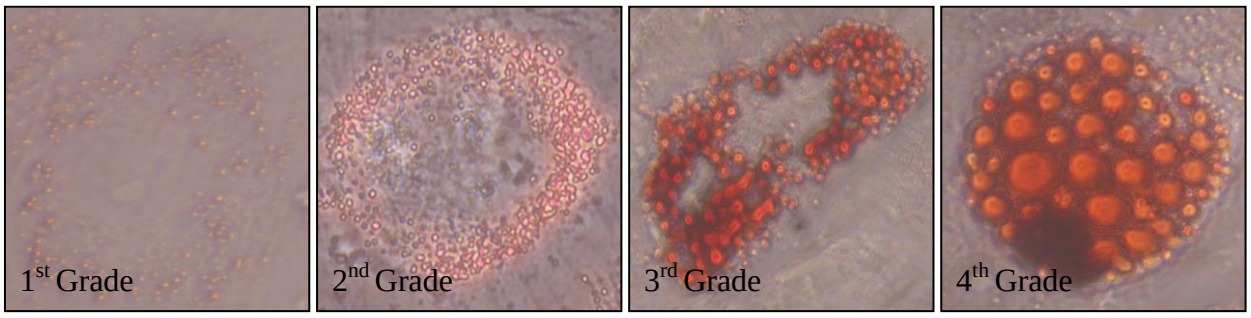


Figure 47: Semi-quantitative scoring system of adipogenic differentiated MSCs' using Oil red-O staining. MSCs were counted and scored in 3 different areas of the well, and ranked into four grades, based on their fat content; Grade 1 (0-24%), Grade 2 (25-49%), Grade 3 (50-74%), and Grade 4 (75-100%). Magnification x200.

From the morphologic point of view, all cultures showed heterogeneous distribution of the differentiated MSCs within the well (Figures 46, 48). Moreover, differences between the fat droplets' size and their distribution within the cells were observed. Furthermore, there were dissimilar differentiation patterns, as well as the failure of differentiation of a subset of MSCs, within the same well.

In particular, regardless the staining technique used, IC AC presented the biggest size of fat droplets with the most uniform distribution within the MSCs. Additionally, the differentiation rate was almost uniform within the culture. VB AC presented a smaller size droplets, compared to IC AC, and slightly more size variety of fat droplets within the MSCs, with an almost uniform differentiation pattern within the culture. Both IC LMP and VB LMP, presented smaller fat droplets (compared to IC AC and VB AC) with a more variable fat droplet size within the same cell and a less uniform differentiation pattern within the culture. VB LMP showed a smaller size of fat droplets within the cells, compared to VB AC. Generally, LMP-harvested MSCs presented a smaller size of fat droplets, which frequently gave a "gold dust" like appearance, especially under fluorescent microscopy (Figure 49).

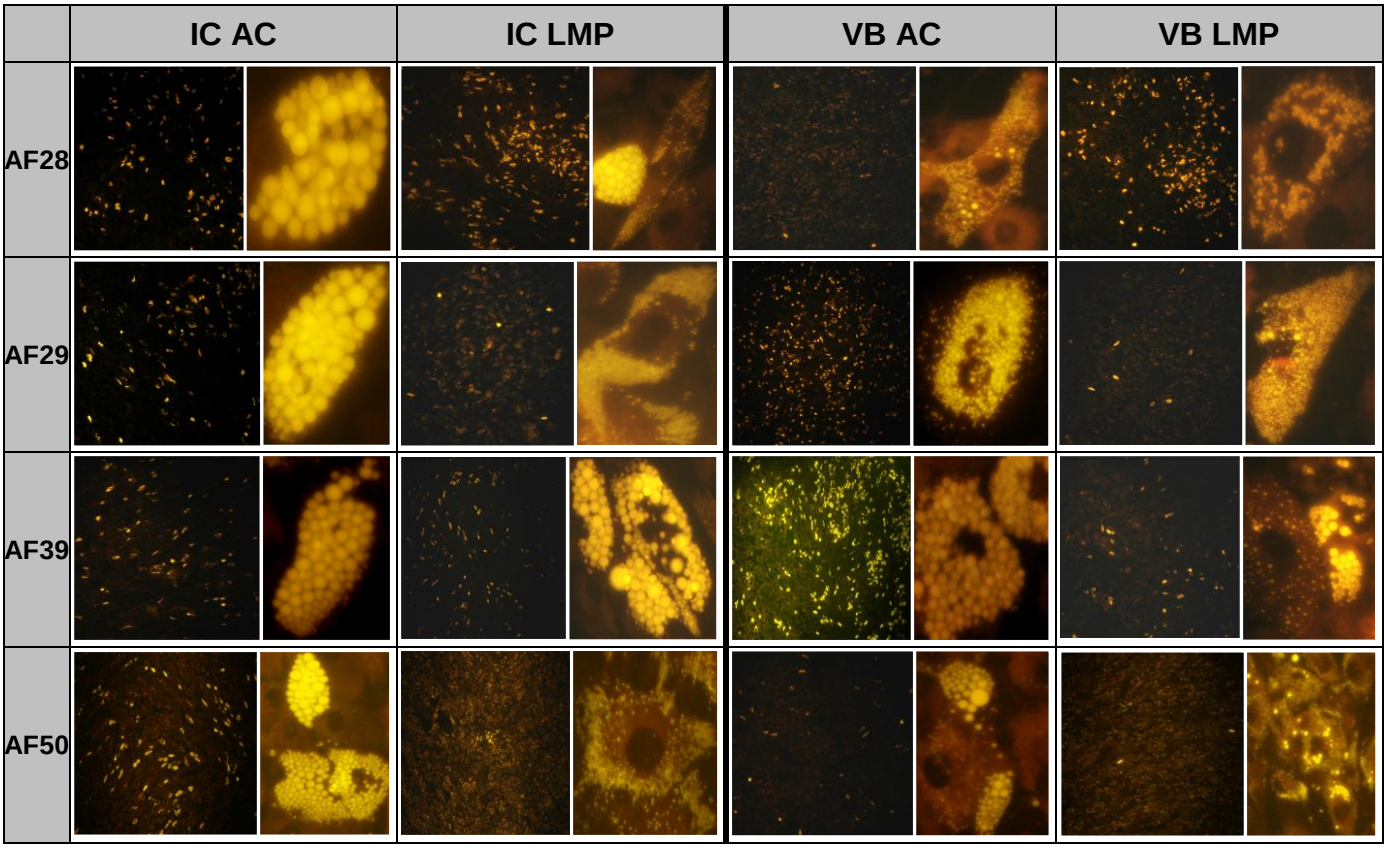


Figure 48: Nile red staining of MSCs under florescence microscopy. Images of differentiated MSCs from all four donors following 21 days of adipogenic induction. Intracellular fat droplets are stained in yellow/gold, and the MSCs' nucleus appear as a dark brown spheroid structure. The bigger and more uniform size of fat droplets is evident in IC AC, followed by VB AC-harvested MSCs, which presented a more varied pattern, with prevalence of medium size fat droplets. Both IC LMP- and VB LMP-harvested MSCs presented prevalently smaller size fat droplets, appearing like "gold dust". Images were taken using an Olympus inverted microscope in x40 and x200 magnifications.

In terms of the staining procedures, all cultures stained positively with Oil red-O and Nile red/DAPI dyes. However, AF50 IC AC culture failed to bind the Oil red-O dye, despite the presence of big intracellular fat droplets visible under microscope even without staining (Figure 46). As it was expected, donor-to-donor variability was also observed. This is a constant characteristic among similar studies and it is widely reported in the literature[51].

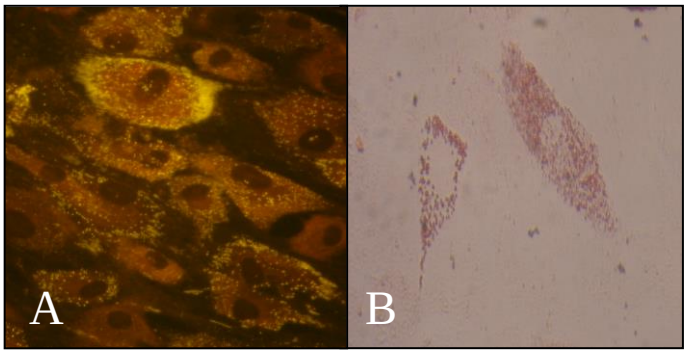


Figure 49: Same type of fine cytoplasmic lipid droplets in MSCs stained with Nile red (A), and Oil red-O (B). A) Characteristic gold dust like appearance of small fat droplets observed mainly in cultures of LMP-harvested MSCs. The nucleus of MSCs appear as dark brown colour. B) LMP-harvested MSCs from the same donor, stained with Oil red-O. Images illustrated are from the same donor (AF 28 IC LMP) and taken using an Olympus inverted microscope at x200 magnification.

Validation of adipogenic assay performance

The variability within the adipogenic assays (Nile red/DAPI duplicates), was assessed to establish its accuracy. The CV within the duplicates was calculated separately for Nile red and DAPI based on the spectrophotometric analysis. The cut-off point of optical density was established at ≤ 10%. Additionally, ratios between Nile red and DAPI were analyzed. The results were also compared to semi-quantitative scoring system of MSCs stained with Oil red-O dye (performed in single well).

Semi-quantitative scoring system for adipogenic differentiated MSCs using Oil red-O staining

For this grading system, 500 differentiated MSCs were measured in a dish following Oil red-O staining, as previously described[227]. The level of adipogenic progression was next ranked between grades 1-4, (Figures 46, 47, Table 39) based on the percentage of fat droplets occupying the cytoplasm of mature adipocytes (grades 2-4) using the following formula:
[# cells (grade 2) + # cells (grade 3) + # cells (grade 4)/500] x 100 = % of mature fat cells.

Correlations between Nile red spectrophotometry and Oil red-O semi-quantitative scoring system were performed. However, given the similar percentages of adipogenesis from all donors (average 88%, range 80%-96%), it was not possible to show any correlation between semi-quantitative scoring system and Nile red absorbance, except for VB LMP (Table 39, Figure 52).

Grading adipocytes it was complex and subjective, because equally graded MSCs can contain different sizes of fat droplets within the cells, which is not accounted by the semi-quantitative scoring system (Figure 50). Additionally the heterogeneous growth pattern within the same culture, increased the degree of complexity of this method (Figure 51).

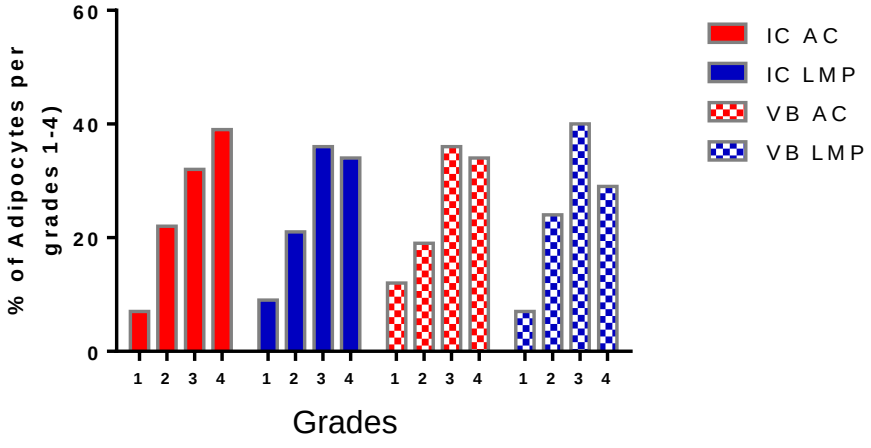
Both semi-quantitative scoring and Nile red absorbance methods could be complementary, to a certain extent, offering important morphologic information. However, Nile red in combination with DAPI staining (microscopic observation and spectrophotometric reading), is faster, consistent and not operator dependent method, hence can be easily reproduced and is comparable between different studies.

Table 39: Semi-quantitative scoring system. Percentages of MSCs' fat content, based on the fat accumulation within the cytoplasm. A) Individual analysis per donor showing the different percentages of fat content per grade. Grades 2-4, represents the mature population of fat-cells within the cultures. Their sum was calculated following 21 days of induction. MSCs from all donors presented an average of 88% (80%-96%) of fat content. B) Summary of overall grade distribution, from all four donors, is illustrated as a bar graphic.

A

AF 28	IC AC	IC LMP	VB AC	VB LMP		**AF 29**	IC AC	IC LMP	VB AC	VB LMP
Grade 1	10	10	20	10		Grade 1	5	10	10	7
Grade 2	10	5	14	30		Grade 2	38	35	20	27
Grade 3	30	30	46	45		Grade 3	43	45	30	47
Grade 4	50	55	20	15		Grade 4	15	10	40	20
Grades 2+3+4	**90**	**90**	**80**	**90**		**Grades 2+3+4**	**96**	**90**	**90**	**94**
AF 39	IC AC	IC LMP	VB AC	VB LMP		**AF 50**	IC AC	IC LMP	VB AC	VB LMP
Grade 1	4	11	4	5		Grade 1	10	5	14	6
Grade 2	20	22	20	20		Grade 2	20	20	20	20
Grade 3	30	44	26	33		Grade 3	24	25	40	34
Grade 4	46	22	50	43		Grade 4	46	50	26	40
Grades 2+3+4	**96**	**88**	**96**	**96**		**Grades 2+3+4**	**90**	**95**	**86**	**94**

B



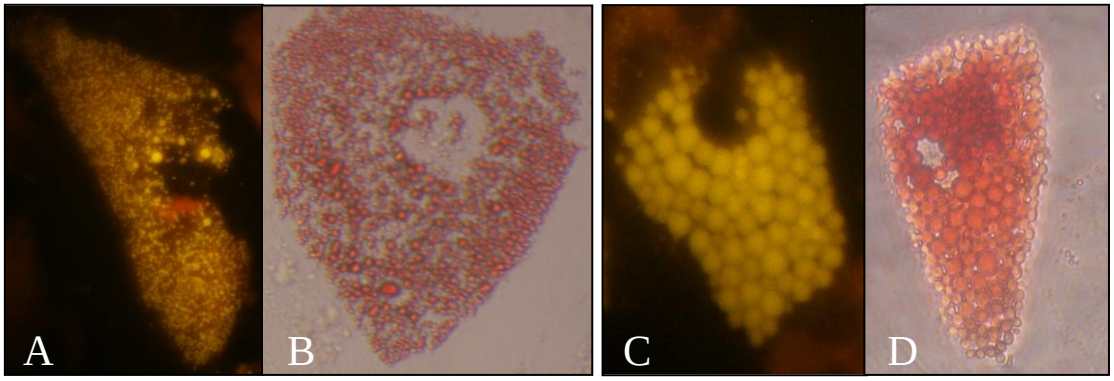


Figure 50: Differences in equally scored adipogenically differentiated MSCs (Grade 4), over the same 21 days induction period. A, B) MSCs from donor (AF28 IC LMP) containing small size fat droplets, mainly associated with LMP-harvested MSCs. C, D) MSCs from donor (AF39 VB AC) containing big size fat droplets, mainly associated with AC-harvested MSCs. The nucleus is visible in all cases as dark brown (A, C) and as spheroid area without fat droplets (B, D). Staining used: Nile red (A, C) and Oil red-O (B, D). Olympus inverted microscope was used at x200 magnification.

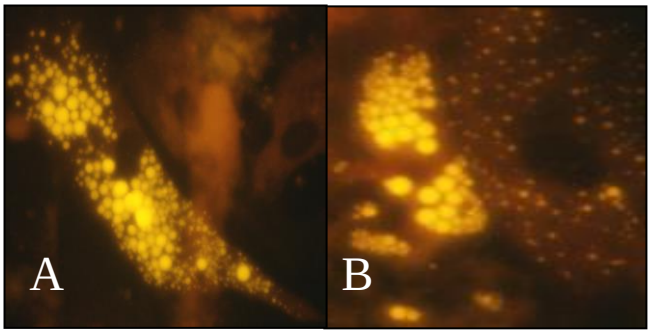


Figure 51: Heterogenic aspect of adipogenesis, using Nile red staining. A) MSCs containing different size of fat droplets within the same cell, were observed across all donors. B) In all four donors, some MSCs were progressing to Grades 3-4, whilst others were remaining at Grades 1-2 within the same culture. Images illustrated are from donors AF50 IC AC (A) and AF39 VB LMP (B). Olympus inverted microscope was used at x200 magnification.

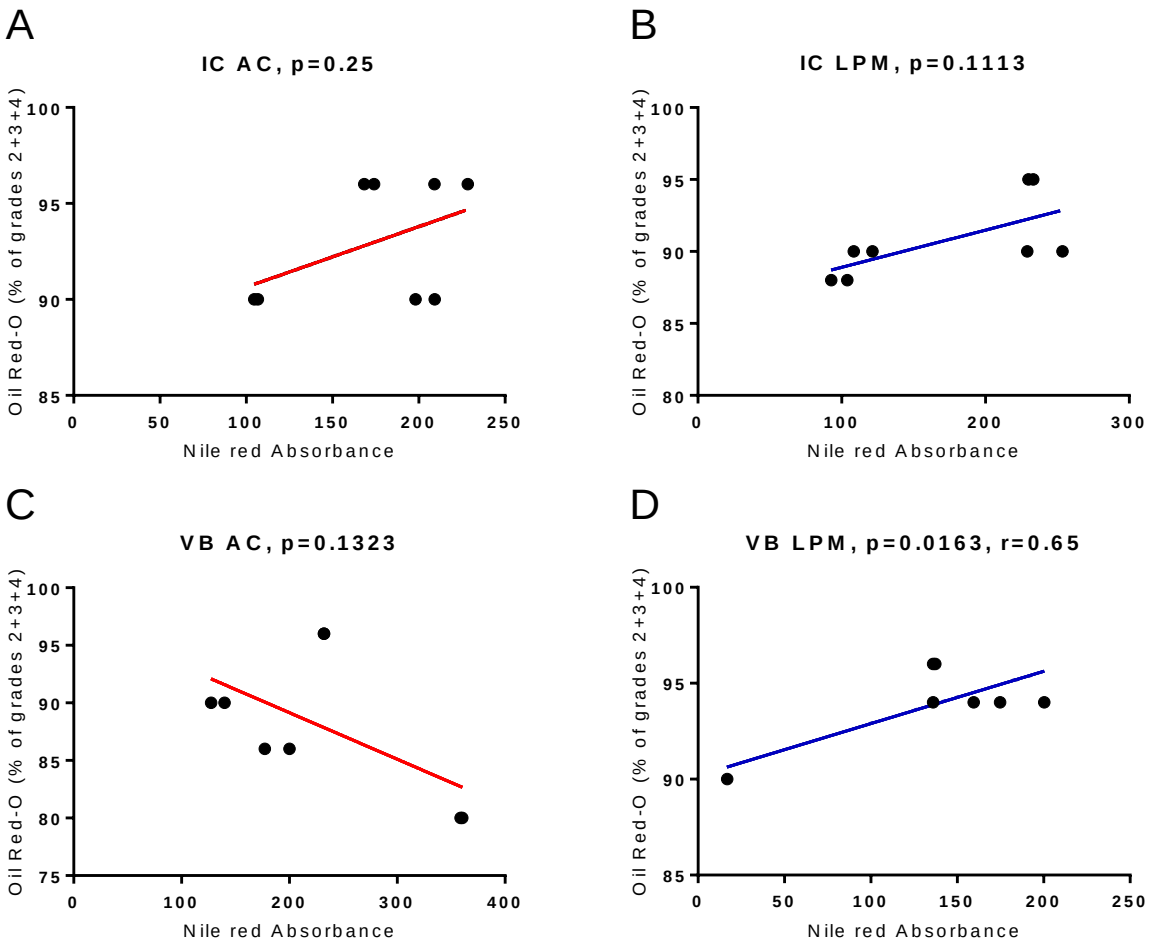


Figure 52: Correlation graphics between the Nile red absorbance and the sum of grades 2-4 of the semi-quantitative scoring system. Values were analysed per group (IC AC, IC LMP, VB AC, VB LMP). A, B, C), showed no statistically significant correlation, with the only exception of D), which was statistically significant.

4.10.2 Individual analysis of each donor

First donor, AF 28, (14.3y, M).

The overall CVs of the duplicates, following spectrophotometry (OD) for Nile red and DAPI were 6.8% and 6.5% respectively, below the cut-off point of 10%, showing satisfactory intra-assay accuracy (Table 40).

The first donor exhibited the highest Nile red OD (differentiation) from IC LMP, followed by IC AC, VB LMP and VB AC with 1.4-fold, 1.6-fold and 1.8-fold difference, respectively. Considering the 6.8% of the overall CV, the differences between the values of IC AC, and VB LMP were above the experimental variability (Table 40).

Also DAPI showed a higher OD (proliferation) from IC LMP, followed by VB LMP, VB AC and IC AC, with 1.2-fold, 1.2-fold and 2-fold difference, respectively. Similarly to Nile red, considering the 6.5% of the overall CV, the differences of IC AC, VB AC, and VB LMP, with respect to IC LMP, were above the experimental variability (Table 40).

Comparing Nile red/DAPI ratios (IC AC 0.3, IC LMP 0.21, VB AC 0.14, VB LMP 0.16), IC AC-harvested MSCs presented the highest adipogenic differentiation/cell, compared to IC LMP, VB LPM and VB AC by 1.4-fold, 1.9-fold and 2.1-fold difference, respectively. Overall, this donor presented a higher adipogenesis/cell from ICBM (Figure 53).

Table 40: First donor, variability between duplicates of adipogenenic assay. ODs in duplicates, were calculated from a 9 spot reading (3x3), for Nile red and DAPI. Mean, SD and CV were calculated per single condition.

AF 28	**Nile red,** Optical density in 48well-plate (x2)				
	1st well	2nd well	**MEAN**	**SD**	**CV (%)**
IC AC	168.44	174.11	**171.28**	4.01	2.34
IC LMP	229.11	253.56	**241.33**	17.28	7.16
VB AC	127.44	139.78	**133.61**	8.72	6.53
VB LMP	136.00	159.56	**147.78**	16.66	11.27
			Total CV Mean		**6.82**
AF 28	**DAPI,** Optical density in 48well-plate (x2)				
	1st well	2nd well	**MEAN**	**SD**	**CV (%)**
IC AC	611.00	533.33	**572.17**	54.92	9.60
IC LMP	1174.00	1150.11	**1162.06**	16.89	1.45
VB AC	890.00	969.78	**929.89**	56.41	6.07
VB LMP	883.11	1004.00	**943.56**	85.48	9.06
			Total CV Mean		**6.54**

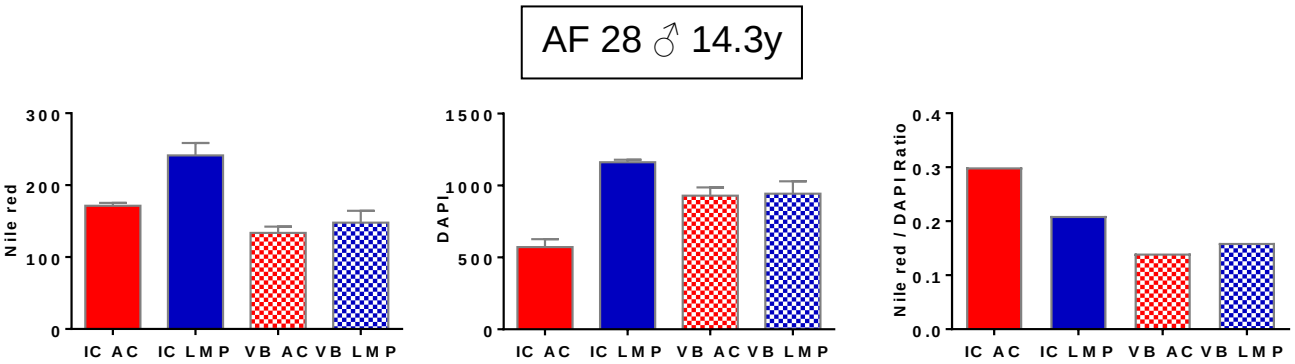


Figure 53: First donor, optical absorbance of Nile red, DAPI and their ratio. Levels of fluorescence were determined, at 21 days of adipogenic induction, calculating the difference of spectrophotometric measurements between pre and post Nile red and DAPI staining. Nile red/DAPI ratios demonstrated a higher adipogenesis/cell for both IC AC and IC LMP by 2.1-fold and 1.9-fold, compared to VB AC and VB LMP, respectively. The text box indicates donor's details. Column-bars graphic shows mean values with SD.

Second donor, AF 29, (15.6y, M).

In this case also, the overall CVs of the duplicates for Nile red and DAPI, following spectrophotometry (OD) were 2.6% and 6.6%, respectively, below the cut-off point of 10%, showing satisfactory intra-assay accuracy (Table 41).

The second donor exhibited the highest Nile red OD (differentiation) from VB AC, compared to IC LMP, IC AC and VB LMP by 3.1-fold, 3.4-fold and 21.4-fold difference respectively. Considering the 2.6% of the overall CV, the differences of IC LMP, IC AC, and VB LMP, were all above the experimental variability (Table 41).

Similarly, DAPI OD (proliferation) was higher from VB AC, compared to IC LMP, IC AC and VB LMP by 2.1-fold, 3-fold and 4.2-fold difference, respectively. Considering the 6.6% of the overall CV, also in this case, the differences of between the values of IC LMP, IC AC, and VB LMP were all above the experimental variability (Table 41).

Comparing Nile red/DAPI ratios, (IC AC 0.22, IC LMP 0.17, VB AC 0.25, VB LMP 0.05) VB AC-harvested MSCs presented the highest adipogenic differentiation/cell, compared to IC AC, IC LMP and VB LMP by 1.1-fold, 1.5-fold and 5-fold difference, respectively. Overall, this donor presented a consistent adipogenic outcome from ICBM with both harvesting methods, whereas VBBM exhibited a higher adipogenesis/cell, but only from AC-harvested MSCs (Figure 54).

Table 41: Second donor, variability between duplicates of adipogenic assay. ODs in duplicates, were calculated from a 9 spot reading (3x3), for Nile red and DAPI. Mean, SD and CV were calculated per single condition.

AF 29	**Nile red,** Optical density in 48well-plate (x2)				
	1st well	2nd well	**MEAN**	**SD**	**CV (%)**
IC AC	106.78	104.67	**105.72**	1.49	1.41
IC LMP	108.44	121.44	**114.94**	9.19	8.00
VB AC	358.67	360.33	**359.50**	1.18	0.33
VB LMP	16.89	16.78	**16.83**	0.08	0.47
			Total CV Mean		**2.55**
AF 29	**DAPI,** Optical density in 48well-plate (x2)				
	1st well	2nd well	**MEAN**	**SD**	**CV (%)**
IC AC	511.00	441.78	**476.39**	48.95	10.27
IC LMP	634.67	723.00	**678.83**	62.46	9.20
VB AC	1385.00	1486.22	**1435.61**	71.57	4.99
VB LMP	334.33	343.67	**339.00**	6.60	1.95
			Total CV Mean		**6.60**

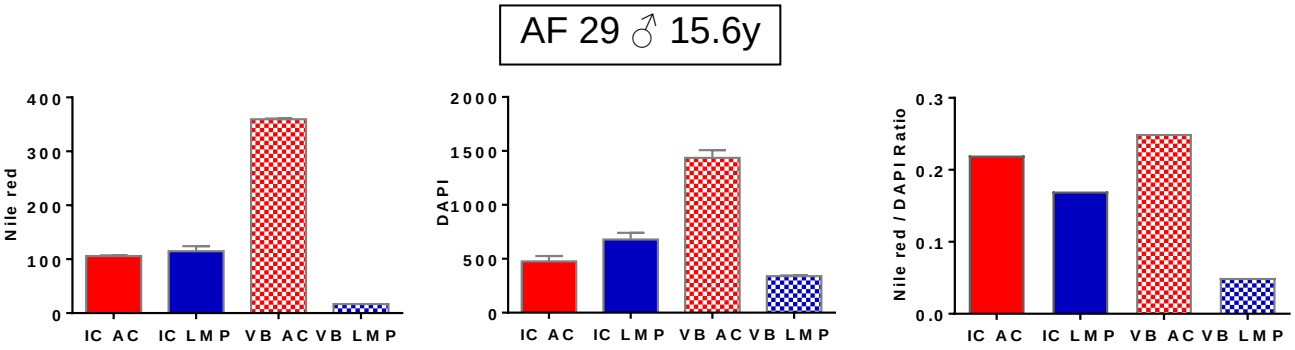


Figure 54: Second donor, optical absorbance of Nile red, DAPI and their ratio. Levels of fluorescence were determined, at 21 days of adipogenic induction, calculating the difference of spectrophotometric measurements between pre and post Nile red and DAPI staining. Nile red/DAPI ratios demonstrated a higher adipogenesis/cell for both IC AC and VB AC, compared to IC LMP and VB LMP, by 1.3-fold and 5-fold deference, respectively. The text box indicates donor's details. Column-bars graphic shows mean values with SD.

Third donor, AF 39, (15.3y, F).

Similarly to the other two donors, in this case the overall CVs of the duplicates for Nile red and DAPI, following spectrophotometry (OD) were 3.7% and 9.6%, respectively, below the cut-off point of 10%, showing satisfactory intra-assay accuracy (Table 42).

The third donor exhibited the highest Nile red OD (differentiation) from VB AC followed by IC AC, VB LMP and IC LMP with 1.1-fold, 1.7-fold and 2.4-fold difference, respectively. Considering the 3.7% of the overall CV, the differences between the values of IC LMP, IC AC, and VB LMP were all above the experimental variability (Table 42).

Similarly, DAPI OD (proliferation) was higher from VB AC followed by IC LMP, IC AC and VB LMP with 1.8-fold, 1.8-fold and 2.1-fold difference, respectively. Considering the 9.6% of the overall CV, the differences of IC LMP, IC AC, and VB LMP, with respect to VB AC, were all above the experimental variability. However, the difference between IC AC and VB LMP, were within the experimental variability (Table 42).

Comparing Nile red/DAPI ratios (IC AC 0.25, IC LMP 0.11, VB AC 0.15 VB LMP 0.11), IC AC-harvested MSCs presented the highest adipogenic differentiation/cell, compared to VB LMP, VB AC and IC LMP by 1.4-fold, 1.7-fold and 2.3-fold difference, respectively. Overall, this donor exhibited the highest adipogenesis/cell from IC AC (Figure 55).

Table 42: Third donor, variability between duplicates of adipogenic assay. ODs in duplicates, were calculated from a 9 spot reading (3x3), for Nile red and DAPI. Mean, SD and CV were calculated per single condition.

AF 39	**Nile red,** Optical density in 48well-plate (x2)				
	1st well	2nd well	**MEAN**	**SD**	**CV (%)**
IC AC	209.11	228.44	**218.78**	13.67	6.25
IC LMP	92.78	103.89	**98.33**	7.86	7.99
VB AC	232.11	232.22	**232.17**	0.08	0.03
VB LMP	137.33	136.00	**136.67**	0.94	0.69
			Total CV Mean		**3.74**
AF 39	**DAPI,** Optical density in 48well-plate (x2)				
	1st well	2nd well	**MEAN**	**SD**	**CV (%)**
IC AC	919.78	806.11	**862.94**	80.37	9.31
IC LMP	814.67	943.67	**879.17**	91.22	10.38
VB AC	1461.78	1678.22	**1570.00**	153.05	9.75
VB LMP	790.56	696.67	**743.61**	66.39	8.93
			Total CV Mean		**9.59**

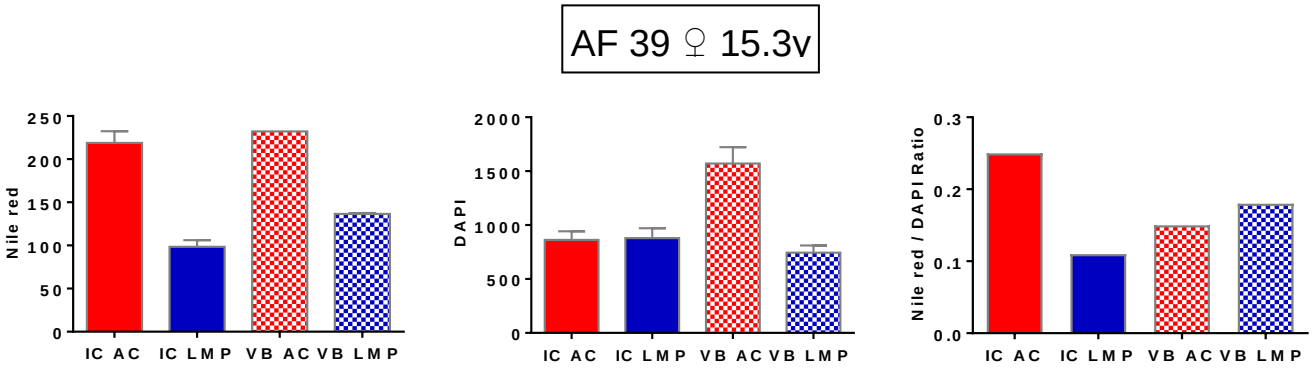


Figure 55: Third donor, optical absorbance of Nile red, DAPI and their ratio. Levels of fluorescence were determined, at 21 days of adipogenic induction, calculating the difference of spectrophotometric measurements between pre and post Nile red and DAPI staining. Nile red/DAPI ratios demonstrated a higher adipogenic differentiation/cell from IC AC, compared to VB LMP, VB AC and IC LMP by 1.4-fold, 1.7-fold and 2.3-fold, respectively. The text box indicates donor's details. Column-bars graphic shows mean values with SD.

Forth donor, AF 50, (17y, M).

In the fourth donor, the overall CVs of the duplicates, following spectrophotometry (OD) for Nile red and DAPI were 5.8% and 5.4% respectively, below the cut-off point of 10%, showing satisfactory intra-assay accuracy (Table 43).

The last donor exhibited similar Nile red OD (differentiation) from both anatomic regions, presenting a slightly higher outcome from ICBM-harvested MSCs. In particular IC LMP presented the highest outcome followed by IC AC, VB AC and VB LMP with 1.2-fold difference, in each case. Moreover, considering the 5.8% of the overall CV, the differences of IC AC, VB AC, and VB LMP, with respect to IC LMP, were above the experimental variability. However, the differences between IC AC, VB AC, and VB LMP, were within the experimental variability (Table 43).

Likewise, DAPI OD (proliferation) presented similar outcomes from both anatomic sites, however, VB LMP showed slightly higher values, compared to VB AC, IC AC and IC LMP, by 1.2-fold, 1.3-fold and 1.3-fold difference, respectively. Considering the 5.4% of the overall CV, the differences of VB AC, IC AC and IC LMP, with respect to VB LMP, were above the experimental variability. However also in this case, the differences between VB AC, IC AC and IC LMP, were within the experimental variability (Table 43).

Comparing Nile red/DAPI ratios, (IC AC 0.15, IC LMP 0.17, VB AC 0.13, VB LMP 0.11) IC LMP-harvested MSCs presented the highest values of adipogenesis/cell compared to IC AC, VB AC and VB LMP, by 1.1-fold, 1.3-fold and 1.6-fold difference, respectively. Overall this donor exhibited the highest adipogenesis/cell from ICBM, for both harvesting methods used (Figure 56).

Table 43: Forth donor, variability between duplicates of adipogenic assay. ODs in duplicates, were calculated from a 9 spot reading (3x3), for Nile red and DAPI. Mean, SD and CV were calculated per single condition.

AF 50	**Nile red,** Optical density in 48well-plate (x2)				
	1st well	2nd well	**MEAN**	**SD**	**CV (%)**
IC AC	198.11	209.22	**203.67**	7.86	3.86
IC LMP	233.33	229.89	**231.61**	2.44	1.05
VB AC	200.22	177.33	**188.78**	16.18	8.57
VB LMP	174.78	200.33	**187.56**	18.07	9.63
			Total CV Mean		**5.78**

AF 50	**DAPI,** Optical density in 48well-plate (x2)				
	1st well	2nd well	**MEAN**	**SD**	**CV (%)**
IC AC	1278.89	1414.22	**1346.56**	95.70	7.11
IC LMP	1284.89	1462.00	**1373.44**	125.24	9.12
VB AC	1479.22	1485.67	**1482.44**	4.56	0.31
VB LMP	1722.00	1848.67	**1785.33**	89.57	5.02
			Total CV Mean		**5.39**

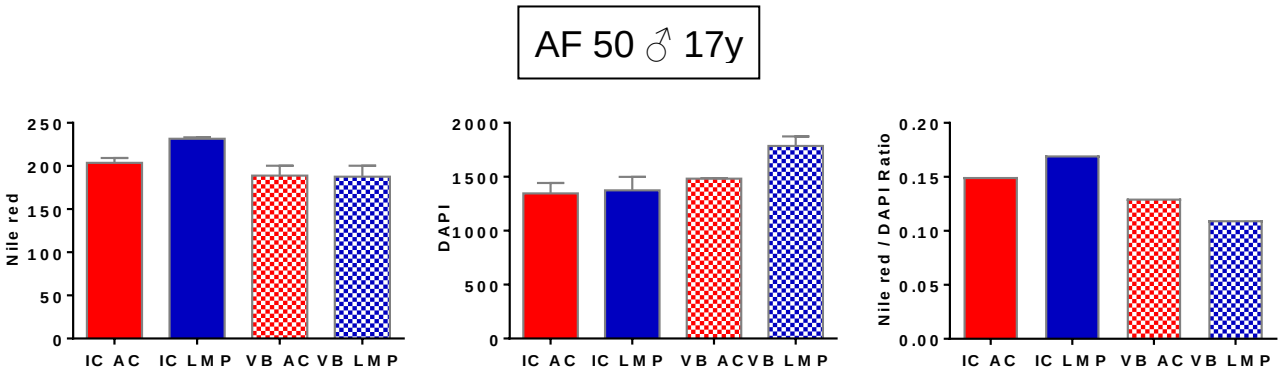


Figure 56: Forth donor, optical absorbance of Nile red, DAPI and their ratio. Levels of fluorescence were determined, at 21 days of adipogenic induction, calculating the difference of spectrophotometric measurements between pre and post Nile red and DAPI staining. Nile red/DAPI ratios showed a higher adipogenic differentiation/cell for both IC LMP and IC AC by 1.6-fold and 1.2-fold, compared to VB LMP and VB AC, respectively. The text box indicates donor's details. Column-bars graphic shows mean values with SD.

4.10.3 Adipogenesis, overall comparison

Regarding Nile red OD, donors AF29 and AF39 presented the highest OD from VBBM, while donors AF28 and AF50 presented the highest OD from ICBM. In terms of DAPI OD three donors (AF29, AF39, AF50) presented the highest OD from VBBM and only donor AF28 showed high OD from ICBM. The overall performance of all four donors is illustrated in Figure 57.

The main conclusions from the summary of Nile red and DAPI ODs, at 21 days post-induction, were related mainly to DAPI OD showing a statistically significant higher outcome from VB AC compared to IC AC-harvested MSCs ($p=0.0078$). Moreover, IC LMP showed a statistically significant result, compared to IC AC ($p=0.039$). With regards to Nile red OD, no statistically significant results were observed, apart from the trend of IC LMP to show higher adipogenesis by 1.3-fold difference, compared to VB LMP (Figure 57).

The overall Nile red/DAPI ratios, (IC AC 0.24, IC LMP 0.17, VB AC 0.15, VB LMP 0.14), showed a superior adipogenic differentiation/cell from IC with both harvesting techniques. In particular adipogenesis/cell from IC AC presented the highest values compared to VB AC, IC AC and IC LMP, however was statistically significant only compared to VB LMP ($p=0.039$). Concluding, overall ICBM, exhibited a higher adipogenesis/cell, compared to VBBM (Figure 57).

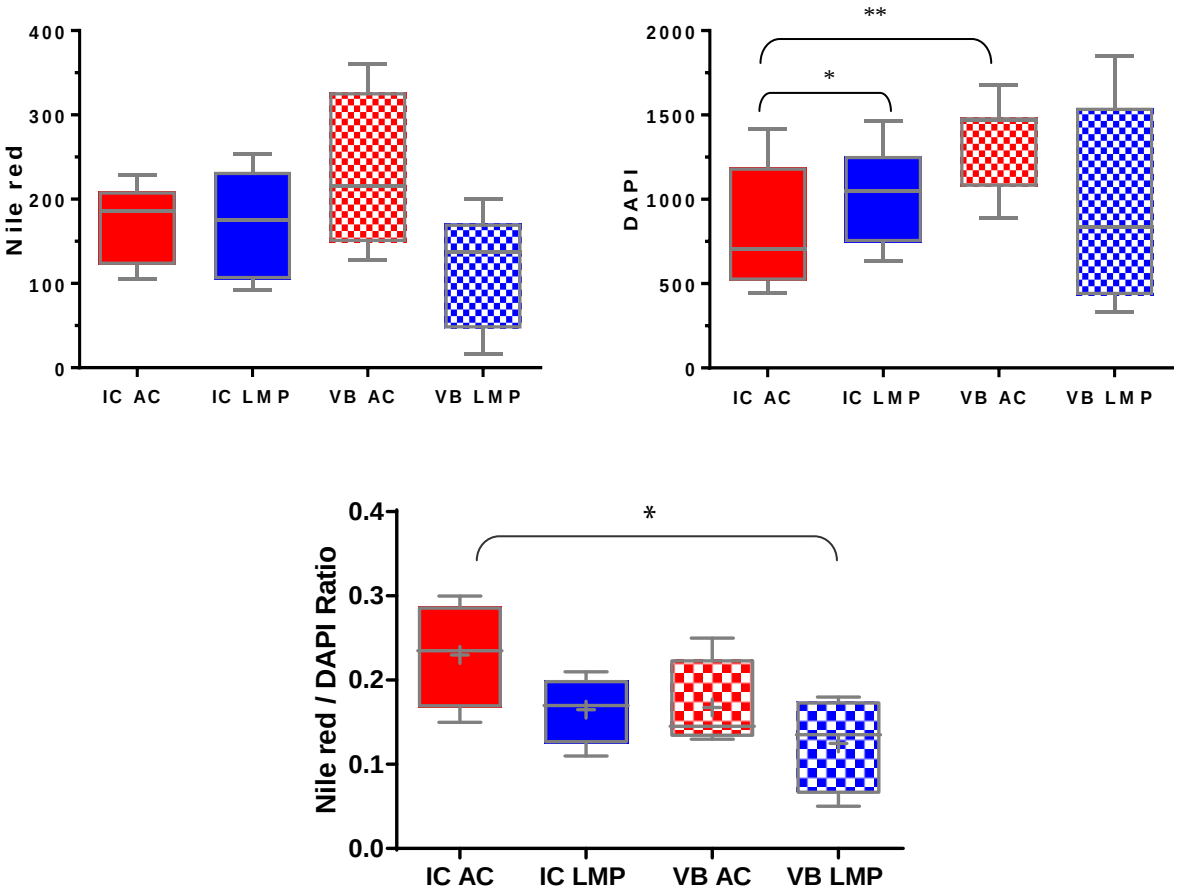


Figure 57: Summarized data from all 4 donors. Nile red and DAPI ODs and their ratios. Average values of OD from Nile red and DAPI, showed a statistically significant difference, in predisposition to proliferation (DAPI), between VB AC- and IC AC-harvested MSCs (**, p=0.0078) and for IC LMP, compared to IC AC (*, p=0.039). Nile red/DAPI ratios demonstrated a higher adipogenesis/cell from IC AC, which was statistically significant, compared to VB LMP (*, p=0.039). Values were processed with Wilcoxon T test and represented as Box & Whiskers graphics. Mean and median are illustrated as "+" and "bar" symbols, respectively.

4.11 ELISA assays

4.11.1 Introduction

The purpose of this chapter was to quantify the serum concentrations and examine the differences in the pro-inflammatory cytokines (IL-6, IL-18, PCT), anti-inflammatory cytokine (IL-10), and biochemical parameters (leucocytes, lymphocytes, neutrophils, platelets, prothrombin time, partial thromboplastin time, and haemoglobin) following AIS, over a week period from surgery and among three different approaches (antero-posterior, posterior with costoplasty and posterior). Furthermore, it was analysed the impact of the duration of surgery and gender on the 4 cytokines over one week period in each different approach, as well as the differences on the biochemical parameters among the three different approaches during the same time period.

Validation of the ELISA assay performance

The ELISA assays were performed in duplicates throughout all the experiments, using 4 kits for every single cytokine tested, for a total of 16x96-well-plates (1.264 readings). Given the high number of tests performed and the use of different ELISA kits per single molecule, intra-assay and inter-assay coefficient of variation (CV) were assessed to establish kits' accuracy. CV is defined as the ratio of the standard deviation (SD) to the mean, which is then multiplied by 100 to allow expression as a percentage. Intra- and inter-assay CVs were calculated at two different stages: 1) between the known values of the standard curves, to assess whether kits are performing similarly and 2) between the OD of the patients' samples examined, as showed in Tables 46,48,50,52.

Cut-off points per assay were established by manufactures as follows: IL-6 high sensitivity (intra-assay CV 7%, inter-assay CV 10%), IL-6 (intra-assay CV 4.5%, inter-assay CV 6.5%), IL-10 (intra-assay CV 5%, inter-assay CV 7.5%), IL-18 (intra-assay CV 11%, inter-assay CV 10%), PCT (intra-assay CV 10%, inter-assay CV 12%) (Appendix 8.).

ELISA assay performance (spectrophotometry)

Standard curves were generated as per manufacturer instructions (Appendix 8.2). Best fit curves were created for all ELISA kits, showing consistent results across the different kits testing for the same molecule. Data were processed with GraphPad Prism software and a four parameter logistic (4-PL) curve was generated for IL-6, IL-10, IL-18. Due to limitations of GraphPad Prism functions, the 4-PL model was working better by plotting the OD on the x-axis against the concentration on the y-axis. Contrarily for PCT, the best fit line was determined linearizing the data by plotting the log of the OD on the y-axis, against the log of the concentrations on the x-axis. Following 1.264 readings, the optical densities were analyzed and the overall and intra- and inter-experimental CVs were 5.4% for both, showing satisfactory performance (Figure 58). Furthermore, for the diluted samples the concentration, determined from the standard curve, was multiplied by the dilution factor.

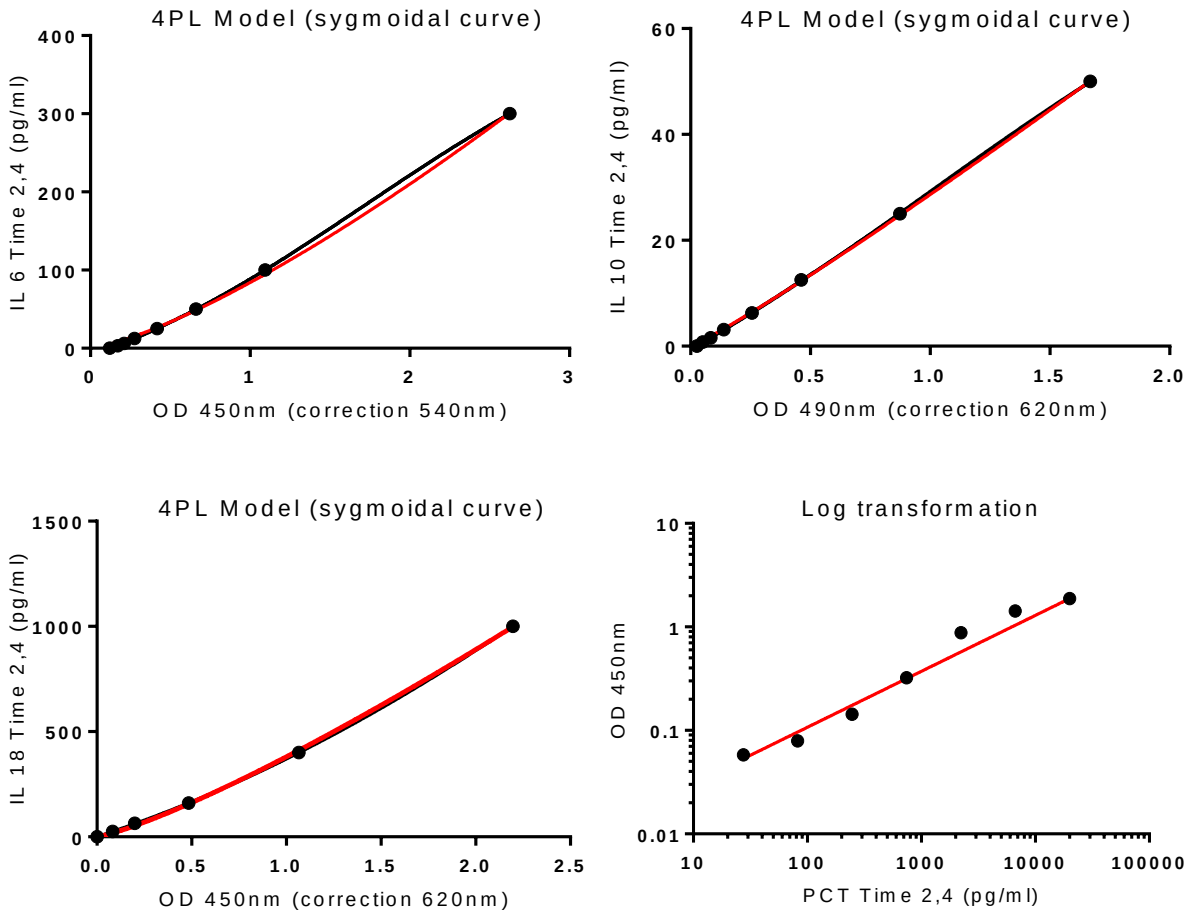


Figure 58: Examples of standard curve calibration for ELISA. Standard curves between OD and IL concentrations for molecules IL-6, IL-10, IL-18 were generated by a 4-PL model, while for PCT were linearized by logarithmic transformation.

4.11.2 Differences of IL-6, IL-10, IL-18, and PCT over one week period, in three different surgical approaches

IL-6 analysis

The overall intra- and inter-assay CVs of the duplicates, following spectrophotometry (OD) were 5.7% and 5.6%, close to the cut-off point of 4.5% and 6.5%, respectively showing satisfactory assay accuracy (Table 45a). Moreover, no statistically significant difference was observed in pre-operative IL-6 concentration, between pre- and post-anaesthetic induction (Table 45c).

Friedman non-parametric tests were conducted to examine for differences in IL-6 serum levels for each surgical approach. Over the period of study, IL-6 concentrations showed statistically significant differences, among groups and time points compared, as showed in Figure 59, Tables 44, 45b (ANOVA: posterior; Friedman=44.76, p<0.0001, antero-posterior; Friedman=28.38, p<0.0001, posterior-costoplasty; Friedman=21.34, p=0.0007).

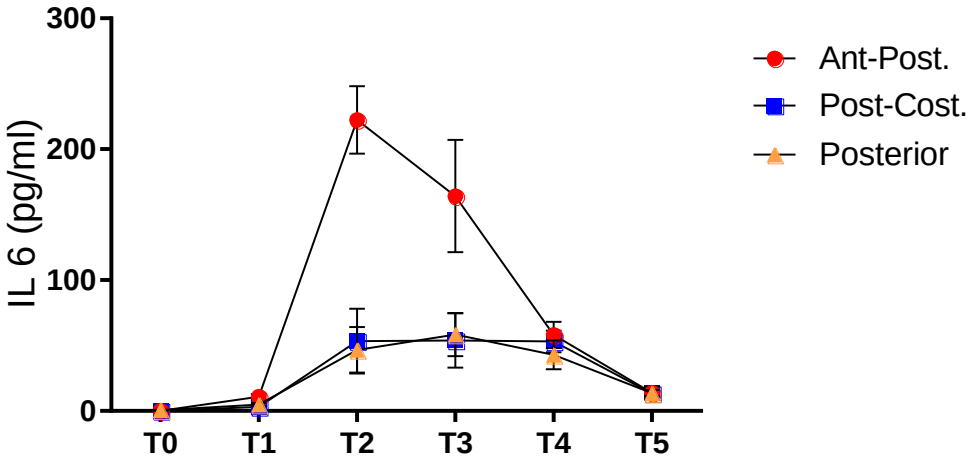


Figure 59: IL-6 concentration over time, comparison between different approaches. IL-6 concentration was significantly elevated mainly at the end of the surgery (T2) and the next 24 hours (T3), in the antero-post, compared to the other approaches. Graphic was presented as mean with SEM.

In the posterior approach, multiple comparisons demonstrated significantly higher concentrations of IL-6 levels at the end of surgery (T2), 24 hours (T3), and 2 days (T4) post-operatively, compared to (T0) and 2 hours intra-operatively (T1), all p<0.001 (Table 44a).

Similarly, in the antero-posterior approach significant differences over time were also found at T2 and T3, where IL-6 concentrations were significantly elevated as compared to T0 and T1, all $p<0.001$ (Table 44a). Finally, in the posterior-costoplasty approach, IL-6 concentration was significantly higher at T2, T3, and T4 compared to T0, all $p<0.05$ (Table 44a).

Table 44: A) Outcome of Friedman non-parametric tests between the time points of each approach and B) outcome of Kruskal-Wallis tests between all three approaches, for every time point.

A	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Ant.-Post.	-	0.0002	0.0009	-	-	0.0506	-	-	-	-	-	-	-	-	-
Post.-Cost.	-	0.0108	0.0352	0.0058	-	-	-	-	-	-	-	-	-	-	-
Posterior	-	<0.0001	<0.0001	<0.0001	-	0.0108	0.0031	0.0108	-	-	-	-	-	-	-

B	T0	T1	T2	T3	T4	T5
Posterior vs. Ant.-Post.	-	-	0.0042	0.048	-	-
Posterior vs. Post.-Cost.	-	-	-	-	-	-
Ant.-Post. vs. Post.-Cost.	-	0.0206	0.0437	-	-	-

A series of Kruskal-Wallis tests were then used to identify possible differences among the three approaches at each time point, which were observed at T1, T2, and T3 (Table 44b). Specifically, IL-6 level in antero-posterior approach was significantly higher than in posterior-costoplasty approach, at T1. At T2, IL-6 level was significantly higher in antero-posterior, compared to the other approaches. Moreover, IL-6 level in the antero-posterior approach remained at higher levels, compared to the posterior approach at T3, all $p<0.05$ (Table 44b).

Therefore, the results indicated that IL-6 concentration was significantly elevated at the end of the surgery (T2) and the level remained elevated at T3 and/or T4 in different approaches. In addition, participants who underwent the antero-posterior approach demonstrated higher levels of IL-6 at T1, T2, and T3.

Table 45: Overall outcome from IL-6 ELISA assay. A) OD with intra- and inter-assay CV, B) final concentrations in pg/ml, C) comparison of pre- and post-anaesthetic induction.

A	IL-6 Optical density in 96well-plate									
	Antero-Post. Approach (n=6)			Post.-Costoplasty Approach (n=5)			Posterior approach (n=12)			Intra-assay overall CV (%)
	Median	SD	Intra-assay CV (%)	Median	SD	Intra-assay CV (%)	Median	SD	Intra-assay CV (%)	
Time 0	0.088	0.006	5.448	0.105	0.011	8.650	0.110	0.003	3.952	6.017
Time 1	0.170	0.004	3.590	0.043	0.001	1.746	0.062	0.002	5.051	3.462
Time "Turn"	0.589	0.026	6.372	-	-	-	-	-	-	6.372
Time 2	2.066	0.132	6.680	0.375	0.017	5.278	0.424	0.024	6.074	6.011
Time 3	1.246	0.056	5.341	0.405	0.025	7.348	0.587	0.019	6.122	6.270
Time 4	0.776	0.034	5.567	0.685	0.038	4.446	0.497	0.017	3.520	4.511
Time 5	0.129	0.013	8.051	0.227	0.013	6.242	0.182	0.008	5.421	6.571
Time 6	0.178	0.011	6.173	0.298	0.024	6.672	0.169	0.010	6.212	6.352
Inter-assay overall CV (Mean)			**5.903**			**5.769**			**5.193**	
Total Inter-assay CV (Mean)			**5.622**				**Total Intra-assay CV (Mean)**			**5.696**

B	IL-6 Final concentration (pg/ml)								
	Antero-Post. Approach (n=6)			Post.-Costoplasty Approach (n=5)			Posterior approach (n=12)		
	Median	SD	Mean	Median	SD	Mean	Median	SD	Mean
Time 0	0.042	0.035	0.044	0.114	0.217	0.215	0.124	1.424	0.575
Time 1	10.834	5.140	10.811	2.798	1.139	3.130	3.952	4.347	4.893
Time "Turn"	41.367	87.318	76.162	-	-	-	-	-	-
Time 2	229.846	63.154	222.340	22.357	55.410	53.460	25.852	60.142	46.746
Time 3	115.317	105.138	164.196	24.829	46.642	53.870	41.196	56.950	58.319
Time 4	62.307	24.583	58.190	52.295	18.419	53.069	33.610	38.202	42.941
Time 5	8.176	11.804	13.893	11.934	6.270	13.452	11.627	10.400	13.645
Time 6	8.103	0.789	8.103	16.649	8.071	16.649	7.574	3.062	8.375

C	Optical density in 96well-plate		
IL-6	Median	SD	CV (%)
Time 0a*	0.106	0.004	3.971
Time 0*	0.130	0.010	7.776

Final concentration (pgr/ml) (n=4)			
IL-6	Median	SD	Mean
Time 0a*	0.087	0.067	0.097
Time 0*	0.234	0.364	0.382

* IL-6 of Time 0a and Time 0, were measured with a high sensibility ELISA kit. The intra-assay CVs of the duplicates, following spectrophotometry (OD) were close the cut-off point of the intra-assay CV 7% of the kit, showing satisfactory assay accuracy.

IL-10 analysis

The overall intra- and inter-assay CVs of the duplicates, following spectrophotometry (OD) were both 6.4%, close to the cut-off point of 5% and 7.5%, respectively showing satisfactory assay accuracy (Table 47a). Moreover, no statistically significant difference was observed in pre-operative IL-10 concentration, between pre- and post-anaesthetic induction (Table 47c).

Friedman non-parametric tests were conducted to examine for differences in IL-10 serum levels for each surgical approach. Over the period of study, IL-10 concentrations showed statistically significant differences, only in posterior and antero-posterior approaches, as showed in Figure 60, Tables 46, 47b, (ANOVA: posterior Friedman=29.38, $p<0.0001$, antero-posterior Friedman=13.56, $p<0.019$, posterior-costoplasty Friedman=9.2, $p=0.1015$).

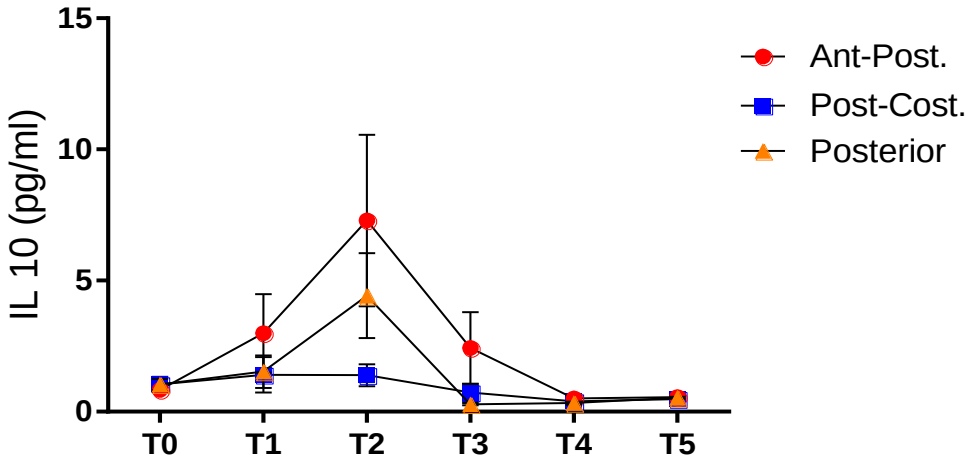


Figure 60: IL-10 concentration over time, comparison between different approaches. IL-10 concentration was significantly elevated mainly at the end of the surgery (T2) and the next 24 hours (T3), in the antero-post and posterior approaches. Graphic was presented as mean with SEM.

In the posterior approach, multiple comparisons demonstrated significantly reduced concentration of IL-10 at T3 and T4, compared to T0 and T1. Furthermore, the IL-10 level at T3 was also lower, compared to T2, all $p<0.05$ (Table 46a). Similarly, in the antero-posterior approach, IL-10 level at T4 was significantly lower, compared to T2, $p=0.014$ (Table 46a).

A series of Kruskal-Wallis tests were then conducted to identify possible differences among the three approaches at each time point. Specifically, IL-10 level in the posterior approach was significantly lower at T3, than in the antero-posterior approach, p=0.006 (Table 46b).

Therefore, it was found that IL-10 concentration was reduced at the baseline at T3 and T4 in the posterior approach, whereas the reduction of IL-10 concentration was only observed in the antero-posterior approach at T4, compared to T2. Furthermore, participants who underwent antero-posterior approach showed a higher level of IL-10, than did those in the posterior approach.

Table 46: A) Outcome of Friedman non-parametric tests between the time points of each approach and B) outcome of Kruskal-Wallis tests between all three approaches, for every time point.

A	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Ant.-Post.	-	-	-	-	-	-	-	-	-	-	0.0136	-	-	-	-
Post.-Cost.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Posterior	-	-	0.0008	0.0108	-	-	0.002	0.0233	-	0.0072	-	-	-	-	-

B	T0	T1	T2	T3	T4	T5
Posterior vs. Ant.-Post.	-	-	-	0.0055	-	-
Posterior vs. Post.-Cost.	-	-	-	-	-	-
Ant.-Post. vs. Post.-Cost.	-	-	-	-	-	-

Table 47: Overall outcome from IL-10 ELISA assay. A) OD with intra- and inter-assay CV, B) final concentrations in pg/ml, C) comparison of pre- and post-anaesthetic induction.

A	IL-10 Optical density in 96well-plate									
	Antero-Post. Approach (n=6)			Post.-Costoplasty Approach (n=5)			Posterior approach (n=12)			Intra-assay overall CV (%)
	Median	SD	Intra-assay CV (%)	Median	SD	Intra-assay CV (%)	Median	SD	Intra-assay CV (%)	
Time 0	0.022	0.002	11.403	0.022	0.001	3.928	0.021	0.001	3.859	6.397
Time 1	0.078	0.007	9.065	0.037	0.004	9.205	0.045	0.001	2.378	6.883
Time "Turn"	0.119	0.011	5.945	-	-	-	-	-	-	5.945
Time 2	0.208	0.005	3.826	0.068	0.005	7.770	0.061	0.002	5.005	5.534
Time 3	0.068	0.003	5.115	0.045	0.002	5.398	0.038	0.002	4.549	5.021
Time 4	0.037	0.001	2.615	0.031	0.001	3.565	0.033	0.002	5.569	3.917
Time 5	0.028	0.005	15.396	0.030	0.001	4.044	0.029	0.002	8.703	9.381
Time 6	0.037	0.004	8.979	0.038	0.004	9.315	0.032	0.002	6.695	8.330
Inter-assay overall CV (Mean)			**7.793**			**6.175**			**5.251**	
Total Inter-assay CV (Mean)			**6.406**				**Total Intra-assay CV (Mean)**			**6.426**

B	IL-10 Final concentration (pg/ml)								
	Antero-Post. Approach (n=6)			Post.-Costoplasty Approach (n=5)			Posterior approach (n=12)		
	Median	SD	Mean	Median	SD	Mean	Median	SD	Mean
Time 0	0.766	0.166	0.828	0.766	0.646	1.046	0.713	0.766	1.042
Time 1	1.806	3.657	2.992	0.757	1.516	1.407	0.958	2.124	1.526
Time "Turn"	2.616	7.269	5.271	-	-	-	-	-	-
Time 2	4.977	7.994	7.288	1.210	0.929	1.387	1.029	5.602	4.423
Time 3	1.152	3.347	2.429	0.477	0.765	0.732	0.291	0.136	0.287
Time 4	0.412	0.337	0.502	0.258	0.278	0.392	0.322	0.194	0.324
Time 5	0.544	0.103	0.548	0.494	0.190	0.473	0.532	0.156	0.524
Time 6	0.249	0.202	0.249	0.277	0.162	0.277	0.149	0.070	0.135

C	Optical density in 96well-plate		
IL-10	Median	SD	CV (%)
Time 0a	0.017	0.001	3.825
Time 0	0.019	0.001	7.277

Final concentration (pgr/ml) (n=4)			
IL-10	Median	SD	Mean
Time 0a	0.528	0.051	0.528
Time 0	0.633	0.031	0.633

IL-18 analysis

The overall intra- and inter-assay CVs of the duplicates, following spectrophotometry (OD) were both 5.4%, below the cut-off point of 5% and 7.5%, respectively showing satisfactory assay accuracy (Table 49a). Moreover, no statistically significant difference was observed in pre-operative IL-18 concentration, between pre- and post-anaesthetic induction (Table 49c).

The results of the Friedman non-parametric tests for IL-18 indicated significant differences over time periods only in the posterior and antero-posterior approaches, as showed in Figure 61, Tables 48, 49b, (ANOVA: posterior Friedman=36.17, $p<0.0001$, antero-posterior Friedman=21.62, $p<0.0006$, posterior-costoplasty Friedman=9.9, $p=0.078$).

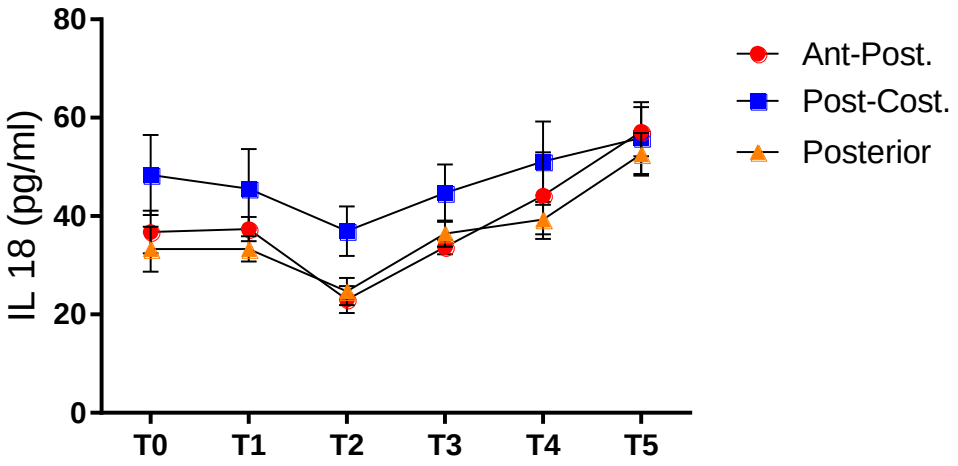


Figure 61: IL-18 concentration over time, comparison between different approaches. IL-18 showed a similar trend between all the approached, with significant differences only within different time-points of the posterior and antero-posterior approach. No significant difference were observed comparing all three approaches. Graphic was presented as mean with SEM.

Multiple comparisons demonstrated significantly elevated concentration of IL-18 in the posterior approach, at T5 in relation to T0, T1, T2, and T3, all $p<0.05$ (Table 48). Furthermore, the IL-18 level at T4 was also significantly higher than that at T2, $p=0.016$ (Table 48). Similarly, in the antero-posterior approach, a significantly higher IL-18 level was observed only at T5, compared to T2, $p=0.0001$ (Table 48).

A series of Kruskal-Wallis tests were then conducted to test for differences among the three approaches at each time point. However, no significant differences were found at any time point.

Therefore, in all the approaches IL-18 slightly dropped after the surgery (T2), for after reach the highest level at five days after surgery (T5). Moreover, only the posterior and the antero-posterior approaches showed statistically significant results at T5, compared to the end of surgery (T2). No significant differences were found among the three approaches at any time point.

Table 48: Outcome of Friedman non-parametric tests between the time points of each approach.

A	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Ant.-Post.	-	-	-	-	-	-	-	-	-	-	-	0.0001	-	-	-
Post.-Cost.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Posterior	-	-	-	-	0.008	-	-	-	0.0048	-	0.0159	<0.0001	-	0.0233	-

Table 49: Overall outcome from IL-18 ELISA assay. A) OD with intra- and inter-assay CV, B) final concentrations in pg/ml, C) comparison of pre- and post-anaesthetic induction.

A	IL-18 Optical density in 96well-plate									
	Antero-Post. Approach (n=6)			Post.-Costoplasty Approach (n=5)			Posterior approach (n=12)			Intra-assay overall CV (%)
	Median	SD	Intra-assay CV (%)	Median	SD	Intra-assay CV (%)	Median	SD	Intra-assay CV (%)	
Time 0	0.134	0.009	8.879	0.164	0.007	4.400	0.093	0.001	4.442	5.907
Time 1	0.112	0.005	4.865	0.137	0.006	4.497	0.099	0.003	3.487	4.283
Time "Turn"	0.089	0.004	5.143	-	-	-	-	-	-	5.143
Time 2	0.064	0.005	6.626	0.109	0.008	6.380	0.068	0.004	6.828	6.611
Time 3	0.101	0.006	6.802	0.147	0.008	5.066	0.118	0.007	6.071	5.979
Time 4	0.115	0.005	5.113	0.151	0.012	8.060	0.129	0.005	4.149	5.774
Time 5	0.173	0.007	3.818	0.173	0.004	2.533	0.156	0.007	3.937	3.430
Time 6	0.170	0.004	2.761	0.116	0.005	4.734	0.139	0.012	9.519	5.671
Inter-assay overall CV (Mean)			**5.501**			**5.096**			**5.491**	
Total Inter-assay CV (Mean)			**5.362**				**Total Intra-assay CV (Mean)**			**5.350**

B	IL-18 Final concentration (pg/ml)								
	Antero-Post. Approach (n=6)			Post.-Approach/Costoplasty (n=5)			Posterior approach (n=12)		
	Median	SD	Mean	Median	SD	Mean	Median	SD	Mean
Time 0	35.723	10.660	36.776	43.966	18.190	48.361	30.725	15.836	33.287
Time 1	36.348	6.067	37.388	44.015	18.018	45.547	32.396	9.374	33.324
Time "Turn"	31.655	5.084	32.395	-	-	-	-	-	-
Time 2	20.706	6.731	23.043	33.941	11.248	36.945	22.717	9.624	24.667
Time 3	33.721	3.542	33.690	41.847	13.005	44.672	36.685	9.230	36.448
Time 4	35.728	21.550	44.162	46.495	18.298	51.079	39.730	10.464	39.295
Time 5	55.262	12.263	57.153	52.981	18.894	55.869	48.600	18.848	52.597
Time 6	46.224	8.729	46.224	36.246	0.372	36.246	40.524	10.758	43.369

C	Optical density in 96well-plate		
IL-18	Median	SD	CV (%)
Time 0a	0.094	0.001	2.008
Time 0	0.093	0.001	4.442

Final concentration (pgr/ml) (n=4)			
IL-18	Median	SD	Mean
Time 0a	25.182	12.497	25.419
Time 0	24.553	17.019	30.901

Procalcitonin (PCT) analysis

The overall intra- and inter-assay CVs of the duplicates, following spectrophotometry (OD) were 4.2% and 4.4%, below the cut-off point of 10% and 12%, respectively, showing satisfactory assay accuracy (Table 51a). Moreover, no statistically significant difference was observed in pre-operative PCT concentration, between pre- and post-anaesthetic induction (Table 51c).

The results of Friedman non-parametric tests for PCT revealed significant differences over time periods only in the posterior and antero-posterior approaches, as showed in Figure 62, Tables 50, 51b, (ANOVA: posterior Friedman=25.71, p=0.0001, antero-posterior Friedman=17.81, p=0.003, posterior-costoplasty Friedman=5.9, p=0.315).

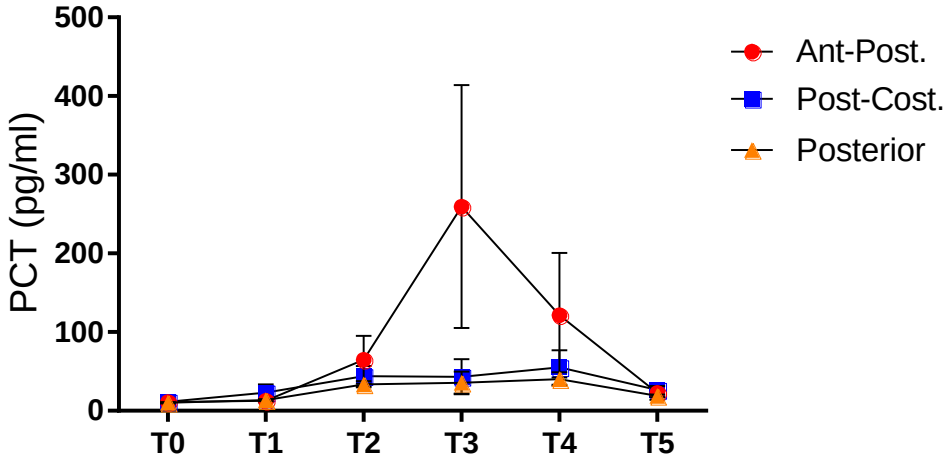


Figure 62: PCT concentration over time, comparison between different approaches. PCT concentration was significantly elevated between the end of the surgery (T2) and the next 48 hours (T4), in the antero-post and posterior approaches. No significant differences were found among the three approaches. Graphic was presented as mean with SEM.

Specifically, multiple comparisons showed significantly elevated PCT concentration in the posterior approach, at T2 and T4, compared to T0 and T1, all $p<0.05$ (Table 50). In the antero-posterior approach, a significantly higher level in PCT was observed at T4, compared to T0, p=0.018 (Table 50).

A series of Kruskal-Wallis tests were then conducted to test for differences among the three approaches at each time point. However, no significant differences of PCT were found at any time point.

Therefore, the results indicate that PCT concentration was significantly elevated 2 days after surgery (T4) in both posterior and antero-posterior approaches.

Moreover, a higher level was also observed at the end of surgery (T2) in the posterior approach. No significant differences were found among the three approaches at any time point.

Table 50: Outcome of Friedman non-parametric tests between the time points of each approach.

	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Ant.-Post.	-	-	-	0.0179	-	-	-	-	-	-	-	-	-	-	-
Post.-Cost.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Posterior	-	0.0008	-	0.0031	-	0.0108	-	0.0338	-	-	-	-	-	-	-

Table 51: Overall outcome from PCT ELISA assay. A) OD with intra- and inter-assay CV, B) final concentrations in pg/ml, C) comparison of pre- and post-anaesthetic induction.

A	PCT Optical density in 96well-plate									
	Antero-Post. Approach (n=6)			Post.-Costoplasty Approach (n=5)			Posterior approach (n=12)			Intra-assay overall CV (%)
	Median	SD	Intra-assay CV (%)	Median	SD	Intra-assay CV (%)	Median	SD	Intra-assay CV (%)	
Time 0	0.035	0.002	4.922	0.038	0.003	8.319	0.036	0.002	5.575	6.272
Time 1	0.045	0.004	7.699	0.057	0.002	5.995	0.044	0.003	6.899	6.864
Time "Turn"	0.036	0.001	1.964	-	-	-	-	-	-	1.964
Time 2	0.043	0.002	3.807	0.039	0.001	2.773	0.041	0.002	4.030	3.537
Time 3	0.065	0.002	2.040	0.049	0.001	2.245	0.058	0.001	2.333	2.206
Time 4	0.046	0.004	7.123	0.044	0.002	3.995	0.044	0.002	4.177	5.098
Time 5	0.047	0.001	2.360	0.063	0.004	6.363	0.054	0.002	4.651	4.458
Time 6	0.048	0.004	7.825	0.035	0.001	1.543	0.037	0.000	0.971	3.446
Inter-assay overall CV (Mean)			**4.718**			**4.462**			**4.091**	
Total Inter-assay CV (Mean)			**4.423**				**Total Intra-assay CV (Mean)**			**4.231**

B	PCT Final concentration (pg/ml)								
	Antero-Post. Approach (n=6)			Post.-Costoplasty Approach (n=5)			Posterior approach (n=12)		
	Median	SD	Mean	Median	SD	Mean	Median	SD	Mean
Time 0	9.339	3.542	10.920	11.433	1.725	11.366	10.208	2.867	10.340
Time 1	12.158	5.314	12.686	19.717	23.160	23.206	11.550	5.118	13.505
Time "Turn"	5.747	5.539	9.249	-	-	-	-	-	-
Time 2	36.154	74.577	64.931	30.809	28.928	44.111	33.795	13.131	33.747
Time 3	21.988	378.350	259.471	11.459	49.973	43.315	16.714	48.332	35.702
Time 4	41.875	193.822	121.506	38.512	48.722	55.041	36.908	29.626	40.218
Time 5	13.263	21.745	22.780	17.479	20.046	26.662	17.634	6.760	18.930
Time 6	11.264	5.057	11.264	5.401	0.488	5.401	5.941	2.717	6.970

C	Optical density in 96well-plate		
PRC	MEDIAN	SD	CV (%)
Time 0a	0.037	0.003	5.800
Time 0	0.037	0.003	7.558

Final concentration (pgr/ml) (n=4)			
PRC	MEDIAN	SD	MEAN
Time 0a	10.893	8.181	13.882
Time 0	11.140	10.698	13.656

4.11.3 Relationship between duration of surgery and IL-6, IL-10, IL-18, PCT concentrations over one week period, in three different surgical approaches

The posterior approach was the most suitable group to perform this analysis, showing a series of advantages over the other approaches. In a first place it was possible to distinguish a clear cut off between a "fast group " (median time 4h, range 2-5h) and a "slow group " (median time 6h, range 5.1-11h), which was statistically significant, p=0.0006. Moreover, the two groups of 6 donors each, presented similar demographics (4/2 M/F, 16 years of age, 6 days LOS). In addition, the posterior approach is the most common in the spinal field and it was the most populated group in this study, allowing a consistent comparison between the different molecules, with only variable, the surgical duration.

IL-6 analysis

The results of Friedman non-parametric tests for IL-6, revealed significant differences over time periods in both fast and slow groups of surgery, as showed in Figure 63, Table 52 (ANOVA: fast group Friedman=21.24, p=0.0007, slow group Friedman=25.05, p=0.0001).

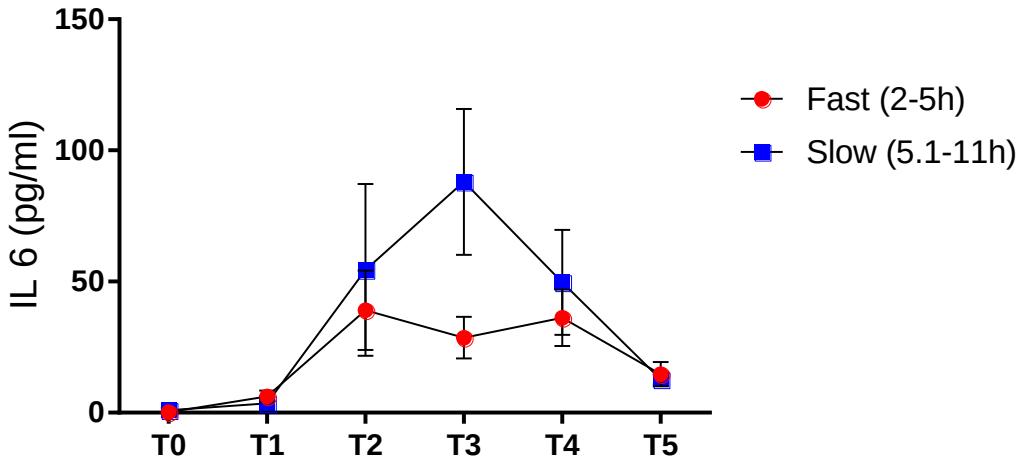


Figure 63: IL-6 concentration over time, comparison between fast and slow duration of surgery in the posterior approach. PCT concentration was significantly elevated between the end of the surgery (T2) and the next 48 hours (T4), in both groups, however the slow group showed significantly higher values with highest 24 hours (T3) post-operatively. Graphic was presented as mean with SEM.

Multiple comparisons demonstrated that IL-6 concentration was significantly elevated in both groups at T2, T3, and T4, compared to T0, $p<0.05$. Moreover, in the slow group the IL-6 level was also significantly higher at T3, compared to T1, $p=0.0103$ (Table 52). A series of Mann-Whitney non-parametric tests showed no significant difference between the two groups at any time point.

In summary, IL-6 concentration was significantly increased at T2 and the level remained elevated throughout T4, in both fast and slow groups. Moreover, donors who had a longer duration of surgery demonstrated a higher level of IL-6 at T3, than did those who had a shorter duration of surgery.

Table 52: Outcome of Friedman non-parametric tests of fast and slow posterior approach, between the different time points (IL-6).

Posterior	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Fast	-	0.0032	0.0179	0.0058	-	-	-	-	-	-	-	-	-	-	-
Slow	-	0.0304	0.0009	0.0179	-	-	0.0103	-	-	-	-	-	-	-	-

IL-10 analysis

The results of Friedman non-parametric tests for IL-10, revealed significant differences over time periods in both fast and slow groups of surgery, as showed in Figure 64, Table 53 (ANOVA: fast group Friedman=12.67, p=0.0267, slow group Friedman=19.62, p=0.0015).

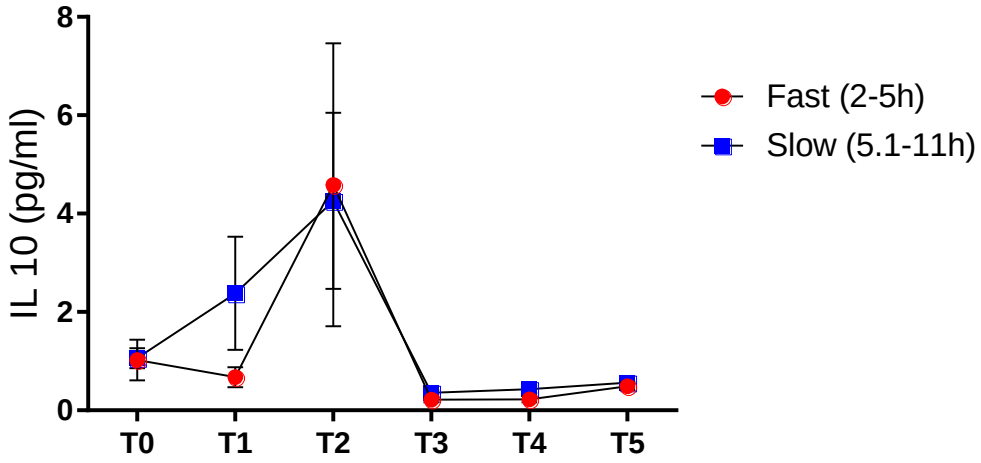


Figure 64: IL-10 concentration over time, comparison between fast and slow duration of surgery in the posterior approach. IL-10 concentration was significantly reduced in both groups 24 hours (T3) post-operatively, without statistical difference between them. The slow group presented a higher level of IL-10 at T1, compared to the fast. Graphic was presented as mean with SEM.

Multiple comparisons demonstrated that IL-10 concentration was significantly reduced in the fast group at T3, compared to T0, p=0.049. In the slow group, IL-10 concentration was significantly reduced at T3, compared to T1, p=0.0058 (Table 53). A series of Mann-Whitney non-parametric tests showed no significant difference between the two groups at any time point. Moreover, donors who had a longer duration of surgery demonstrated a higher level of IL-10 at T1 and T3, than did those who had a shorter duration of surgery.

Table 53: Outcome of Friedman non-parametric tests of fast and slow posterior approach, between the different time points (IL-10).

Posterior	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Fast	-	-	0.049	-	-	-	-	-	-	-	-	-	-	-	-
Slow	-	-	-	-	-	-	0.0058	-	-	-	-	-	-	-	-

IL-18 analysis

The results of Friedman non-parametric tests for IL-18, revealed significant differences over time periods in both fast and slow groups of surgery, as showed in Figure 65, Table 54 (ANOVA: fast group Friedman=19.16, p=0.0018, slow group Friedman=17.81, p=0.0032).

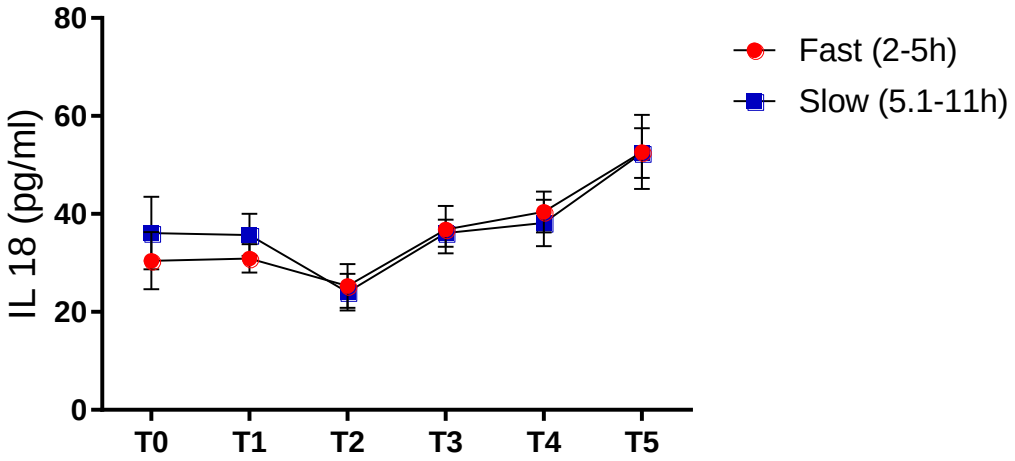


Figure 65: IL-18 concentration over time, comparison between fast and slow duration of surgery in the posterior approach. IL-18 showed a similar trend between both approached, with significant differences at 5 days post-operatively (T5) for both groups. Graphic was presented as mean with SEM.

Multiple comparisons demonstrated that IL-18 concentration was significantly higher in the fast group at T5, compared to T0 and T2, $p<0.05$. In the slow group, IL-18 concentration was significantly elevated at T5, compared to T2, p=0.0005 (Table 54). Therefore, IL-18 concentration was significantly raised above the baseline, 5 days after surgery (T5) in both groups. Despite, IL-18 presents the lowest level at T2 for both groups, it was not statistically significant. Also, no significant differences were found between the two groups at any time point using Mann-Whitney non-parametric tests.

Table 54: Outcome of Friedman non-parametric tests of fast and slow posterior approach, between the different time points (IL-18).

Posterior	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Fast	-	-	-	-	0.0167	-	-	-	-	-	-	0.0012	-	-	-
Slow	-	-	-	-	-	-	-	-	-	-	-	0.0005	-	-	-

PCT analysis

The results of Friedman non-parametric tests for PCT, revealed significant differences over time periods only for the slow groups of surgery, as showed in Figure 66, Table 55 (ANOVA: fast group Friedman=9.71, p=0.084, slow group Friedman=16.95, p=0.0046).

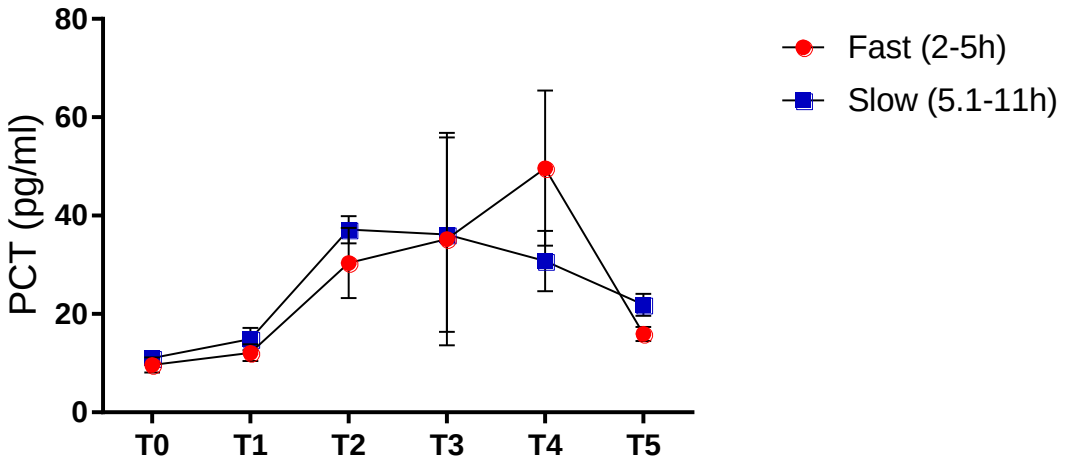


Figure 66: PCT concentration over time, comparison between fast and slow duration of surgery in the posterior approach. PCT concentration was significantly higher only in the slow group at T2. A significant difference among groups was found at T5 with slow group presenting a higher concentration. Graphic was presented as mean with SEM.

Multiple comparisons demonstrated that PCT concentration was significantly higher only in the slow group at T2, compared to T0, p=0.0103 (Table 55). Moreover, a series of Mann-Whitney non-parametric tests were conducted and the results showed a significative difference only at T5, with the slow group presenting higher levels of PCT, than the fast group, p=0.039 (Figure 66).

Table 55: Outcome of Friedman non-parametric tests of fast and slow posterior approach, between the different time points (PCT).

Posterior	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Fast	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Slow	-	0.0103	-	-	-	-	-	-	-	-	-	-	-	-	-

4.11.4 Relationship between gender and IL-6, IL-10, IL-18, PCT concentrations over one week period, in three different surgical approaches

Given the gender distribution and the cohort demographics (Tables 1, 2, 9, 10), the posterior (8M/4F) and the antero-posterior (3M/3F) approaches were the most suitable groups to perform this comparison, considering also that the posterior-costoplasty approach was composed only by females. Although the size of this cohort and the gender distribution represented the limiting factor for this comparison, it was important to identify possible gender differences at this stage.

IL-6 analysis

Friedman non-parametric tests were conducted in antero-posterior and posterior approach groups to examine for differences in IL-6 levels over one-week time period in each gender, as showed in Figure 67.

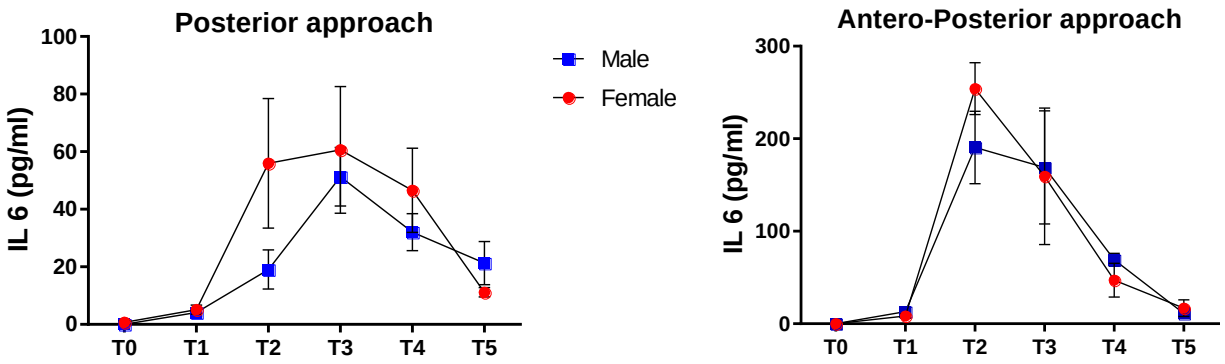


Figure 67: IL-6 concentration over time, comparison between genders in the posterior and antero-posterior approach. Although both approaches presented significant differences between T2-T4, compared to the base line, no significant differences, were detected between genders. Graphic was presented as mean with SEM.

Specifically, in the posterior approach, the overall time effect was significant in both genders (ANOVA: female Friedman=33.95, $p<0.0001$, male Friedman=14.24, $p<0.0001$). Moreover, in females IL-6 concentration was significantly increased at T2, T3, and T4, compared to T0, all $p<0.005$. IL-6 level at T2 was also significantly higher, compared to T1, $p=0.0100$. Within the males, the only significantly higher level of IL-6 was at T3, compared to T0, $p=0.0338$ (Table 56).

Similarly in the antero-posterior approach, an overall significant time effect was found in both genders (ANOVA: female Friedman=14.24, p=0.0001, male Friedman=14.24, p<0.0001), with IL-6 concentration presenting a significant increment at T2, compared to T0, p=0.0338 (Table 56).

A series of Mann-Whitney non-parametric tests were conducted to measure the differences between genders at each time point. However, no significant differences, were detected on either approaches, all p>0.05.

Therefore, in the posterior approach, IL-6 increased significantly at T2, T3, and T4 in females, but within the males an increment was found only at T3. In the antero-posterior approach, IL-6 presented higher concentrations, compared to posterior approach, however both genders showed an elevated level of IL-6 only at T2.

Table 56: Outcome of Friedman non-parametric tests for both genders in posterior and antero-posterior approach, between the different time points (IL-6).

Posterior	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Male	-	-	0.0338	-	-	-	-	-	-	-	-	-	-	-	-
Female	-	<0.0001	0.0005	0.0014	-	0.0100	-	-	-	-	-	-	-	-	-

Antero-Posterior	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Male	-	0.0338	-	-	-	-	-	-	-	-	-	-	-	-	-
Female	-	0.0338	-	-	-	-	-	-	-	-	-	-	-	-	-

IL-10 analysis

Friedman non-parametric tests were conducted in antero-posterior and posterior approach groups to examine for differences in IL-10 levels over one-week time period in each gender, as showed in Figure 68.

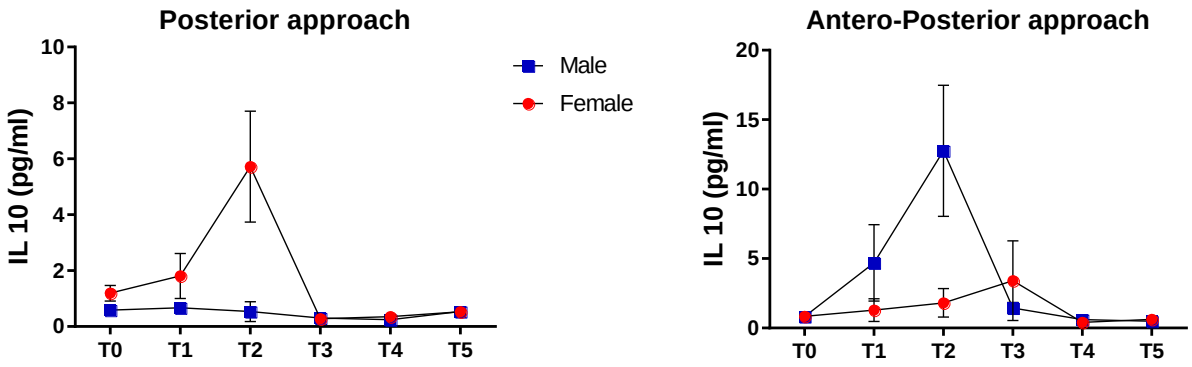


Figure 68: IL-10 concentration over time, comparison between genders in the posterior and antero-posterior approach. Posterior approach presented an overall significant difference only in females, and the antero-posterior approach presented significant differences only in males, compared to base line. Were no detected significant differences between genders. Graphic was presented as mean with SEM.

Specifically, in the posterior approach, the overall time effect was significant only in females (ANOVA: female Friedman=26.52, $p<0.0001$, male Friedman=6.24, $p=0.32$). Moreover, for females IL-10 concentration was significantly reduced at T3, compared to T0, T1 and T2, all $p<0.01$ (Table 57).

Contrarily, in the antero-posterior approach, an overall significant time effect was found only in males (ANOVA: female Friedman=5, $p=0.47$, male Friedman=11.95, $p=0.0084$) (Table 57). However, multiple comparisons failed to yield significance between any time points, hence it could not be determined the exact time-points.

A series of Mann-Whitney non-parametric tests were also used to measure gender differences at each time point, but no significant differences were found, all $p>0.05$.

Table 57: Outcome of Friedman non-parametric tests for both genders in posterior approach, between the different time points (IL-10).

Posterior	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Male	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Female	-	-	0.0024	-	-	-	0.0063	-	-	0.0024	-	-	-	-	-

IL-18 analysis

Friedman non-parametric tests were conducted in antero-posterior and posterior approach groups to examine for differences in IL-18 levels over one-week time period in each gender, as showed in Figure 69.

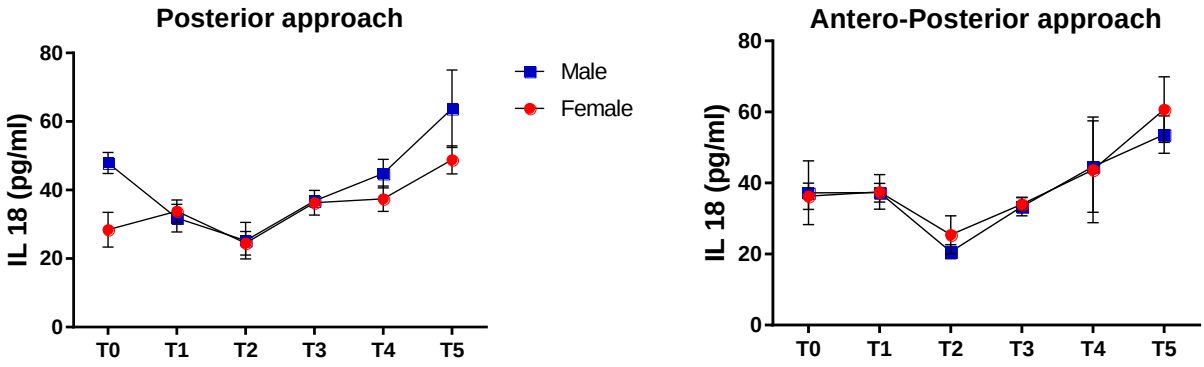


Figure 69: IL-18 concentration over time, comparison between genders in the posterior and antero-posterior approach. In both approaches and genders IL-18 dropped at the end of the surgery (T2), however it was not statistically significant. IL-18 was significantly elevated 5 days after surgery (T5) in both approaches and genders. Graphic was presented as mean with SEM.

Specifically, in the posterior approach, the overall time effect was significant in both genders (ANOVA: female Friedman=27.8, p<0.0001, male Friedman=14.24, p=0.0001). Furthermore, in females, IL-18 concentration was significantly higher at T5, compared to T0 and T2, all p<0.001, whereas in males, IL-18 concentration was significantly higher at T5, but only compared to T2, p=0.159 (Figure 69, Table 58)

Similarly, in the antero-posterior approach, an overall significant time effect was found in both genders (ANOVA: female Friedman=11.76, p=0.0095, male Friedman=11.57, p=0.0117). In both groups, the only significantly higher level of IL-

18 was observed at T5, compared with T2, p<0.05. A series of Mann-Whitney non-parametric tests were also used to measure gender differences at each time point, but no significant differences were found, all p>0.05.
Therefore, in both approaches IL-18 concentrations dropped at the end of the surgery (T2), for both genders, however, it was not statistically significant. Moreover, IL-18 was significantly elevated 5 days after surgery (T5) for both approaches and genders, mainly compared to T2.

Table 58: Outcome of Friedman non-parametric tests for both genders in posterior and antero-posterior approaches, between the different time points (IL-18).

Posterior	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Male	-	-	-	-	-	-	-	-	-	-	-	0.0159	-	-	-
Female	-	-	-	-	0.0005	-	-	-	-	-	-	< 0.0001	-	-	-

Antero-Posterior	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Male	-	-	-	-	-	-	-	-	-	-	-	0.0338	-	-	-
Female	-	-	-	-	-	-	-	-	-	-	-	0.0159	-	-	-

PCT analysis

Friedman non-parametric tests were conducted in antero-posterior and posterior approach groups to examine for differences in PCT levels over one-week time period in each gender, as showed in Figure 70.

Specifically, in the posterior approach, the overall time effect was significant in both genders (ANOVA: female Friedman=15.22, p=0.0095, male Friedman=12.33, p=0.0052). Furthermore in females, PCT was significantly increased at T2 and T4, compared to T0, all p<0.05. In males, however, multiple comparison was not able to determine where the differences were (Figure 75, Table 59).

In the antero-posterior approach, an overall significant time effect was found only in males (ANOVA: female Friedman=7.76, p=0.168, male Friedman=11.38, p=0.0136), however, multiple comparisons were not able to determine where the differences were.

Additionally, there were no significant differences between genders at any time points using Mann-Whitney non-parametric tests.

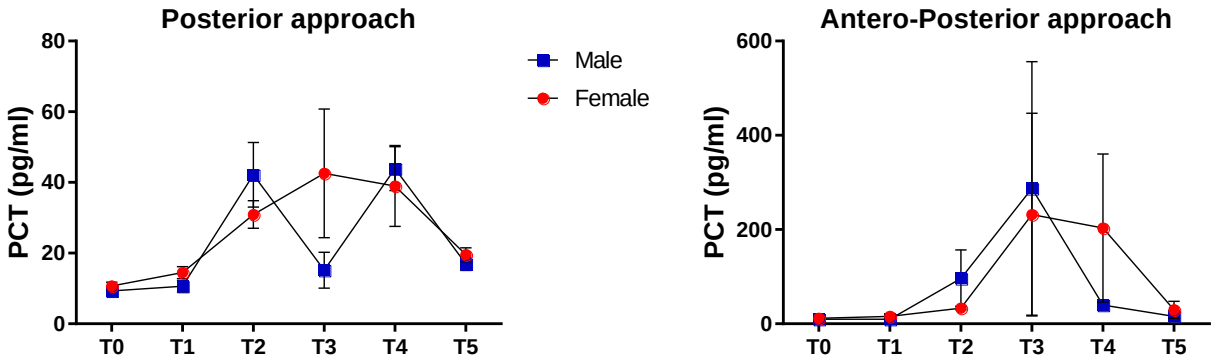


Figure 70: PCT concentration over time, comparison between genders in the posterior and antero-posterior approach. PCT levels presented a statistically significant increment at the end of surgery (T2) and 2 days post-operatively (T4), only in the posterior approach in females. Were no detected significant differences between genders. Graphic was presented as mean with SEM.

Table 59: Outcome of Friedman non-parametric tests for both genders in posterior approach, between the different time points (PCT).

Posterior	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Male	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Female	-	0.0147	-	0.0338	-	-	-	-	-	-	-	-	-	-	-

4.11.5 Differences between biochemical parameters over one week period, following three different surgical approaches

Biochemical parameters (leucocytes, lymphocytes, neutrophils, platelets, PT, PTT and Hb) were obtained from the Leeds Teaching Hospitals Haematology laboratory, to match with the blood samples' (ELISA) time points (Table 60). A comparison between the three approaches has not been identified in the current literature. Therefore the purpose of this section was to quantify the variation of biochemical parameters following AIS, over a week period, among three different approaches (antero-posterior, posterior- costoplasty, posterior).

Table 60: Overall biochemical parameters collected over a week period

Blood analysis	Approach	Time 0	Time 2	Time 3	Time 4	Time 5
Leucocytes	Antero-posterior	5.84	9.93	10.86	8.14	6.63
	Post.+Costoplasty	6.01	12.25	11.36	7.51	6.52
	Posterior	5.79	15.43	11.69	12.11	6.67
Lymphocytes	Antero-posterior	2.21	0.56	0.61	1.17	1.10
	Post.+Costoplasty	2.41	0.82	0.98	1.02	1.23
	Posterior	1.94	1.14	1.24	0.97	1.30
Neutrophils	Antero-posterior	3.26	8.93	8.17	6.18	4.74
	Post.+Costoplasty	3.29	10.67	8.97	5.45	4.60
	Posterior	3.12	13.65	8.97	6.16	4.67
Platelets	Antero-posterior	248.33	111.00	104.83	122.33	225.33
	Post.+Costoplasty	266.20	195.60	168.40	191.25	215.50
	Posterior	268.67	195.83	167.00	166.55	293.63
Prothrombin Time	Antero-posterior	11.67	15.00	17.33	12.80	12.20
	Post.+Costoplasty	12.60	14.00	14.40	13.50	12.00
	Posterior	12.17	14.50	14.08	13.50	13.20
Partial Thromboplastin Time	Antero-posterior	33.85	24.52	25.82	29.54	30.34
	Post.+Costoplasty	33.78	28.58	28.00	31.00	33.40
	Posterior	33.02	30.66	27.44	30.89	32.00
Haemoglobin	Antero-posterior	14.90	8.72	8.63	9.18	10.10
	Post.+Costoplasty	13.80	11.28	10.44	9.30	10.85
	Posterior	14.15	11.14	9.48	9.45	9.71

Leukocytes analysis

Friedman non-parametric tests were conducted to test for leukocyte concentration differences over one-week time period. As showed in Figure 71, the results indicated significant differences over time in all three approaches (ANOVA: posterior Friedman=35.20, p<0.0001, posterior-costoplasty Friedman=14.56, p=0.0057, antero-posterior approach Friedman=13.73, p<0. 0082).

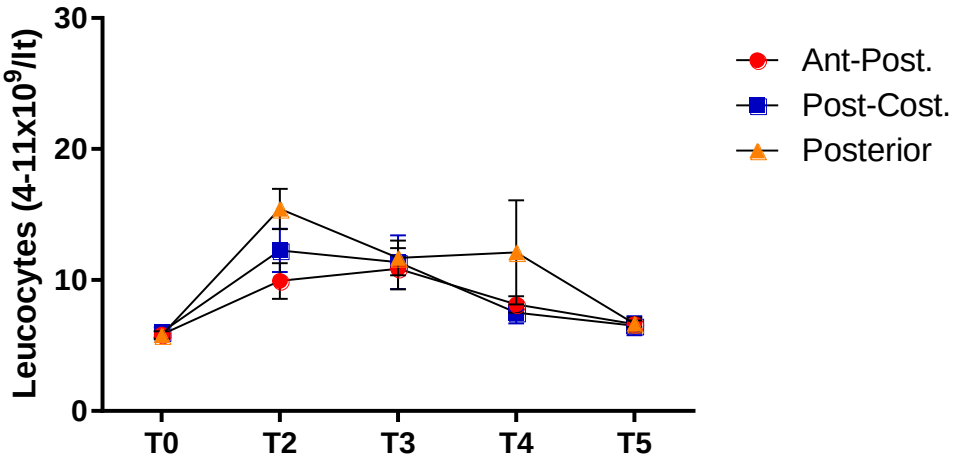


Figure 71: Leukocytes concentration over time, comparison between different approaches. Leukocytes concentrations were significantly increased at T2 and T3 and then dropped to basal level at T5 in all the approaches. No statistical differences at any time point were observed among al the approaches. Graphic was presented as mean with SEM.

In the posterior approach, multiple comparisons showed higher leukocytes concentration at T2 and T3, compared to T0, followed by a drop to a lower level at T5, compared to T2 and T3, all p<0.05. In the antero-posterior approach, leukocytes only showed a higher level at T3, compared to T0, p =0.0102. Also, in the posterior-costoplasty approach, leukocytes concentrations were significantly elevated compared to baseline (T0), only at T2, p=0.0137 (Table 61).

A series of Kruskal-Wallis tests were then conducted to test for differences among the three approaches at each time point, but no significant differences were observed, all p>0.05

Therefore, it appears that leukocytes concentrations were increased at T2 and T3 and then dropped to basal level at T5 in the posterior approach. Leukocytes were also elevated at T2 and T3 above the baseline in the posterior-costoplasty and the antero-posterior approaches, respectively. No differences between approaches were found at any time point.

Table 61: Outcome of Friedman non-parametric tests between the time points of each approach (Leucocytes).

	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Ant.-Post.	-	0.0102	-	-	-	-	-	-	-	-	-	-	-	-
Post.-Cost.	0.0137	-	-	-	-	-	-	-	-	-	-	-	-	-
Posterior	< 0.0001	0.0001	-	-	-	-	-	-	-	-	0.0030	-	0.0451	-

Lymphocytes analysis

A series of Friedman non-parametric tests were conducted to examine whether lymphocytes level changed over time in each approach. In the Figure 72, the results revealed significant differences of lymphocyte concentrations over time in all three approaches (ANOVA: posterior Friedman=25.07, p<0.0001, posterior-costoplasty Friedman=12.97, p=0.0114, antero-posterior approach Friedman=19.06, p=0.0008).

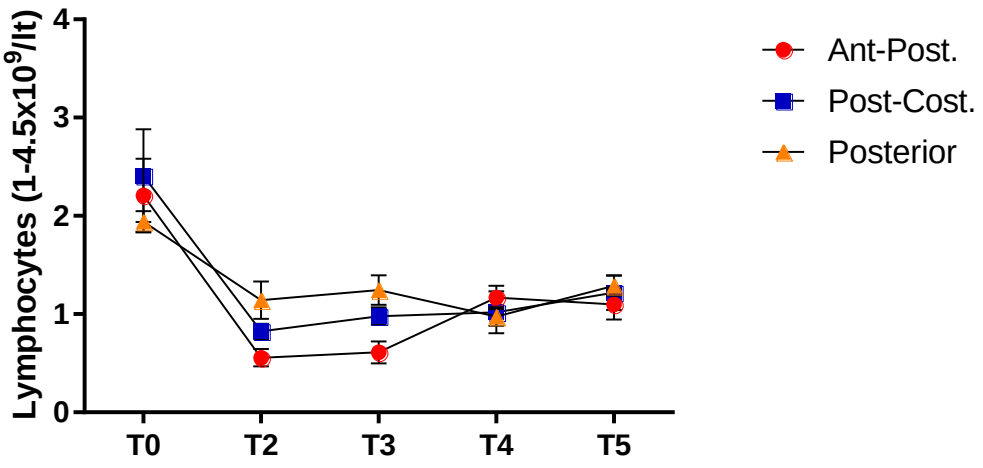


Figure 72: Lymphocytes concentration over time, comparison between different approaches. Lymphocytes were significantly decreased between T2-T4 in all the approaches, however the antero-posterior approach presented significantly lower levels at T2 and T3, compared to the posterior approach. Graphic was presented as mean with SEM.

In the posterior approach, lymphocytes were significantly decreased below T0 at T2, T3, and T4, all $p<0.05$. Similarly, in the antero-posterior approach, lymphocytes were significantly decreased at T2 and T3, compared to T0, all $p<0.05$. Lastly, in the posterior-costoplasty approach, lymphocytes were lower at T2 and T4, compared to T0, all $p<0.05$ (Table 62a).

A series of Kruskal-Wallis tests were conducted, showing significant differences among the posterior and antero-posterior approaches at T2 and T3, with lymphocytes being significantly higher in the posterior approach, at both time points, all $p<0.05$ (Table 62b).

Therefore, lymphocytes were decreased at T2, T3, T4 in the posterior approach, at T2, T3 in the antero-posterior approach, and at T2, T4 in the posterior-costoplasty approach. Moreover, lymphocytes showed significantly lower levels in antero-posterior approach at T2 and T3, compared to posterior approach.

Table 62: A) Outcome of Friedman non-parametric tests between the time points of each approach and B) outcome of Kruskal-Wallis tests between all three approaches, for every time point (Lymphocytes).

A	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Ant.-Post.	0.0006	0.0140	-	-	-	-	-	-	-	-	-	-	-	-
Post.-Cost.	0.0270	-	0.0373	-	-	-	-	-	-	-	-	-	-	-
Posterior	0.0011	0.0195	< 0.0001	-	-	-	-	-	-	-	-	-	-	-

B	T0	T1	T2	T3	T4	T5
Posterior vs. Ant.-Post.	-	-	0.0141	0.0296	-	-
Posterior vs. Post.-Cost.	-	-	-	-	-	-
Ant.-Post. vs. Post.-Cost.	-	-	-	-	-	-

Neutrophils analysis

The results from a series of Friedman non-parametric tests, revealed significant neutrophils concentrations differences over time in all three approaches (Figure 73). (ANOVA: posterior Friedman=42.40, $p<0.0001$, posterior-costoplasty Friedman=16.85, $p=0.0021$, antero-posterior approach Friedman=12.93, $p=0.0116$).

More specifically, as illustrated in Figure 73, Table 63, in the posterior approach, neutrophil concentration reached a significantly higher level at T2 and T3 compared

to T0, T4, T5 and T0, T5, respectively, all p<0.05. In the antero-posterior approach, neutrophil concentration was only significantly increased at T2, compared to T0, p=0.0102. In the posterior-costoplasty approach, significantly higher levels were found at T2 and T3, compared to T0, all p<0.05 (Table 63).

The results from a series of Kruskal-Wallis tests suggested no significant differences among the three approaches at any time point, all p>0.05.

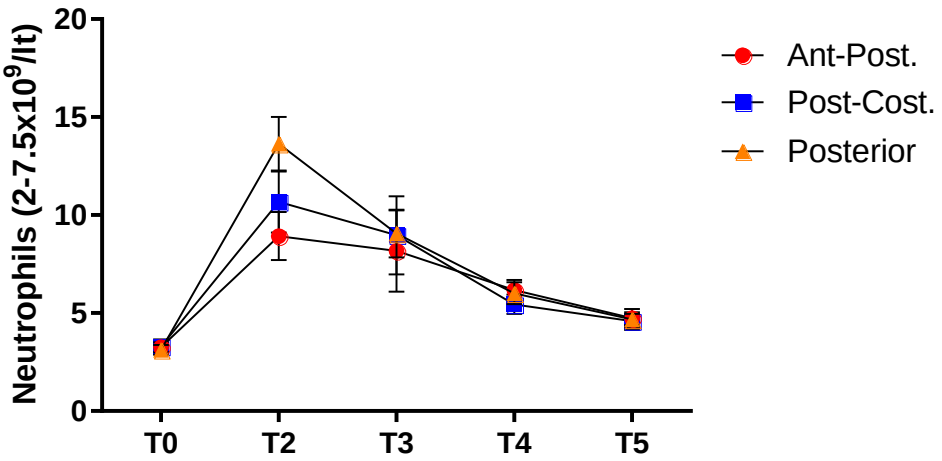


Figure 73: Neutrophils concentration over time, comparison between different approaches. Neutrophils were significantly elevated at the end of the surgery (T2) in all the approaches, however without significant differences between them. Graphic was presented as mean with SEM.

Therefore, neutrophils were significantly elevated at the end of the surgery (T2) in all the approaches. In the posterior and posterior-costoplasty approaches, concentrations remained elevated at T3. Moreover, no significant differences were observed among the three approaches at any time point.

Table 63: Outcome of Friedman non-parametric tests between the time points of each approach (Neutrophils).

	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Ant.-Post.	0.0102	-	-	-	-	-	-	-	-	-	-	-	-	-
Post.-Cost.	0.0032	0.0137	-	-	-	-	-	-	-	-	-	-	-	-
Posterior	< 0.0001	< 0.0001	-	-	-	-	-	-	-	0.0125	0.0006	-	0.0451	-

Platelets analysis

A series of Friedman non-parametric tests, revealed significant platelet concentrations differences over time in all three approaches (ANOVA: posterior Friedman=33.13, p<0.0001, posterior-costoplasty Friedman=16.12, p=0.0029, antero-posterior approach Friedman=16.67, p=0.0022) (Figure 74).

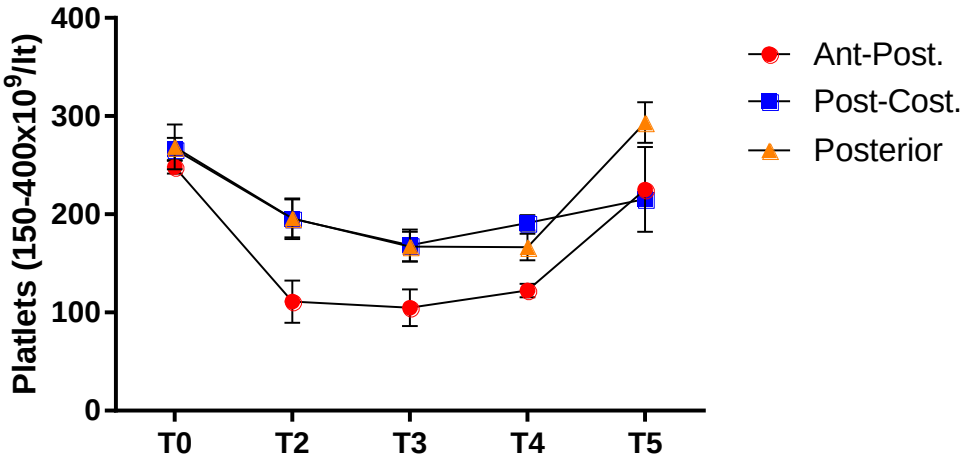


Figure 74: Platelets concentration over time, comparison between different approaches. Platelets presented the lowest concentration at T3 in all the approaches. The antero-posterior approach presented significantly lower levels of platelets, compared to the other approaches. Graphic was presented as mean with SEM.

More specifically, in the posterior approach, platelets concentration presented significantly lower levels at T3 and T4, compared to T0 and T5, all p<0.005. Similarly, in the antero-posterior approach significantly lower levels of platelets were found at T2 and T3, compared to T0, all p<0.05. In the posterior-costoplasty approach, platelet only showed a lower level at T3, compared to T0, p=0.0014 (Table 64a).

A series of Kruskal-Wallis tests were conducted between the three approaches at each time point, showing significant differences of platelets concentration among the approaches at T2 (p=0.0415), T3 (0.0414), and T4 (p=0.0161). However, multiple comparisons demonstrated a relation only between antero-posterior and posterior-costoplasty approaches at T4, where platelets concentration was significantly lower in the antero-posterior approach, p=0.0189 (Table 64b).

In summary, platelets levels were significantly decreased at T3 in all the approaches and additionally at T2 and T4, T5 in the antero-posterior approach and posterior approach, respectively. Furthermore, antero-posterior approach presented significantly lower levels of platelets, compared to the other groups.

Table 64: A) Outcome of Friedman non-parametric tests between the time points of each approach and B) outcome of Kruskal-Wallis tests between all three approaches, for every time point (Platelets).

A	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Ant.-Post.	0.0349	0.0191	-	-	-	-	-	-	-	-	-	-	-	-
Post.-Cost.	-	0.0014	-	-	-	-	-	-	-	-	-	-	-	-
Posterior	-	0.0030	0.0002	-	-	-	-	-	-	-	-	-	0.0018	0.0001

B	T0	T1	T2	T3	T4	T5
Posterior vs. Ant.-Post.	-	-	-	-	-	-
Posterior vs. Post.-Cost.	-	-	-	-	-	-
Ant.-Post. vs. Post.-Cost.	-	-	-	-	0.0189	-

Prothrombin (PT) analysis

There were significant changes of PT over time in all three approaches using a series of Friedman non-parametric tests (ANOVA: posterior Friedman=21.50, p=0.0003, posterior-costoplasty Friedman=16.73, p=0.0022, antero-posterior approach Friedman=19.52, p=0.0006) (Figure 75).

In both posterior and antero-posterior approaches, PT was significatively elevated at T2 and T3, compared to T0, all $p<0.05$. Also, in posterior and posterior-costoplasty approaches, PT dropped significantly at T5, compared to T3, all $p<0.05$ (Figure 80, Table 65a).

A series of Kruskal-Wallis tests were then conducted to test for differences among the three approaches, detecting a statistically significant higher PT, for the posterior approach only at T5, compared to the other two approaches, all $p<0.05$ (Table 65b).

Therefore, the results suggested that PT significantly increased to a higher level at T2 and T3, compared to T0, in both posterior and antero-posterior approaches, for after return to base line quite simultaneously. Moreover, antero-posterior and

posterior-costoplasty approaches presented a significantly lower PT, compared to posterior approach at T5.

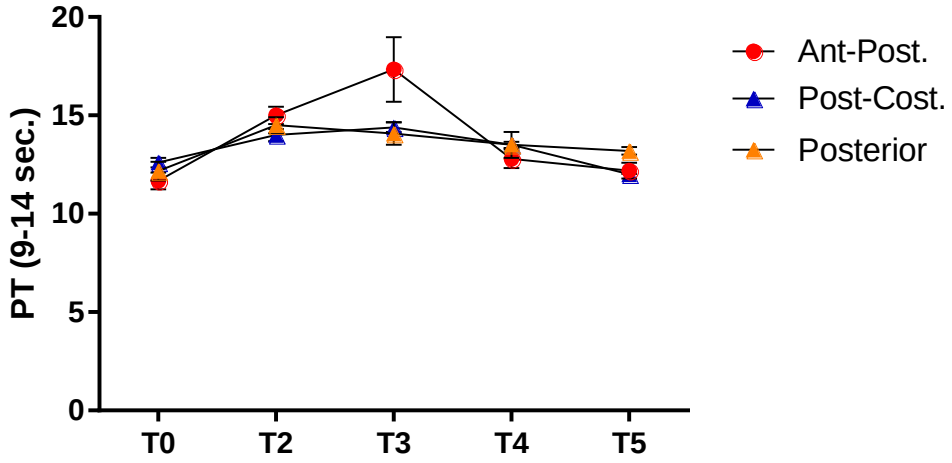


Figure 75: PT concentration over time, comparison between different approaches. PT was significantly increased at T2 and T3, in all approaches, compared to T0. The antero-posterior and posterior-costoplasty approaches presented a significantly lower PT, compared to posterior approach at T5. Graphic was presented as mean with SEM.

Table 65: A) Outcome of Friedman non-parametric tests between the time points of each approach and B) outcome of Kruskal-Wallis tests between all three approaches, for every time point (PT).

A	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Ant.-Post.	0.0191	0.0037	-	-	-	-	-	-	-	-	-	-	0.0466	-
Post.-Cost.	-	-	-	-	-	-	-	-	-	-	-	-	0.0137	-
Posterior	0.0005	0.0023	-	-	-	-	-	-	-	-	-	-	-	-

B	T0	T1	T2	T3	T4	T5
Posterior vs. Ant.-Post.	-	-	-	-	-	0.0421
Posterior vs. Post.-Cost.	-	-	-	-	-	0.0361
Ant.-Post. vs. Post.-Cost.	-	-	-	-	-	-

Partial thromboplastin (PTT) analysis

A series of Friedman non-parametric tests were conducted to examine time effect in each approach, showing significant changes of PTT over the time in all three approaches (ANOVA: posterior Friedman=24.02, p=0.0001, posterior-costoplasty Friedman=14.67, p=0.0054, antero-posterior Friedman=13.33, p=0.0098) (Figure 76).

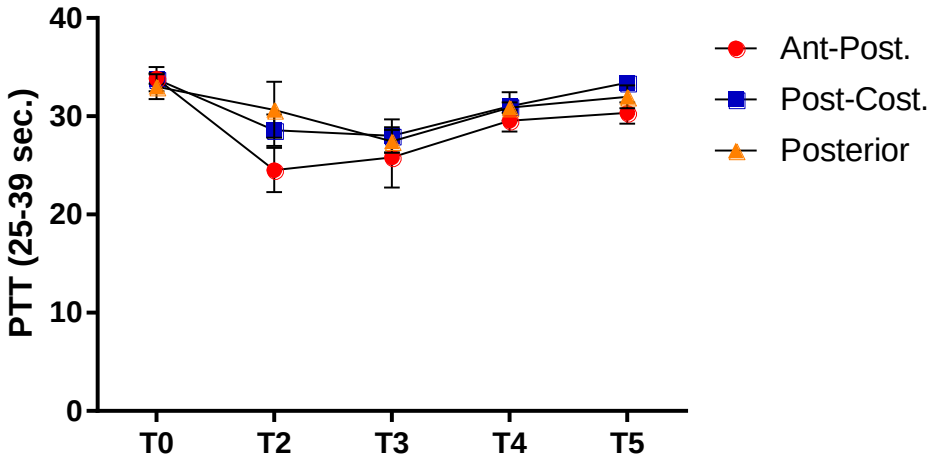


Figure 76: PTT concentration over time, comparison between different approaches. PTT was lower at T2 and T3 in all the approaches, compared to T0. No significant differences were found among the three approaches at any time point. Graphic was presented as mean with SEM.

In the posterior approach, PTT was significantly lower at T3, compared to T0 and T5, all p<0.005. In both antero-posterior and posterior-costoplasty approaches, PTT was significantly reduced at T2 and T3, compared to T0, all p<0.05 (Table 66). Moreover, no significant differences among the three approaches at any time point were observed using Kruskal-Wallis tests, all p>0.05.

Table 66: Outcome of Friedman non-parametric tests between the time points of each approach (PTT).

	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Ant.-Post.	0.0102	0.0349	-	-	-	-	-	-	-	-	-	-	-	-
Post.-Cost.	0.0373	0.0373	-	-	-	-	-	-	-	-	-	-	-	-
Posterior	-	0.0002	-	-	-	-	-	-	-	-	-	-	0.0018	-

Haemoglobin (Hb) analysis

Intraoperative blood-loss and Hb concentration are important elements able to influence the post-operative outcome, requiring constant peri-operative monitoring. Typical blood loss for AIS correction procedures may exceed the 50% of patient's total blood volume and is directly related to the number of vertebrae involved, surgical techniques utilised, duration of surgery and anaesthetic factors (blood pressure, assisted ventilation)[232]. The intent of this section was to study the variation of intra- and post-operative Hb concentrations, in three different approaches. However, due to the characteristics of this cohort and the clinical priorities, the Hb concentration analysed is the final result following intra-operative and post-operative red cells transfusion (intra-operative: from blood bank and/or auto-transfusion from cell saver device, post-operative from blood bank only) (Table 67).

Table 67: Blood loss and blood transfusion over time, for all the approaches.

Posterior approach (Total)	**T0 (ml)**	**T1 (ml)**	**T2 (ml)**	**T3 (ml)**	**T4 (ml)**
Blood transfusion	-	55	75	50	75
Auto-transfusion (cell saver)	-	136	576	-	-
Blood loss	-	Not recorded	1.998 Total	-	-
Posterior approach (Fast)	**T0 (ml)**	**T1 (ml)**	**T2 (ml)**	**T3 (ml)**	**T4 (ml)**
Blood transfusion	-	0	0	86	-
Auto-transfusion (cell saver)	-	221	570	-	-
Blood loss	-	Not recorded	1.964 Total	-	-
Posterior approach (Slow)	**T0 (ml)**	**T1 (ml)**	**T2 (ml)**	**T3 (ml)**	**T4 (ml)**
Blood transfusion	-	100	129	0	129
Auto-transfusion (cell saver)	-	52	689	-	-
Blood loss	-	Not recorded	1.752 Total	-	-
Post.-costoplasty approach	**T0 (ml)**	**T1 (ml)**	**T2 (ml)**	**T3 (ml)**	**T4 (ml)**
Blood transfusion	-	0	240	-	120
Auto-transfusion (cell saver)	-	0	318	-	-
Blood loss	-	Not recorded	1.239 Total	-	-
Antero-posterior approach	**T0 (ml)**	**T1 (ml)**	**T2 (ml)**	**T3 (ml)**	**T4 (ml)**
Blood transfusion	-	0	200	50	50
Auto-transfusion (cell saver)	-	0	653	-	-
Blood loss	-	Not recorded	2.478 Total	-	-

The results from a series of Friedman non-parametric tests revealed significative changes of haemoglobin concentrations over time in all three approaches (ANOVA: posterior Friedman=26.83, p<0.0001, posterior-costoplasty Friedman=17.58, p=0.0015, antero-posterior Friedman=14.27, p=0.007) (Figure 77).

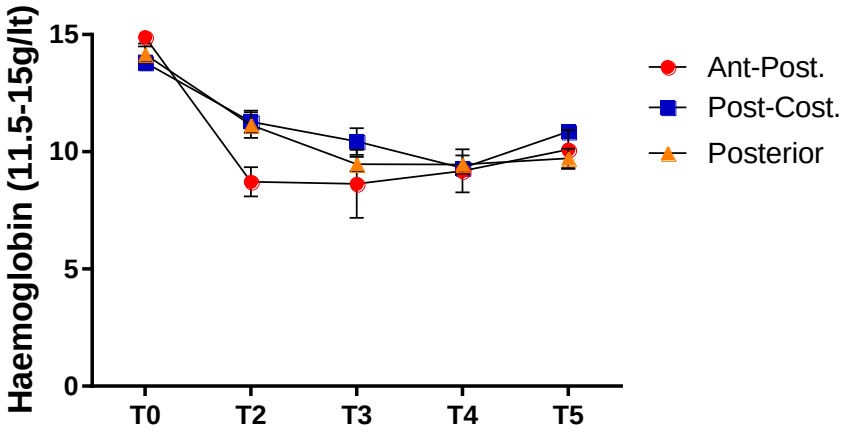


Figure 77: Hb concentration over time, comparison between different approaches. In all approaches Hb level was significantly reduced between T3 and T5. The antero-posterior approach presented the lowest Hb level, at the end of surgery (T2) and for the next 24 hours (T3). Graphic was presented as mean with SEM.

In particular, the posterior approach, showed significantly lower Hb levels at T3, T4, and T5, compared to T0, all p<0.005. Similarly, in the antero-posterior approach, significantly lower Hb levels were presented at T3 and T4, compared to T0, all p<0.04. Contrarily, the posterior-costoplasty approach presented significantly reduced Hb levels only at T4, compared to T0, p=0.001 (Table 68a).

A series of Kruskal-Wallis tests were performed to test for differences of Hb levels among the approaches in each time point, presenting statistically significant differences only at T2 (Table 68b). At the end of the surgery, the antero-posterior approach showed the lowest Hb level, compared to the other two approaches, despite the red cells transfusion received, all p<0.05 (Tables 67, 68b).

Therefore in both antero-posterior and posterior approaches, Hb level was significantly reduced at T3, T4 and T5, respectively. In the posterior-costoplasty approach, Hb level was significantly decreased only at T4. In addition, at the end of surgery (T2) and for the next 24 hours (T3), participants who underwent the antero-

posterior approach demonstrated a significantly lower level of haemoglobin, compared to the other two approaches.

Table 68: A) Outcome of Friedman non-parametric tests between the time points of each approach and B) outcome of Kruskal-Wallis tests between all three approaches, for every time point (Hb).

A	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5	T1 vs T2	T1 vs T3	T1 vs T4	T1 vs T5	T2 vs T3	T2 vs T4	T2 vs T5	T3 vs T4	T3 vs T5	T4 vs T5
Ant.-Post.	-	0.0349	0.0052	-	-	-	-	-	-	-	-	-	-	-
Post.-Cost.	-	-	0.0010	-	-	-	-	-	-	-	-	-	-	-
Posterior	-	0.0002	0.0002	0.0018	-	-	-	-	-	-	-	-	-	-

B	T0	T1	T2	T3	T4	T5
Posterior vs. Ant.-Post.	-	-	0.0274	-	-	-
Posterior vs. Post.-Cost.	-	-	-	-	-	-
Ant.-Post. vs. Post.-Cost.	-	-	0.0220	-	-	-

4.11.6 Cytokines dynamics, inflammatory response and surgical outcome, overall comparison

Concerning the cytokine differences over a week period, in the antero-posterior approach IL-6 and IL-10 presented significantly elevated concentrations at T2 and the level remained elevated at T3 (Figures 60, 61). Contrary, IL-18 slightly dropped at T2, in all the approaches, for after reach the highest level at T5, however only the posterior and the antero-posterior approaches showed statistically significant results at T5, compared to T2. PCT, level was significantly elevated at T3 and T4 in both posterior and antero-posterior approaches. PCT kinetics were similar to IL-6 and IL-10, but PCT concentration became higher at T3, T4 time-points, instead of T2 and T3 of IL-6 and IL-10. With respect to the differences among the three approaches at each time point, IL-6 level was significantly higher in the antero-posterior, compared to the other approaches. IL-10 was significantly higher in antero-posterior, but only compared to posterior approach and IL-18 and PCT showed no significant differences at any time point.

The duration of surgery affected the kinetics of all the cytokines examined. In particular, although IL-6 did not show any significant difference between the two groups at any time point, concentration was higher in the slow group from T2 to T4. Moreover, IL-10 presented higher levels at T1 and T3 in the slow group and both

groups gave a higher pick at T2. Similarly to IL-6, IL-18 concentration presented very similar kinetics for both groups, with no significant differences between them. In both groups IL-18 was significantly raised at T5. Lastly, although PCT concentration was high at T2 for both groups, the increment was significant only in the slow group. Furthermore, the slow group presented a significative higher PCT concentration at T5, compared to the fast group. Concluding, regardless the degree of significance, all cytokines presented higher concentrations in the slow group of surgery.

Concerning the comparison between genders and cytokine concentrations, IL-6 in the posterior approach increased significantly from T2 to T4 in females, contrary males presented only a pick at T3. In the antero-posterior approach, IL-6 presented overall higher concentrations, compared to posterior approach with both genders showing a pick only at T2. IL-10 concentrations, although appeared higher in the posterior approach at T1 and T2 in females, inversely in the antero-posterior approach appeared higher in males at T1 and T2. Similarly to IL-6, the overall IL-10 concentration was higher in the antero-posterior approach, compared to posterior approach. IL-18 concentrations, similarly to previous comparisons, presented a characteristic drop at T2 in both genders and approaches, which however it was not statistically significative. Moreover, IL-18 was significantly elevated at T5 for both approaches and genders, compared to T2. PCT levels presented a statistically significant increment at T2 and T4, only in the posterior approach in the female group. Furthermore, similarly to IL-6 and IL-10, PCT concentrations were higher in the antero-posterior approach, compared to posterior approach. In none of the cases, were detected significant differences on either genders between approaches.

Leukocytes concentrations in the posterior approach were increased at T2 and T3 for after return to basal level at T5. In the posterior-costoplasty and the antero-posterior approaches leukocytes increased at T2 and T3, respectively, compared to T0. Contrarily, lymphocytes were decreased at T2 to T4 in the posterior approach. Similarly, they were decreased at T2, T3 and at T2, T4 in the antero-posterior and posterior-costoplasty approaches, respectively. Moreover, lymphocytes presented significantly lower levels in the antero-posterior approach, compared to posterior approach. Neutrophils were significantly elevated at T2 in all the approaches and in both posterior and posterior-costoplasty approaches, concentrations remained elevated at T3. Moreover, with the exception of lymphocytes, no significant differences were observed among the three approaches at any time point, concerning leukocytes and neutrophils.

Regarding coagulation markers, platelets levels were significantly decreased at T3 in all three approaches and at T2 and T4, T5 of the antero-posterior approach and posterior approach, respectively. As expected, the antero-posterior approach presented the lowest level of platelets, compared to the other approaches. PT increased significantly at T2 and T3, from baseline in both posterior and antero-posterior approaches. Moreover, antero-posterior and posterior-costoplasty approaches presented a significantly lower PT at T5, compared to posterior approach. PTT was significantly decreased at T3 in all three approaches. Furthermore, the antero-posterior and posterior-costoplasty approaches, presented a lower level of PTT at T2. Moreover, no significant differences were observed among the three approaches at any time point.

Hb level was significantly reduced, in both antero-posterior and posterior approaches, at T3, T4 and T5, respectively. Hb level of the posterior-costoplasty approach, was significantly decreased only at T4. As expected at T2 and T3, the antero-posterior approach showed the lowest level of Hb, compared to the other approaches.

With regards to spinal infections, from a cohort of 23 patients studied, two developed a late spinal infection (few months after the discharge), which did not present any apparent signs or symptoms during their in-hospital stay. Donor AF22 was tested positive for staphylococcus aureus, however, for donor AF26 it has not been possible to identify the pathogen despite the clinical signs of infection. Both donors were treated successfully with six weeks of antibiotics. Although their cytokines, in addition to clinical and biochemical parameters were investigated, in an attempt to identify any possible elements for early signs of infections, it was not possible to reach a clear conclusion. Both clinical and biochemical parameters were within the physiologic ranges and despite cytokine levels presented at few time-points higher concentrations, compared to the average, this element in isolation was not sufficient to use it as early predictive marker of infection (Tables 69, 70).

Concerning the spinal fusion, it was ascertained radiologically, considering fused a spinal segment which present bridging callus across the treated vertebrae on two orthogonal views on plain X-ray films. As it was expected, given the young age, all patients presented satisfactory healing at 12 months following AIS correction, regardless the approach performed.

Table 69: Comparisons between median concentrations of cytokines, with the single concentration of the two infected donors. Differences were calculated for every time-point. Concentrations are expressed in pg/ml.

	Posterior-Costoplasty			**Posterior**		
IL-6	Median values	**AF22** values	Fold-difference	Median values	**AF26** values	Fold-difference
Time 0	0.1	0.0	-0.1	0.1	0.1	0.0
Time 1	2.8	2.7	-0.1	4.0	2.1	-1.8
Time 2	22.4	70.4	48.0	25.9	5.5	-20.3
Time 3	24.8	17.6	-7.2	41.2	43.7	2.5
Time 4	52.3	37.7	-14.6	33.6	23.5	-10.1
Time 5	11.9	22.3	10.4	11.6	Discharged	
Time 6	16.6	10.9	-5.7	7.6	Discharged	
IL-10						
Time 0	0.8	0.6	-0.2	0.7	0.6	-0.1
Time 1	0.8	0.8	0.0	1.0	0.7	-0.3
Time 2	1.2	0.3	-0.9	1.0	1.2	0.2
Time 3	0.5	0.6	0.1	0.3	0.4	0.1
Time 4	0.3	0.2	-0.1	0.3	0.1	-0.2
Time 5	0.5	0.5	0.0	0.5	Discharged	
Time 6	0.3	0.2	-0.1	0.1	Discharged	
IL-18						
Time 0	44.0	43.1	-0.8	30.7	51.0	20.2
Time 1	44.0	45.6	1.5	32.4	39.7	7.3
Time 2	33.9	33.9	0.0	22.7	33.6	10.9
Time 3	41.8	45.7	3.8	36.7	37.2	0.5
Time 4	46.5	44.7	-1.8	39.7	42.6	2.8
Time 5	53.0	64.5	11.5	48.6	Discharged	
Time 6	36.2	36.5	0.3	40.5	Discharged	
PCT						
Time 0	11.4	12.7	1.3	10.2	10.2	0.0
Time 1	19.7	7.6	-12.1	11.6	8.5	-3.0
Time 2	30.8	29.4	-1.4	33.8	58.2	24.4
Time 3	11.5	10.4	-1.0	16.7	23.1	6.4
Time 4	38.5	38.5	0.0	36.9	38.5	1.6
Time 5	17.5	16.3	-1.2	17.6	Discharged	
Time 6	5.4	5.1	-0.3	5.9	Discharged	

With regards to the cohort demographics and surgical outcome, the antero-posterior group presented an equal gender distribution and as it was expected the surgical time was longer, compared to the other approaches. Given the surgical complexity, these donors spent more time in intensive or higher dependency care units and required a longer in hospital stay before discharged (Table 70). The posterior-costoplasty group, considering also that costoplasty was mainly performed as an aesthetic correction rather than a functional necessity, was made only by females. Furthermore, the duration of surgery was similar to posterior approach, donors presented the shorter length of hospital stay and they did not require intensive or higher dependency care (Table 70). Lastly, the posterior approach was the most populated and gender distribution presented 1:2 M/F ratio. Moreover, appeared that the faster group of surgery, had higher requirements of intensive dependency care (Table 70). Additionally, for all three approaches were used similar amounts of pedicle screws and a similar number of vertebrae were involved in the correction (Table 70).

Table 70: Demographics and outcomes for the three different approaches.

	F/M	**Age**	**HDU/ICU LOS** (days)	**Total LOS** (days)	**Duration of Surgery** (hours)	**Infections**	**N. of screws**	**Number of vertebrae involved**
Antero-posterior	3F/3M	14.7	3	8.5	10	0	15	11
Post.-costoplasty	5F	15.9	0	4	5.6	1	16	10
Posterior (Total)	8F/4M	15.8	1.5	6	5.1	1	16	10
Posterior (Fast)	4F/2M	15.9	2	6	4.1	1	16	11
Posterior (Slow)	4F/2M	15.6	0	6	6.1	0	18	13

5. Discussion

This thesis is composed by two main units, the comparison of MSCs populations from ICBM and VBBM, isolated using two different processing methods (density gradient concentration and red cells lysis) and the immuno-inflammatory response following surgery.

The purpose of the MSC component of the study, using a well-established methodology and a robust cohort, was to determine whether the VB, a well known hematopoietic stem cells reservoir[229-231], could provide comparable MSCs' numbers, with those in IC, in donor-matched samples. Hence, providing an additional source of MSCs for grafting augmentation during spinal procedures. Moreover, a second hypothesis tested was whether VB-MSCs had a similar or even better proliferation and differentiation potentials compared to IC-MSCs. Furthermore, the third hypothesis tested in the study, was to investigate the effectiveness of red cells lysis technique (AC) compared to density gradient concentration technique (LMP) for MSCs isolation for both ICBM and VBBM.

The potential benefit of these findings, would be to minimize the need for IC harvest, in many cases, and to provide an additional source of MSCs both for patients requiring extensive grafting (spinal revision surgery, extended spinal instrumentation) and for those with limited availability of ICBM due to anatomic variability or in case they had previous irradiation of the pelvis (due to cancer), where may not be an adequate volume of ICBM to complete the spinal fusion.

Spinal arthrodesis is the ideal treatment for severe or progressive spinal conditions like spinal instability following extensive spinal decompression, spondylolisthesis, and several degenerative diseases. The success of the operation, often depends from the quality of the bone fusion; its failure (pseudoarthrosis) may lead to failure of metalwork, possible revision surgery, higher risk of infection, and pain[2, 98].

Among the factors that spinal surgeons can control to maximize the surgical outcome, is a robust bone fusion through bone graft augmentation. Currently, in their armamentarium there are mainly bone morphogenetic proteins, allograft scaffolds and autograft possibilities[8-12]. The first is an expensive alternative, which continue to show promising results, however, their application present limitations, are not devoid of complications and also several aspects of their mechanisms of action need to be

further clarified[8-12]. Corticocancellous autograft is the most common solution for bone graft augmentation and IC harvesting is considered the "gold standard" source. However, harvesting can become laborious, generate post-operative pain and could predispose patient to post-operative complications[8,11].

A valid alternative is represented by allograft material, especially when used in combination with BM-MSCs showing spinal fusion rates similar to autograft[8,11-13]. Furthermore, in terms of therapeutic concentration of MSCs and bone healing, it was reported by Hernigou et al, that grafting over 50.000 MSCs in bone defects was able to ensure a particularly effective bone healing[13]. Considering VB enumeration assays' results, the present study demonstrated that VBBM is comparable to ICBM, able to provide sufficient concentration of MSCs to use in graft augmentation.

Despite the fact that VBBM is a well known hematopoietic stem cells source, from cadaveric donors[229,231], for the last 50 years, and is used routinely in transplantation field[230]; its use as autologous bone graft in spinal surgery is not yet considered as a routine procedure despite the great benefits it can offer.

On technical ground, while aspiration from IC takes only a few minutes, aspiration from pedicle adds virtually no extra time, because pedicles have to be approached as part of the procedure for the pedicle screws implantation. Additionally, the practical difference between both procedures, is that VBBM harvesting can be virtually extended as far as the metalwork goes. BM can be aspirated from both pedicles of each vertebra, without adding extra time or resulting more painful for the patient in the post-operative period, as it does for IC aspirate, in case of multiple aspirations for optimal MSCs concentration with low haemodilution.

5.1 Bone Marrow: Harvesting

BM-MSCs from 19 donors were isolated using two different methods and selected though their ability to adhere on plastic surface under standard culture conditions[219]. MSCs were able to proliferate from single cells into colonies presenting the expected fibroblastic-like morphology with long spiky tentacles and growing together in a radial-growth pattern. CFU-F, flow cytometry and trilineage differentiation assays were performed in order to confirm the MSC nature of the harvested cells, fulfilling the ISCT criteria[69], for the definition of MSCs.

Consistency of anatomic structures involved, aspiration and isolation techniques are deemed as the most important aspects of a study, because they can influence the quality of all the forthcoming results. Isolating the maximum number of cells with the minimum blood dilution is crucial, therefore, 1-4ml of BM has been described as the optimum aspiration volume[170,173,229]. However, bigger quantities (8-10ml) with similar results, in terms of cell concentration, were also reported[7,53,173]. Haemodilution is a well documented condition, however in a clinical scenario, the priority is to harvest the highest number of MSCs per singe aspirate using the most effective technique. To a certain extent the haemodilution factor can be overcame intraoperatively, using bone marrow aspirate centrifugation devices, to concentrate the MSCs obtaining a high quality sample[233-235] and allowing direct MSCs application into the defect area. In this study 10ml of BM were harvested, from IC and VB, in order to fulfil the experimental requirements. Considering also the high BM cellularity, due to the young age of the subjects[51,173], a sufficient number of cells was isolated.

Additionally, concerning the safety of the BM harvesting procedure, 10ml of BM is far below the recommended aspiration volumes based on the bone marrow transplantation guide lines, which recommend a total volume of 1,000-1,200 ml from adult patients and 500ml from children (15-20ml/kg in adults and 10-15ml/kg in children). Moreover, following bone marrow aspiration, abnormalities were documented with skeletal scintigraphy, CT and MRI, mainly in patients undergoing conspicuous aspirations (1000ml approx.) for transfusion purposes. Signal changes in MRI, showing oedema, hyperaemia and proliferative activity were eventually detected at the aspiration sites 4 weeks after harvest[236] and the great majority of those donors experienced prolonged discomfort. Although, tissue repair was achieved, commonly after a year in a significant percentage of cases signal abnormalities were still persisting[236], however, without necessary a pathological

relevance. Nevertheless, the documentation of these findings is important for differential diagnosis purposes[237]. In this study, only 5 patients reported a transient mild discomfort from the IC, lasted 3-4 days. However, this discomfort could be also caused by the intra-operative positioning and not necessarily from the aspiration procedure itself. Moreover, all patients recovered satisfactorily as expected, during the in-hospital stay and the follow-up period.

Many studies have reported significant MSC heterogeneity *in vitro*, identifying three main factors:
1) harvesting technique used[51], culture aspects[68] (cell seeding density, media used) and donor's related factors[51,238,239] (age, gender, health status and different anatomic architecture of the cancellous bone where the cells where harvested);
2) existence of different subpopulations[185,221,240] within the BM-MSCs' aspirate, however not all of them may have a therapeutic relevance[241];
3) most recently, through studies investigating gene expression profiles and cellular markers[241-243], became more clear that native micro-environment from the donor site (topography), is one of the most important aspects, particularly concerning MSCs' differentiation[241,242,244].

Furthermore, it is important to consider that despite the different micro-environment of every native tissue[245], MSCs (including BM-MSC harvested from different bones[246]) are generally studied using identical methods for harvesting, selection expansion and induction. This, raises the question whether important subpopulations[242,247] of MSCs are lost, due to the ways they are isolated and expanded.

The mechanisms of higher osteogenicity/chondrogenicity of VBBM MSCs versus ICBM MSCs have not been analyzed here, however a variation in the genetic/epigenetic factors regulating the differentiation, could be proposed. A distinctive epigenetic signature has been reported explaining the variable differentiation potential between MSCs from BM and adipose tissue[268]. Moreover, to better explain the MSCs' function, Hox codes[248] could play an important role in determining a "genetic identification profile" unique to every MSC-type based on its function and topography.

In this study, despite the restricted age range and the same harvesting technique used, it was not possible to limited the donor-to-donor CV, which was ranging

between 85%-89% for both anatomic sites (85%IC AC, 89%IC LMP, 87%VB AC, 86%VB LMP). The interindividual variation in MSCs/ml of BM, it was reported by different authors and was consistent with previous studies[11,239] for both anatomic regions.

5.2 Bone Marrow: Comparison between LMP and AC

Two different methods were used to isolate potential MSCs from bone marrow. The first method (LMP), has been well characterised and uses density gradient centrifugation to separate the mononuclear layer from BM, which is next isolated through further washes and centrifugation. The second alternative method, uses AC salt to lyse the erythrocytes contained in the BM sample, selecting the leucocytes which are concentrated through centrifugation in the cell pellet.

Despite the well documented effectiveness of AC in ICBM[54-56], all available studies in the literature comparing IC vs VB, were using density gradient concentration technique (Table 44). Furthermore, LMP is able to isolate only MSCs that can "fit" within its density features (1.077 ± 0.001 g/ml), which is similar to MNC (monocytes and lymphocytes) density (≤ 1.077 g/ml)[54-56]. Moreover, LMP is characterized by a higher number of steps, compared to AC[57] and is a more operator-dependent technique, introducing potentially more variability.

Sufficient evidence is available from several authors[55,56,216,217], showing a significative loss of MSCs following use of LMP from ICBM, compared to AC. A possible explanation of these results, could be related to the presence of at least two subpopulations[185,221,240] of MSCs. Other authors suggested that MSCs may have a different grade of maturation, presenting different size/weight and level of activity, at the moment of aspiration[58]. Thus, LMP is not able to "hold trapped" within the interlayer all different MSCs and therefore a part of them, most likely the heaviest cells, are lost within the discarded component[54,55,57] (erythrocytes and granulocytes).

Contrarily, AC isolates MSCs using the principle of red cells lysis concentrating all leucocytes and not only MNCs. Hence, the potential loss of MSCs following AC procedure is significantly lower. Taking into account these findings, it appears more accurate to use AC for comparisons between different anatomic sites, and suboptimal the use of LMP. Up to date, there are several studies, comparing both

cells isolation methods from ICBM[56,57], but no data exist analysing VBBM using both techniques.

5.3 Comparison of MSCs findings with the literature

Summarising the enumeration assays findings of this study, cells count (total cells/ml), presented a statistically higher number of cells when AC was used for both IC ($20x10^6$/ml) and VB ($15x10^6$/ml), compared to LMP (IC $7.23x10^6$/ml, VB $5x10^6$/ml). Moreover, prevalence (CFU-F/10^6) of MSCs, showed that both IC and VB yielded slightly higher number of colonies when LMP was used (IC 40/10^6, VB 57/10^6), compared to AC (IC 34/10^6, VB 44/10^6), but without statistical significance. Most importantly from a clinical perspective, concentration of MSCs (CFU-F/ml), was statistically higher when AC was utilized for both IC (916/ml) and VB (840/ml), compared to LMP (IC 357/ml, VB 417/ml). However, no statistically significant difference was found between anatomic sites.

Comparison of these results with the current literature (Table 71) shows slightly higher total cells concentrations from ICBM, however, a clear trend was not observed, with respect to the anatomic site. Nevertheless, regarding MSCs concentration, authors Ahrens, *et al*[62], McLain, *et al*[52], Defino, *et al*[172], and Woo-Kie, *et al*[68], similarly to this study, showed higher MSCs/ml from VBBM. However, the difference was not always statistically significant, because they all used LMP.

A critical determinant of BM-MSCs capacity to enhance bone healing, is the ability to generate quickly a sufficient number of proliferating colonies able to promote chondrogenesis and osteogenesis. Risbud, *et al*[171], measured the CFU-F size from both IC and VB donor-matched samples, classifying colonies in three different sizes (small, medium, large), showing a statistically significant higher number of smaller colonies in VB, whilst IC presented a more uniform distribution of bigger colonies. This could suggest that VB-MSCs may have better mitogenic properties, justifying the higher number of smaller colonies. Furthermore, heterogeneous groups of MSCs and a statistically significative faster doubling rate of VB-MSCs has been also observed by authors Defino, *et al*[172] and Barbanti Brodano G, *et al*[173]. In this thesis a statistically significant difference in colonies size was showed between IC and VB, but only when AC was used; which confirms further the presence of more subpopulations which are not entirely selected by LMP.

Concerning gender-related differences in BM-MSCs, despite several reports and the recently discovered androgenic receptors on both genders' MSCs[242], given the uneven gender distribution of this cohort, due to the demographics of scoliosis, it was not possible to perform robust statistics in this respect. Nevertheless, in this study, but also from the IC vs VB comparison studies examined (Table 71), no significant difference between genders were observed.

With regards to the left or right side for the ICBM and VBBM aspiration, as it was also investigated by other authors[7,242], there is no anatomic basis to suggest a possible difference. Hence, this information was not recorded in this study.

Concerning donors' age, BM-MSCs are involved in the bone remodeling process not only following an injury, but also physiologically during the entire life span, therefore it is expected that their concentrations and functions are adapted accordingly. Also aging is associated with a significant age-related decline in MSCs concentration per single aspirate in both genders[250] and additionally with a significant decrement of BM osteoblastic progenitors in women, but not in men[250]. Scope of this research was to eliminate the age variability factor by restricting the age range. There are not well codified data on this subject, however the experience of Leeds MSCs group is consistent with previous studies reporting an age-related decline[51,173].

Different weaknesses have been observed, analysing the current literature (Table 71). Generally, the number of patients recruited was relatively limited[68,170-173]. Moreover, the BM harvesting technique and the anatomic site were not always constant, with the exception of only one study where BM-aspiration was performed consistently for the same vertebra[68]. Furthermore, despite the evidence of better results with AC[56,57,216], all the studies used LMP method for cells isolation[52,62,68,170,171,173]. In addition, in two studies VBBM and ICBM was not donor-matched[62,173]. In the great majority of the studies, donor's age and gender were given, however health status was rarely reported.

To complicate matters further, comparisons among BM harvested from patients and from cadavers were made, increasing the variability of the results[62,173]. Considering also, BM variability, based on the donors age[238,239], none of the studies performed a further analysis of the results in age-groups, despite the broad age range of patients analysed (~3-80 years old).

Table 71: Overview of the literature, comparing ICBM and VBBM.

Elements examined per paper / Papers listed in chronologic order	N. of patients	Mean age	Cadaveric study	Donor-matched VB / IC	Same vertebra used	BM harvesting technique (ml of BM)	Cell isolation	Cells counting/ml	CFU-F/10^6 VB vs IC	CFU-F/ml VB vs IC	Differentiation assays	Proliferation assays	Flow-cytometry (standard markers)	PCR (Gene analysis)
2004 Transplantation. Ahrens N[62] Mesenchymal stem cell content of human vertebral bone marrow.	3 VBBM 24 ICBM	n/a for all donors	Mixed	No	No	Transabdominal access (washout of VB)	Density gradient	$5.7x10^8$ (whole VB)	Not reported	11.6 VB 1.5 IC	Performed (No more info)	Not performed	IC=VB	Not performed
2005 Eur Spine J. Romih M[170] The vertebral interbody grafting site's low concentration in osteogenic progenitors can greatly benefit from addition of iliac crest bone marrow. (scoliosis correction)	12	31	No	Yes	No	Transpeduncolar access (2ml) (10ml syringe)	Density gradient	$3x10^6$ VB $4x10^6$ IC	7.7 VB 9.9 IC	23.1 VB 39.6 IC	Osteo IC>VB	Not performed	Not performed	Not performed
2005 J Bone Joint Surg Am. McLain RF[52] Aspiration of osteoprogenitor cells for augmenting spinal fusion: comparison of progenitor cell concentrations from the vertebral body and iliac crest.	21	59	No	Yes	No	Transpeduncolar access (8ml) (10ml syringe)	Density gradient	$19.8x10^6$ VB $16.9x10^6$ IC	19.62 VB 17.76 IC	465 VB 356 IC	Not performed	Not performed	Not performed	Not performed
2006 Spine (Phila Pa 1976). Risbud MV[171] Osteogenic potential of adult human stem cells of the lumbar vertebral body and the iliac crest.	15	48.1	No	Yes	No	Transabdominal or Transpeduncolar access (6ml)	Density gradient	Not reported	92 VB 23.5 IC	Not reported	Osteo IC=VB	IC≤VB	IC=VB	IC<VB Alkaline Ph. IC=VB Runx-2, Osteocalcin
2009 Spine (Phila Pa 1976). Defino HL[172] In vitro proliferation and osteoblastic phenotype expression of cells derived from human vertebral lamina and iliac crest.	3	Not reported	No	Yes	No	Lamina (bone chips)	Type II collagenase	IC=VB	Not reported	IC>VB (No more info)	Osteo IC>VB	IC<VB	Not performed	IC<VB Alkaline Ph, Runx-2 Osteocalcin, MSX2 BMP7, ICAM1 RANKL, OPG
2010 Eur Spine J. Min WK[68] Proliferation and osteoblastic differentiation of bone marrow stem cells: comparison of vertebral body and iliac crest.	14	54.7	No	Yes	Yes	Transpeduncolar access (4.5ml)	Density gradient	$3.65x10^7$VB $3.97x10^7$ IC	Yes (No more info)	$3.7x10^3$ VB $3.2x10^3$ IC	Osteo IC<VB	IC=VB	IC=VB	IC<VB Runx-2, Osteocalcin
2013 Eur Spine J. Barbanti Brodano G[173] Mesenchymal stem cells derived from vertebrae (vMSCs) show best biological properties.	Not reported	Not reported	Mixed	Not reported	No	Transpeduncolar access (10ml)	Density gradient + Erythrolysis	Not reported	Not reported	Not reported	Osteo IC<VB Adipo IC<VB Chondro IC<VB	IC<VB	IC=VB	Not performed

5.4 Flow cytometry, discussion

Currently the use of MSCs in orthopaedics to promote bone healing is being progressed and developed due to the unique characteristics of these cells, in terms of proliferation, differentiation and growth factor secretion at the application site (e.g. osseous defects).

CFU-F assay represent the most common method for MSCs analysis, however is a lengthy procedure and as it was highlighted from other authors[225,227], by being a plastic adherence–based cultivation method, may select only a portion of the MSCs population, leading to the loss of some poorly adherent, but highly potent MSCs.
Flow cytometry is a faster method and is based on extra- and intra-cellular markers detection. It was performed with the aim to confirm and study further MSCs nature, as per ISCT criteria[69,222], in addition to differentiation and CFU-F assays.

Although AC- and LMP-harvesting methods are commonly accepted for BM-MSCs sample preparation, for flow cytometric characterization little data exist regarding the effects of these procedures[226] on the physical and biochemical properties of the MSCs. Furthermore, from the literate review performed, a comparison between harvesting methods, was reported by few authors[224,226,251] based only on ICBM-MSCs, but not on VBBM-MSCs. Additionally, to increase the complexity, it is established that different subpopulations of BM-MSCs exist[223,225,251] and also is known that there is an intra-donor variability regarding BM-MSCs harvested from different anatomic sites[51] of the same donor. Despite CD271 represent an ideal marker for the identification of all the subpopulation of MSCs, its use is mainly indicated for fresh-harvested MSCs, as shown in previous publications[191,193,223,224] and not in frozen MSCs used in this study.

Moreover, on a technical ground, MSCs' size can influence the light scatter signals (large cells will refract more light, leading to high forward scattered light and vice versa)[224]. Also, changes in the cell membrane (smoothness) due to the sample preparation technique used, can influence the index of the light's refraction[224]. Additionally, side-scattered light can be also affected by the internal complexity of the cells[224]. Hence, the aim of the flow cytometry was also to investigate whether ICBM-MSC differs from VBBM-MSCs especially when different isolation methods were employed.

Moreover, studying the ALP intracellular marker[187,188] and MSCA1 extracellular marker[182-186], it was possible to investigate to some extent the osteogenic predisposition of MSCs. This method can provide a "proof of principle" that the whole BM-MSCs population can be directly phenotypically characterized, without necessarily utilising cell-culture techniques.

All the MSCs standard characterization markers (CD105, CD90, CD73), presented a similar MFI pattern with CD90 showing always the highest intensity, for both VB and IC, regardless the harvesting method used. Moreover, CD90 from IC AC-harvested MSCs, was the only marker to present a statistically significant higher MFI ($p=0.048$), compared to IC LMP.

Regarding the overall outcome from all 4 donors, the intracellular ALP[187,188] staining, presented a higher MFI from LMP-harvested MSCs from both anatomic sites and the highest outcome from ICBM by 1.34-fold, compared to VBBM. Whereas the extracellular marker MSCA1[182-186], presented the opposite outcome with the highest MFI from AC-harvested MSCs, equally from both anatomic sites. For the number of donors examined, it was not observed any statistically significant difference between anatomic sites or harvesting method used, in terms of osteogenic predisposition in non-osteogenically differentiated MSCs.

SUSD2 extracellular marker for an additional MSCs characterization[181-183], presented similar results for both VB and IC. Nevertheless, LMP-harvested MSCs from VBBM presented a slightly higher MFI by 1,24-fold, however without any statistic significance.

Although different techniques were used (flow cytometry, CFU-F and calcium assays), in order to study MSCs' function and osteogenic ability, it was not always possible to perform a direct comparison between techniques. Moreover, whilst flow cytometry analyses mainly the presence of markers, colourimetric assays used, are based on cellular cultures, which are more dynamic, however can influence MSCs' behavior. In addition, MSCs proliferation rate and MSCs confluency, is one of the most important elements which can influence osteogenic differentiation[252] *in vitro*. Moreover, for same amount of incubation time, VB-MSCs were growing slightly faster compared to IC, as it was also observed from other authors[171-173], therefore VB cultures could had virtually "more time" to produce a higher calcium concentration.

5.5 Trilineage differentiation, discussion

The aim of the trilineage differentiation, was to prove retrospectively, the tripotenciality of the isolated MSCs, as per ISCT's position statement[69,222] and also to compare mainly the osteo-chondral capacity of BM-MSCs from VB and IC. In order to understand better the VBBM potential in a clinical setting, the MSCs' tripotenciality has to be compared as a whole, especially the chondro-osteogenesis axis and not only focusing on the osteogenic capacity on MSCs, as often happens.

However, before the final conclusions in this chapter, it is of paramount importance to highlight briefly the different embryogenic steps, regarding the enchondral ossification of the spine, in order to better appreciate the deep relation between the three components of the differentiation assays performed, especially between chondrogenesis and osteogenesis.

The development of the axial skeleton is derived from a synchronised migration of sclerotomal cells both along a rostral to caudal and towards the midline directions, surrounding the spinal cord and notochord and forming a long mesenchymal column through the process of the endochondral ossification[253]. This process, takes place in 3 stages (pre-cartilage, chondrification, and ossification), involving the gradual replacement of a temporary structure with a permanent one and is completed at about 25 years of age[253]. A less extensive version of the last two steps of the same process, is reactivated every time that a traumatic or surgical fracture occurs.

During this complex process, a sequence of events in chondrocyte behaviour coordinated with the actions of blood vessels, osteoclasts, osteoblasts and other factors (receptors, signalling molecules, transcription factors and cytoskeletal components regulating the chondrocytes' behaviour) occur, in such a way to prepare the cartilage matrix for the "invasion" by the cells of the ossification front[253,254].

Chondrocytes, following proliferation, pass through a transition stage called ‘pre-hypertrophic, to hypertrophic, increasing their volume and secreting, at the same time, extracellular matrix. Hypertrophic chondrocytes then die, via apoptosis or other unspecified mechanism, allowing to the invading elements of the ossification front (blood vessels, osteoclasts, osteoblasts and bone marrow cells), to colonise and mineralise the extracellular cartilaginous matrix, transforming it into bone[254].

Osteogenic induction for calcium assay, matrix mineralization AR assay and ALP activity was performed during 14 days instead of 21[200-202]. This decision was based on a pilot experiment performed before setting the differentiation assay, using the same cell density, but 2 different induction periods of 14 and 21 days. After 2 weeks the cell-layer reached confluency and cells started to lift off from the well, risking to alter the outcome of the assay. Hence, due to the continuous proliferation in combination with the mineralisation, which appear to have potential to continue even beyond the 21 days period, it was decided that 14 days of induction it was a safe period able to guarantee valid results, without compromising the outcome of the assay.

The ALP activity data of this study, supported also by AR and calcium assays, showed an overall superior osteogenic capacity from VBBM, compared to ICBM. Matrix mineralization is a result of continuous mineral deposition due to ALP activity, which has a critical role in osteogenesis. Moreover, ALP is involved in the cleavage of calcium phosphate groups and also contributes to a better colonization and consequent mineralization of the scaffolds *in vitro*[254].

With regards to chondrogenesis, it was performed using a three-dimensional-type of culture during 21 days of induction, compacting together a large number of cells in a spheroid-like shape following micro-centrifugation in eppendorf tubes. GAG accumulation was used as a marker of chondrogenesis and was evaluated spectrophotometrically and through pellet staining. For pellet cryo-preservation and sectioning OCT was employed. It is physically less stable, compared to paraffin or resin embedded sections, however it was preferred as provides a satisfactory definition of the fine details of the specimen[206,207]. Additionally, it is faster/simpler to set up, does not involve dehydration and also preserves better the antigenicity of the specimen, therefore the detection of antigens under microscope for future studies[207].
All 4 donors, showed statistically higher GAG concentrations from VB-MSCs, compared to IC-MSCs (VBBM: AC 44.3µg/ml, LMP 43.5µg/ml, ICBM: AC 11.7µg/ml, LMP 22.8µg/ml).

Adipogenic differentiation presented also a certain donor-to-donor heterogeneity and variability within the same well. Despite the homogeneous culture condition in the well, a small subset of MSCs did not show any fat droplets accumulation even at 21 days of induction. The concentration of differentiated MSCs within the well, appeared to be higher in the outer perimeter rather than the centre of the well, presenting areas

with a higher concentration of adipogenic MSCs and others less differentiated. Possibly, communication between adipocytes may had a role in this pattern of growth or merely it was for mechanical reasons due to the initial location of the cells when seeded on the well.

Moreover, based on Nile red/DAPI ratios, ICBM showed a higher adipogenesis/cell for both harvesting methods used, compared to VBBM. Even though adipogenesis assay is not an deal method to measure MSCs-proliferation, VBBM presented a certain predisposition to proliferation, based on DAPI outcome, which was higher compared to ICBM. Regarding the harvesting method used, LMP appeared to be related to higher predisposition to proliferation for both VB and IC-harvested MSCs, showing a smaller size of fat droplets. Contrarily, AC appeared to be related to an increment of adipogenesis for both anatomic sites, also associated to bigger fat droplets size.

Oil red-O semi-quantitative scoring system which provides a visual method of adipogenic quantification, it was attempted in this study, however, it was proven to be subjective (operator dependent), time consuming and not always correlating between Oil red-O and Nile red OD measurements. Additionally, given the heterogeneity of the cultures, it was not always straightforward to establish the appropriate grade. Furthermore, semi-quantitative scoring system does not take into account the intracellular variability of the fat droplets (different size droplets in the same cell), hence, this can influence the final scoring decision.

Alternatively, spectrophotometric measurement of adipogenesis, using Nile red/DAPI dyes, it was faster, not operator dependent and also it was not influenced by the morphologic characteristics of the fat droplets or the heterogeneity of the cultures. Moreover, the dual examination of the same wells, through fluorescent imaging and spectrophotometric readings, offered an additional level of control, regarding the MSCs' fat content.

Despite the thorough tissue culture techniques, all the trilineage differentiations shown a certain heterogeneous distribution of the staining within the same well, which was believed to be due to the heterogenic nature of MSCs[238,256].
In the available literature examined (Table 44), a complete trilineage differentiation assay was performed only in one study[173] showing better differentiation from VBBM for all three lineages. Osteogenic assay was performed in five studies, showing

better osteogenic performance from VB in two studies[68,173], even results in one[171] and better performance from IC in two[170,172]. Adipogenic and chondrogenic differentiation was performed only in one study[173] showing higher outcome from VBBM, however, without reporting a detailed analysis of the results. A better VB osteogenesis, was also supported by a higher runx-2, osteocalcin and alkaline phosphatase gene activity[68,171,172], which may be related to better bone healing results *in vivo*. Furthermore, the LMP outcomes from the literature are similar to the LMP-results of the present study. However, the use of AC-harvested MSCs added a more define perspective, offering a more realistic outcome considering the better efficiency of AC in isolating more MSCs.

An additional element for reflection, is whether VB-MSCs are genuinely more osteogenic/chondrogenic, compared to IC, or maybe VBBM contains more subpopulations of MSCs[257] which are possibly contributing to the higher VBBM performance. A supportive element towards this idea, in an attempt to explain the different results between AC and LMP; could be related to AC ability to isolate more efficiently the different subpopulations contained in the VBBM. Nonetheless, authors who compared single-cell expanded populations of donor-matched MSCs, from different tissues, showed that MSCs' differentiation ability were influenced by their tissue of residence[255]. Additionally, in these studies, all cultures were compatible with the ISCT criteria. Regardless the fact that the question on VBBM performance is still open, VBBM offers a series of advantages from a biological and technical perspective, for the spinal surgeon, compared to ICBM.

Nowadays becomes more clear that the ISCT definition of MSCs, despite the fact that represents a milestone in MSCs' field, its unable to "capture" the subtle differences in otherwise similar behaving MSCs. On the other hand, MSCs' tripotenciality is classically tested retrospectively, following *in vitro* exposure of MSCs to specific culture supplements, which are far from simulating the physiological environment. Also these methods, despite the considerable amount of knowledge provided, are not supplying information as to what MSCs look like in their naturally residence tissues. To address this issue, in addition to what already exist, better surface phenotype markers, potentially subdividing MSCs based on their subtle characteristics, together with improved experimental methods such as microarray, transgenic animal technologies, human and animal genomes, could provide precious information towards this direction, in the years to come.

5.6 Immuno-inflammatory component of the study: discussion

The purpose of the immuno-inflammatory component of the study, was to analyse the post-operative responses of the three most common approaches of AIS operation and correlate the pro-inflammatory (IL-6, PCT, IL-18) and the anti-inflammatory (IL-10) cytokines with clinical and biochemical parameters, during a week period. Moreover, the second aim was to analyse the effects of surgical duration and gender, in the three different approaches.

Based on the current literature review performed in this thesis, focusing mainly on the immuno-inflammatory aspect of the spinal surgery, the great majority of authors analysed the host defence response in adult polytrauma patients, associated eventually with spinal injuries[91-93]. Moreover, other authors investigated the local and systemic effects of herniated discs[101-107] and only few studies analysed the immuno-inflammatory response following AIS correction via posterior approach[99,100,102,261]. However, despite the pioneering work performed and the essential information provided, none of the studies analysed the antero-posterior and the posterior-costoplasty approaches, especially under the prism of IL-18 and PCT in addition to IL-6 and IL-10. Considering the above, the potential benefit of this component of the thesis, could be to provide new insight in a field where still little is known.

In patients undergoing scoliosis operation, surgical trauma is known to produce a transient impairment of immuno-competence affecting both the cellular and humoral arms of the immune system[99,100,261]. Nevertheless, in order to interpret better cytokines' behavior, data needed to be correlated with the biochemical parameters. Specifically, surgical stress in the antero-posterior approach resulted to be higher, followed by the posterior-costoplasty and next by the posterior approach. This outcome was consistent with author Suzuki *et al*[99], showing a transient reduction of lymphocytes concentrations between T2-T4, followed by a gradual return to the base line concentrations. Moreover, during the same period of time, leukocytes and neutrophils showed in contrast, an increment between T2-T3, however without differences between the approaches. Both these responses were in balance, showing a well maintained biological homeostasis[91,99].

IL-6 was selected as the most consolidated and reliable marker for the measurement of the magnitude of a surgical insult[91]. Moreover, it is a major mediator of the acute-phase response and presents a long half-life which makes IL-6 less transient and

readily measurable. In this study, consistently with other authors[163,261], IL-6 was increased 2 to 4 hours intra-operatively (T1), reached a peak within 6 hours (T2) and then maintained a plateau for up to 24-36 hours (T3,T4), followed by a gradual decrement to baseline between T5-T6. Similar to PCT, in the antero-posterior approach, IL-6 first reached its peak at T2 and then started decreasing gradually without presenting a plateau. Contrarily to PCT and IL-18, IL-6 increment in the antero-posterior approach it was statistically significant, compared to the other approaches, possibly also due to the discectomies performed (average 4), which could had contributed to the IL-6 increment[101-107].

IL-10 was selected as one of the most consolidated and reliable antiinflammatory markers, producing mainly immunosuppressive[123] and also protective effects against conspicuous cytokine release during a shock[123]. In the antero-posterior and posterior approaches, IL-10 level increased 2 to 4 hours intra-operatively (T1), reached a peak within 6 hours (T2), followed by a rapid decrement to baseline between T3-T4. Contrarily posterior-costoplasty approach, following a mild peak of IL-10 at T1, started decreasing gradually, returning to baseline at T3. Similarly to IL-6, IL-10 presented a statistically significant higher concentration, compared to the other approaches. IL-10 kinetics correlated with the severity of the operation and was consistent with other authors[163,266]. Moreover, the fast return to baseline could be explained due to the lack of other mechanisms (infection, shock) which could have triggered the IL-10 de novo synthesis, as suggested by author Peronnet *et al*[267].

PCT increases prevalently following bacterial infection, thus, it was selected as an element of differential diagnosis between a bacterial from another infection (virus)[150-157]. In contrast with current literature[265], suggesting that PCT levels did not increase following elective spinal surgeries in infection-free patients, this study provided additional evidence suggesting that non-infectious inflammatory stimuli, like major spinal surgery, when extremely severe can increase PCT levels[150-157]. In this study, PCT level increased 2 to 4 hours intra-operatively (T1), reached a peak within 6 hours (T2) and then maintained a plateau for up to 24-48 hours (T3,T4), followed by a gradual return to baseline between T5-T6. These findings were consistent with the literature[146-149], with the only variation of the antero-posterior approach where PCT concentration reached its peak at 24 hours (T3) and started decreasing gradually afterwards without presenting a plateau. This different behaviour of the antero-posterior approach, was consistent throughout the majority of the experiments performed, confirming the higher immuno-inflammatory stress, compared to the other

two approaches. However, no statistically significant differences were detected between the approaches.

IL-18 was selected as an early predictive factor in case of lethal postoperative sepsis[137] or respiratory complications like interstitial lung disease and pneumonia [139], which could be an important post-operative complication particularly in posterior-costoplasty and antero-posterior approaches. However, this cohort of patients did not present such complications, hence, although all approaches and genders presented significantly elevated levels at T5, compared to T2, it was not correlated to any clinical symptoms, including the two donors presenting late onset spinal infection. Furthermore, all approaches presented similar concentrations throughout, with a characteristic drop at T2, which it was not statistically significative.

Blood loss, transfusion requirements and clotting profile during spinal fusion for AIS remain a concern, despite the anaesthetic protocols[258,259], the use of cell saver devices and tranexamic acid to control bleeding[260]. The two arms of the coagulation cascade[165,166], external (PT) and intrinsic (PTT) pathways, behaved in an inversely proportional manner, with PT presenting a mild increment at T2 and T3, whilst contrarily PTT was decreased at the same time points in all three approaches. Also in this case, antero-posterior approach, presented the highest PT and the lowest PTT, followed by the other two approaches, but without any statistic significance between the lesser two. Moreover, concerning the platelets concentration, as it was expected, the antero-posterior approach presented a significative lower concentration at T2-T4, compared the other two. Also, the surgical stress often induced a hypercoagulable state, which was more frequent within the antero-posterior group. The outcomes were consistent with author Bosch *et al*[262], although in his study analysed only the posterior approach.

Concerning the comparison between genders and cytokine concentrations, although were not statistically significant differences between genders in the posterior and antero-posterior approach, few trends observed deserve further discussion. IL-6 contrarily to the current literature[91,97,263,264], suggesting elevated levels in males due to the estrogens down-regulation of the IL-6 function, in this study however, no gender-related differences were observed. Nevertheless, a high IL-6 concentration throughout all donors was observed between T2-T4, consistent with the literature[100,102,261]. IL-18, presented for both approaches and genders similar concentrations throughout, with a characteristic drop at T2, which however, it was not

statistically significant. IL-10 concentrations, although appeared higher in the posterior approach at T1 and T2 in females, and in males at T1 and T2 in the antero-posterior approach, the variation was not statistically significant. PCT levels presented higher concentrations at T2 and T4, only in the posterior approach in the female group, however, no differences were observed between the genders. Furthermore, the antero-posterior approach presented always higher concentrations of IL-6, IL-10 and PCT.

AIS correction is never an emergency operation, hence there is often time to optimise patients pre-operatively and adapt the anaesthetic procedure accordingly, in order to prevent post-operative complications, especially in patients with significant comorbidities. Based on patients' necessities and individual decision of the anaesthetist involved, different drugs were used intra-operatively[258,259] (propofol, remiphentanil, ketamine, phentephedrine, vecuronium, diazepam, ondansetron dexamethasone, midazolam). Additionally, antibiotics, as per trust protocol, (gentamicin, Flucloxacillin) and tranexamic acid to control bleeding[260], were also administered during the procedure. Moreover, according to post-operative patients' clinical responses, further medication was prescribed (fentanyl pump, paracetamol, diclophenac, tramadol, morphine, ibuprofen)[259,260]. As a result of all the poly-therapy prescribed, the intra- and post-operative clinical signs were affected and all appeared to be similar and within physiological ranges for all three groups, hence they did not show substantial differences which could have been used for comparisons.

5.7 Limitations of the MSCs component of the study

In this study the cells used for culture, enumeration, differentiation assays and flow cytometry, were cryopreserved rather than used fresh. This decision was made considering the prolonged duration of the surgeries, the lengthy MSCs-harvesting and culturing procedures, and logistic issues related to the hospital and laboratories. Nonetheless, consistent procedures of cryopreservation/thawing during the whole study were applied to minimise bias.

A relatively small cohort of patients (n=4) was used for both flow cytometry and differentiation assays. Despite the low number of donors, 4 different conditions were tested per donor (IC AC, IC LMP, VB AC, VB LMP), for a total of 16 sets of experiments. Cells were expanded for differentiation assays with scope to use them also for flow cytometry, in order to integrate the outcomes. However, during the planning phase of the experiments, leading role had mainly the degree of their complexity and the high cost of reagents for both differentiation assay and flow cytometry. This resulted in the need to downsize the number of donors to a minimum. Nevertheless, similar strategies were observed in other studies comparing ICBM with VBBM (Table 71).

In spite the use of same donors for both CFU-F and flow cytometry, was not possible to perform direct comparisons between colony numbers from CFU-F and MSCs' count from flow cytometry. Thawed P3 expanded MSCs were used for flow cytometry, whereas for CFU-F assay, fresh-frozen leucocytes (AC) and MNC (LMP) were cultured. Moreover, these two sets of experiments and the MSCs' samples used, were not performed in a synchronized manner to allow direct comparisons. Ideally, direct comparisons between CFU-F assay and flow cytometry, should give more accurate results, when are performed in tandem, using the same sample of fresh-harvested MSCs.

Although the importance of gene analysis for better comparison of the different behaviour between VB- and IC-MSCs, at the current stage of this research, information on gene expression was not performed. It has to be acknowledged however, that indirectly the findings of this research confirmed the higher osteogenic, chondrogenic and proliferation ability of VBBM, especially from AC-harvested MSCs.

Inevitably, time was a limiting factor in these experiments and despite the important information that RT-PCR could have added to this work, it could not be completed at the current stage. It would be interesting to confirm from a genetic perspective why VB-MCS are more osteogenic and or whether are present more subpopulations between them which are more efficiently selected by AC. These unanswered questions, remain to be addressed in future studies.

5.8 Limitations of the immuno-inflammatory component of the study

Blood samples were collected in different time-points within a week period, thus any variation of cytokines which may occurred beyond that period of time were not taken into account. Furthermore, due to patients' clinical priorities and their in-hospital management, blood samples were not obtained at a standard time during the day, hence, values may be influenced by any diurnal variation of cytokines that might exist. Additionally, scoliosis surgery is an uncomfortable experience for the great majority of the patients, which are often becoming emotionally vulnerable. Hence, in those circumstances, patients' dignity and clinical necessities were prioritised, becoming challenging to keep always a rigorous timetable of peripheral blood sampling.

Concerning the biochemical parameters, it must be noted that data derived from the hospital laboratory. As these tests were carried out based on the individual patients' needs, measurements did not cover with the same consistency the last time point (T6), as measured for the cytokines. Moreover, ideally for the immuno-inflammatory component of the study, serial blood samples should be taken for up to two weeks following an operation. However, the average duration of the in-hospital stay, was approximately 6 days, ranging between 4-24 days, restricting consequently the peripheral blood sampling. Therefore, the analyses were performed excluding T6 data. However despite this, approximately a third of the patients' blood samples were obtained at T6 time-point showing consistent trend over time, compared to patients with the missing data, hence despite the incomplete data set, it was possible to finalise the overall analysis.

This study could potentially be limited with regards to the number of patients recruited, although the trends showed by these molecules, to a certain extent implied that the sample size was sufficient for analysis. However, a more even

gender distribution throughout the cohort could have provided a more define picture between genders. Moreover, with a significative bigger cohort of patients and ideally a serial blood samples taken up to two weeks, more infection cases could have been included in the study and possibly early markers and correlations between infected patients, cytokines and biochemical parameters could have been identified.

Samples were stored at −80°C, and the possibility of protein variation cannot be excluded. However, this potential effect, if present, it should be extremely low and the cytokines' concentration in this study, to a certain extent, were consistent to other studies used similar cryopreservation protocols[100,102,163].

In this study it was applied the internal control concept (T0-base line values pre-operatively) as control group, mainly for two reasons. Firstly, given the lack of international agreement on the physiological concentration range of cytokines and the variety of factors that can affect their levels among an apparently healthy cohort, it was considered more reasonable to use the preoperative sample as base line value. Second, this research is focused on minors and peripheral blood sampling can be often an uncomfortable experience for them. It was not conceived from the authors as a necessary step at this stage, to recruit an external control group of healthy minors, merely for blood sampling without any clinical reason. ,.

Cytokines were measured in peripheral blood only and not in drained blood nor from the cell saver devices. This reduced the amount of information gathered, on the amount of cytokines released locally. Nonetheless, scope of this study was to analyse the systemic effect of the cytokines on the human body and correlate with the clinical outcome.

Furthermore, anaesthetic drugs and post-operative pain management may have affected the cytokines levels. However, given the fact that all patients were treated applying the same clinical protocols, was not possible to analyze separately the effects of a single drug on the cytokines' concentration taken in exam.

6. Future study

Would be interesting to investigate further the genetic setup of VB and IC MSCs, in an attempt to clarify whether their differences are related to different subpopulations of MSCs or a different embryogenic background based on their anatomic location. Moreover, another practical aspect requiring further investigation could be whether different vertebrae of the same donor present comparable MSCs concentrations. In addition could be interesting to identify whether any correlation exists between the volume of the vertebras and their MSCs content.

From a transitional point of view, the MSCs findings of this research could be used intra-operatively together with the technology currently available (MSCs concentrators, portable flow cytometry), to monitor prospectively the MSCs numbers yielded from every anatomic site, allowing consistent MSCs concentrations and potentially standardising better the clinical outcome.

Concerning the immuno-inflammatory component of this research, will be useful to study bigger groups with an even gender distribution and ideally be able to monitor cytokines, biochemical and clinical data for two weeks period of time. This will determine better their kinetics and in particular of those patients undergoing longer and more aggressive procedures. The intra-operative cell saver device is commonly used in spinal field. However, still little is know regarding its impact on the biochemical parameters and cytokines in different surgical approaches, based on its efficiency and erythrocytes preparation prior retransfusion.

The great majority of research performed in the spinal field, is based on the posterior approach. Consecutively, the protocols related to the post-operative management, as well as the use of antibiotics, are both based on the posterior approach. Moreover, also for more traumatic and prolonged procedures (antero-posterior approach), presenting longer immunosuppression periods post-operatively, the protocols are still following the posterior approach model. The transitional aspect of the immuno-inflammatory component of this research could be to review and possibly amend the current protocols, in an attempt to optimise and better adapt them into individual patients' requirements, based on a number of elements, like: type and duration of surgery, metabolic conditions, age and other factors predisposing for infection.

7. References

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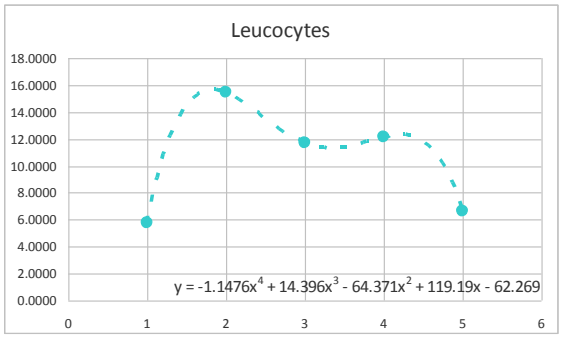
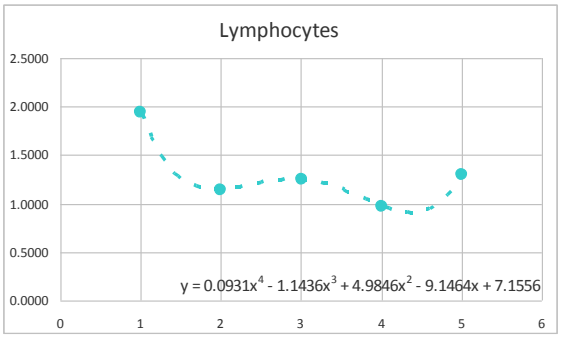
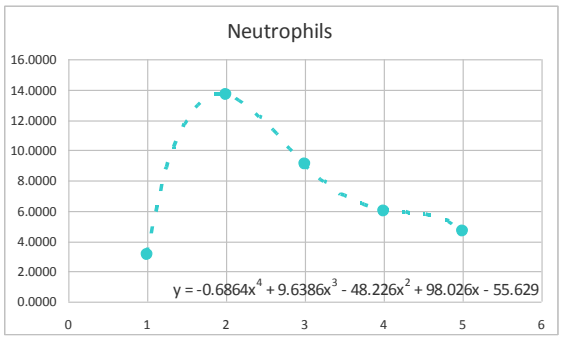
8. Appendices

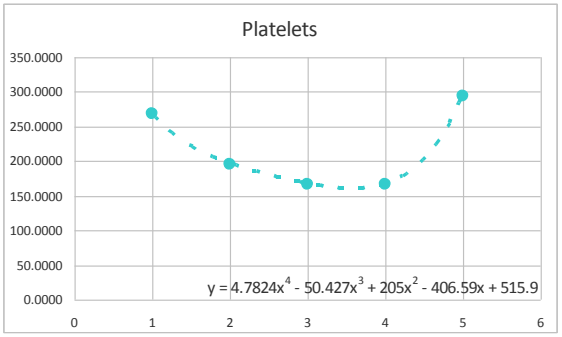
8.1 Regression mean imputation for missing data recovery. Regression trends were generated based on mean scores over time for the known values (blood parameters). Formulas were generated accordingly, using IBM SPSS software, in order to calculate the missing data and allowed GraphPad Prism software to perform the statistic analysis. A) Posterior approach, B) Antero-posterior approach, C) Posterior approach and costoplasty

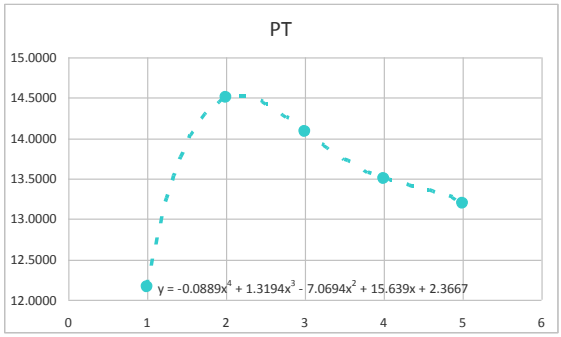
A

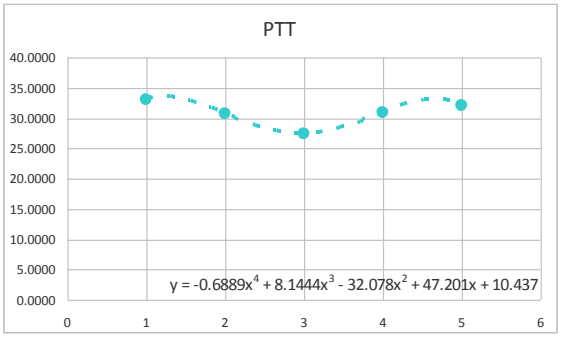


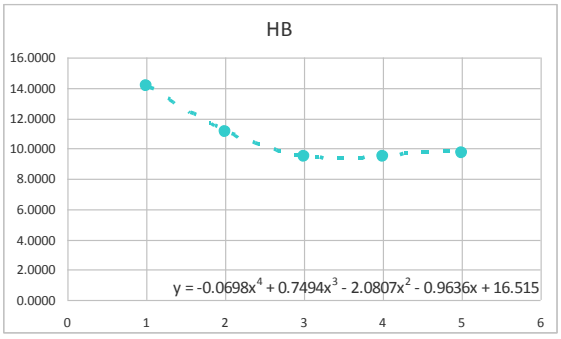






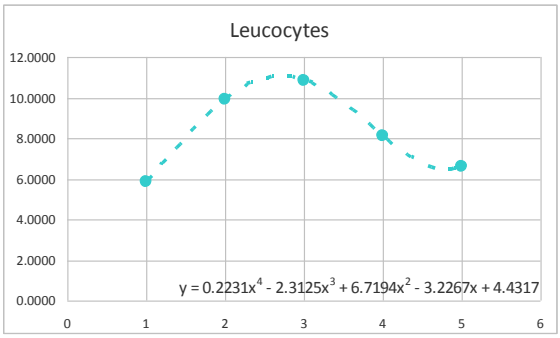


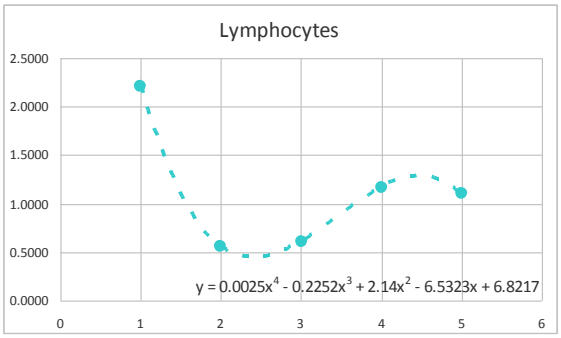


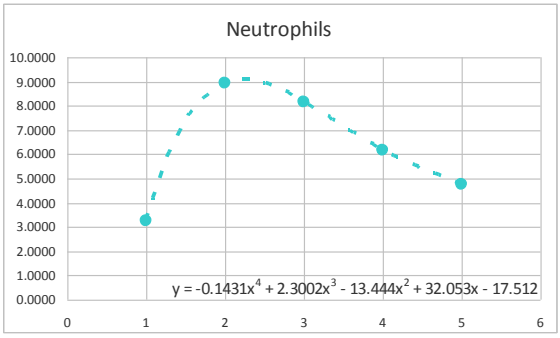


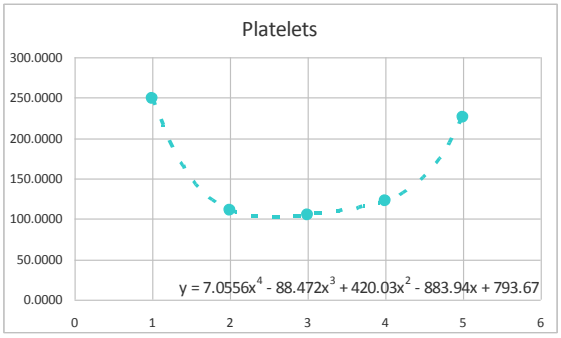
A1

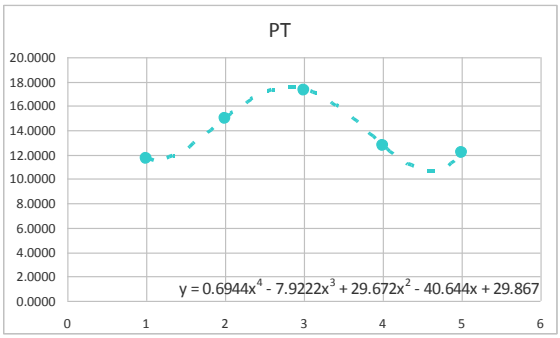
B

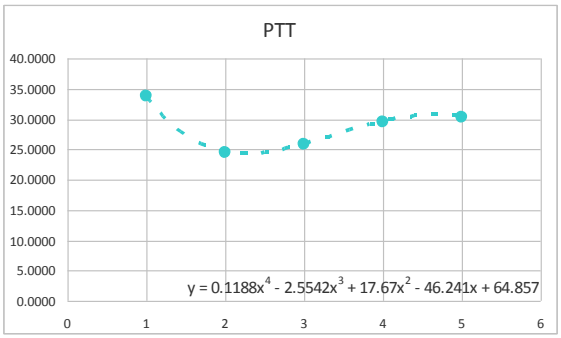

Leucocytes
y = 0.2231x4 - 2.3125x3 + 6.7194x2 - 3.2267x + 4.4317

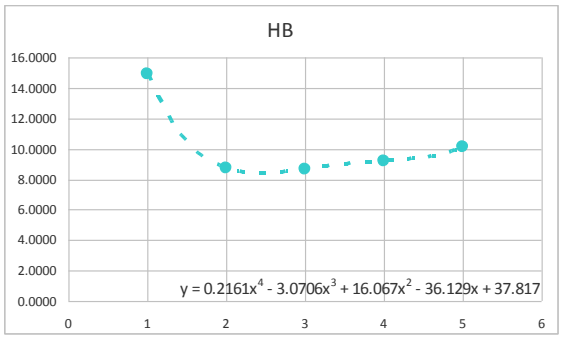

Lymphocytes
y = 0.0025x4 - 0.2252x3 + 2.14x2 - 6.5323x + 6.8217


Neutrophils
y = -0.1431x4 + 2.3002x3 - 13.444x2 + 32.053x - 17.512

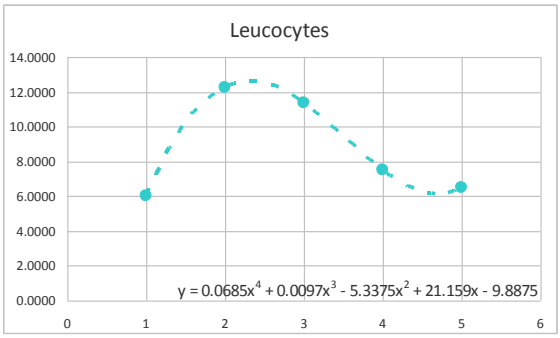

Platelets
y = 7.0556x4 - 88.472x3 + 420.03x2 - 883.94x + 793.67

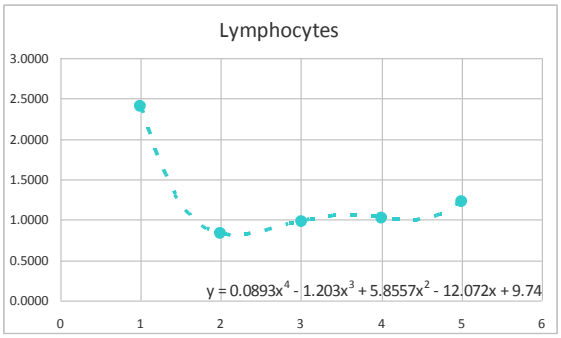

PT
y = 0.6944x4 - 7.9222x3 + 29.672x2 - 40.644x + 29.867

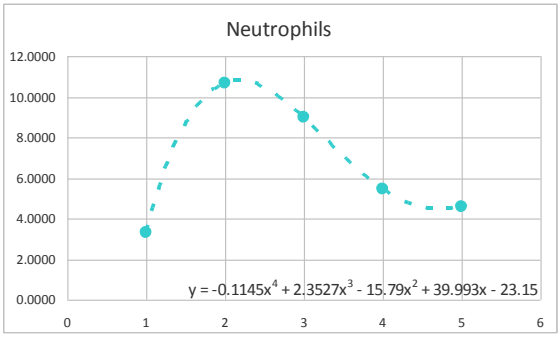

PTT
y = 0.1188x4 - 2.5542x3 + 17.67x2 - 46.241x + 64.857

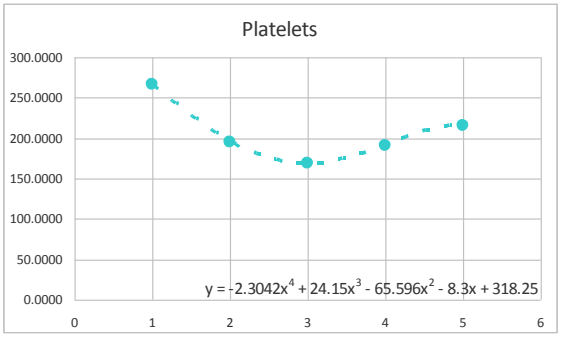

HB
y = 0.2161x4 - 3.0706x3 + 16.067x2 - 36.129x + 37.817

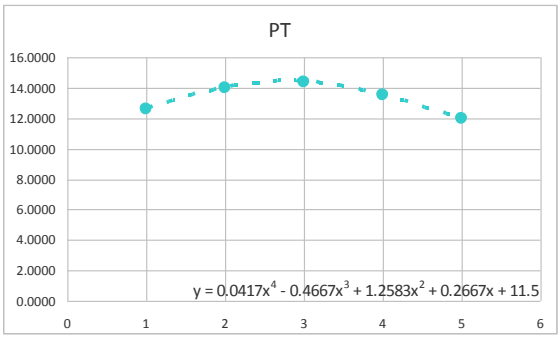
C

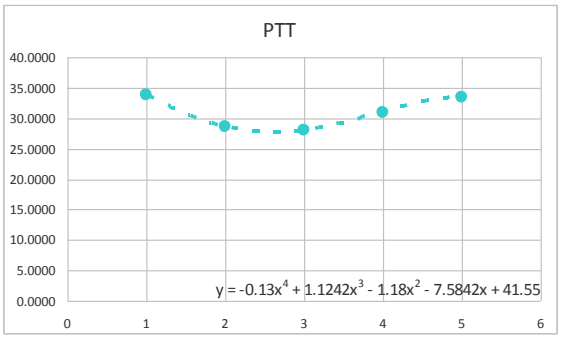

Leucocytes
y = 0.0685x^4 + 0.0097x^3 - 5.3375x^2 + 21.159x - 9.8875

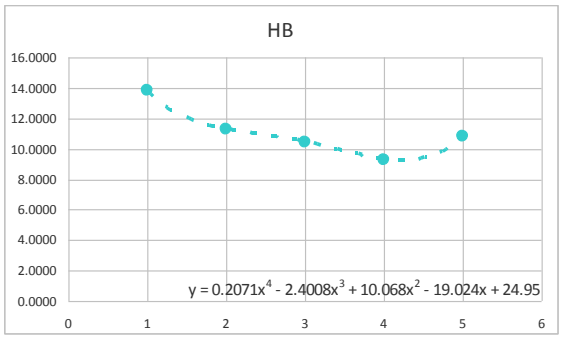

Lymphocytes
y = 0.0893x^4 - 1.203x^3 + 5.8557x^2 - 12.072x + 9.74


Neutrophils
y = -0.1145x^4 + 2.3527x^3 - 15.79x^2 + 39.993x - 23.15


Platelets
y = -2.3042x^4 + 24.15x^3 - 65.596x^2 - 8.3x + 318.25


PT
y = 0.0417x^4 - 0.4667x^3 + 1.2583x^2 + 0.2667x + 11.5


PTT
y = -0.13x^4 + 1.1242x^3 - 1.18x^2 - 7.5842x + 41.55


HB
y = 0.2071x^4 - 2.4008x^3 + 10.068x^2 - 19.024x + 24.95

8.2 Materials, consumables and equipment

For samples collection and processing, the following laboratory consumables and equipment were used, in addition to what had been already described in the relative sessions of the manuscript. :

Media and Reagents

Media / Reagents	Product code	Company
Dulbecco's modified eagle's medium DMEM(1X) + GlutaMAX™-I	61965-026	Gibco®, Invitrogen
Stem macs MSC expansion media	130-091-680	Miltenyi Biotec
Dulbecco's phosphate buffered saline DPBS(1X)	14190-094	Gibco®, Invitrogen
Dimethyl sulfoxide (DMSO)	M81802	Sigma
Trypsin/EDTA solution	15400-054	Invitrogen
Penicillin/Streptomycin solution	14140	Gibco®, Invitrogen
Lymphoprep™	1114545	Axis-Shield
Ammonium chloride (NH_4Cl)	12125-02-9	Vickers Laboratories
Boric acid	B0252	Sigma
Tryphan blue	T8154	Sigma
Methylene blue stain	66720	Fluka
Fetal calf serum (FCS)	A3059-500g	Sigma
Formaldehyde	BP531-500	Fischer Scientific
DNAse	D4513	Sigma
Disodium tetracarbonate decahydrate	106308-0500	Merck
Calcium assay kit	17667	Senitial Diagnostics
Sulfated glycosaminoglycan assay kit	B1000	Blyscan™
Quantikine ELISA Human IL-6 Immunoassay	D6050	R&D Systems
Quantikine ELISA Human IL-6 Immunoassay	HS600B	R&D Systems
Quantikine ELISA Human IL-10 Immunoassay	HS100C	R&D Systems
Human IL-18 Immunoassay	7620	MBL International corporat.
Human procalcitonin	RAB0037	Sigma-Aldrich
As2P	A8960	Sigma-Aldrich
Sodium Pyruvate 100x concentrated stock 5mL	S8636	Sigma-Aldrich
PBS	14190-169	Gibco LifeTech
BSA	7906	Sigma-Aldrich
Proline	P5607	Sigma-Aldrich
Dexamethasone	D1756	Sigma-Aldrich
Papain	P4762	Sigma-Aldrich
TGFβ3	243-B3-002	R&D System
ITS+ (1:100 50µL)	12521	Sigma-Aldrich
Beta-2-Glycerophosphate	G6251	Sigma-Aldrich
Fast blue RR Salt	FBS25-10CAP	Sigma-Aldrich
Naphthol AS-MX phosphate alkaline solution	855-20ML	Sigma-Aldrich
Citrate concentrated solution	83273-250ML-F	Sigma-Aldrich

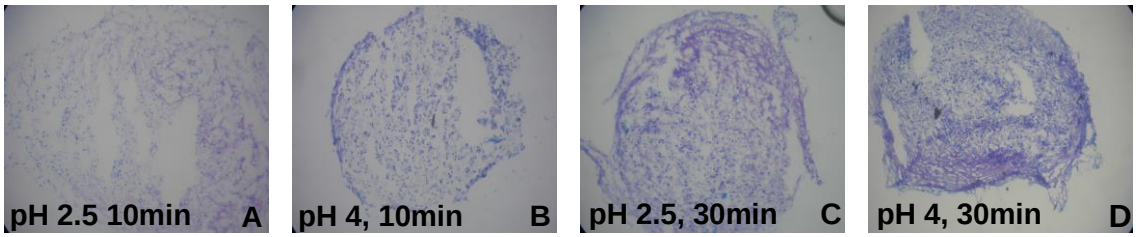
Isobutylmethylxantine	I5879	Sigma-Aldrich
Hydrocortisone	H2270	Sigma-Aldrich
Horse Serum	06750	Stem Cell Technologies
Oil Red	O0625	Sigma-Aldrich

Equipment	**Product code**	**Company**
Inverted microscope	DMIL090-135.001	Leica Microsystems
Inverted microscope	CKX41	Olympus
Digital camera	C-7070	Olympus
Mechanical freezer -80°C	ULTF 80	Dairel
Mechanical freezer -150°C	MDF-C2156 VAN	Sanyo
Liquid nitrogen	NA	NA
Centrifuge	PK 130 R	ACL
Centrifuge	5810 R	Eppendorf
Centrifuge	Z 206 A	Hermle
Multiskan EX photometer	VWR-14230-392	Thermo Scientific
Biological safety cabinet, class II	AC2-4E1	ESCO
Incubated at 37 ^{o}C, 5% CO_2	50100783	Sanyo
Haemocytometer	BS.748	Hawksley
37 ^{o}C water bath		Grant

Plastic devices

Plastic-ware	**Product code**	**Company**
Pasteur pipettes sterile 1ml	1170915	Gilson
Pipette tips (1000 µl)	F167104	Gilson
Pipette tips (200 µl)	F167103	Gilson
Pipette tips (10 µl)	F167101	Gilson
Stripette (25 ml)	4251	Corning
Stripette (10 ml)	4101	Corning
Stripette (5 ml)	4051	Corning
96 well plate (round bottom)	3799	Corning
6 well plates	3516	Corning
Flask (25 cm2)	430639	Corning
Flask (75 cm2)	430641	Corning
Petri-dish (10cm diameter)	430167	Corning
Cryovials (2 ml)	72.380	Sarstedt
Cryovials (1.2 ml)	72.377.005	Sarstedt
Cryo boxes	93.872.625 and 93.876.281	Sarstedt
Syringe (10 ml)	302188	BD
Syringe (50 ml)	300867	BD
Centrifuge tubes (15 ml)	430790	Corning
Centrifuge tubes (50 ml)	430828	Corning
Vacuette (K2EDTA) 4ml	454023	GBO
Vacuette (z serum clot activator) 6ml	456092	GBO
MF-millipore^{TM} membrane (0.45 µm)	SLHA033SS	Millex – HA
Freezing container,nalgene86mm tall X	C1562-1EA	Sigma
13G match-ground bevel tip introduction needle 5"	306-131	Stryker

8.3 Differentiation assay, optimization of chondro-pellets staining technique



Toluidine blue staining of chondro-pellets under different conditions. A, B) Toluidine blue was adjusted on pH2.5 and 4 and both were incubated for 10min. C, D) Toluidine blue was adjusted to pH2.5 and 4, respectively and both were incubated for 30min. A better colour appearance was observed for the combination of pH4 at 30min. of incubation (D). (The staining optimization was performed on a secondary selection pellets, Magnification 64X.)

8.4 Flowcytometry, histograms of the BM-MSCs markers

For all four donors, antigens and fluorochromes used, are illustrated in each histogram. Histograms were gated between 10^2-10^5 being deemed as positive staining. Population of interest (POI) was stained positively for markers APL, CD105, CD90, CD73, SUSD2, MSCA1, and negatively for CD14, CD19, CD34, HLA-DR and CD45 confirming MSCs' nature.

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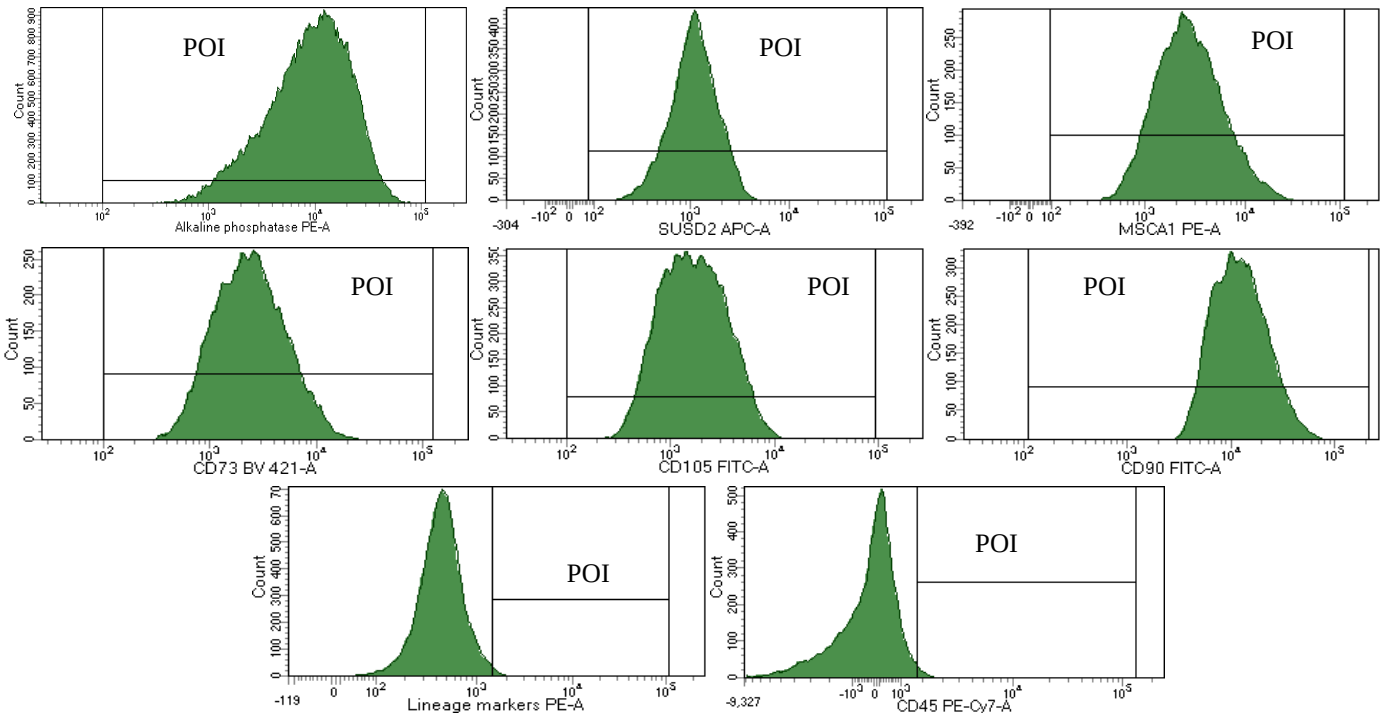

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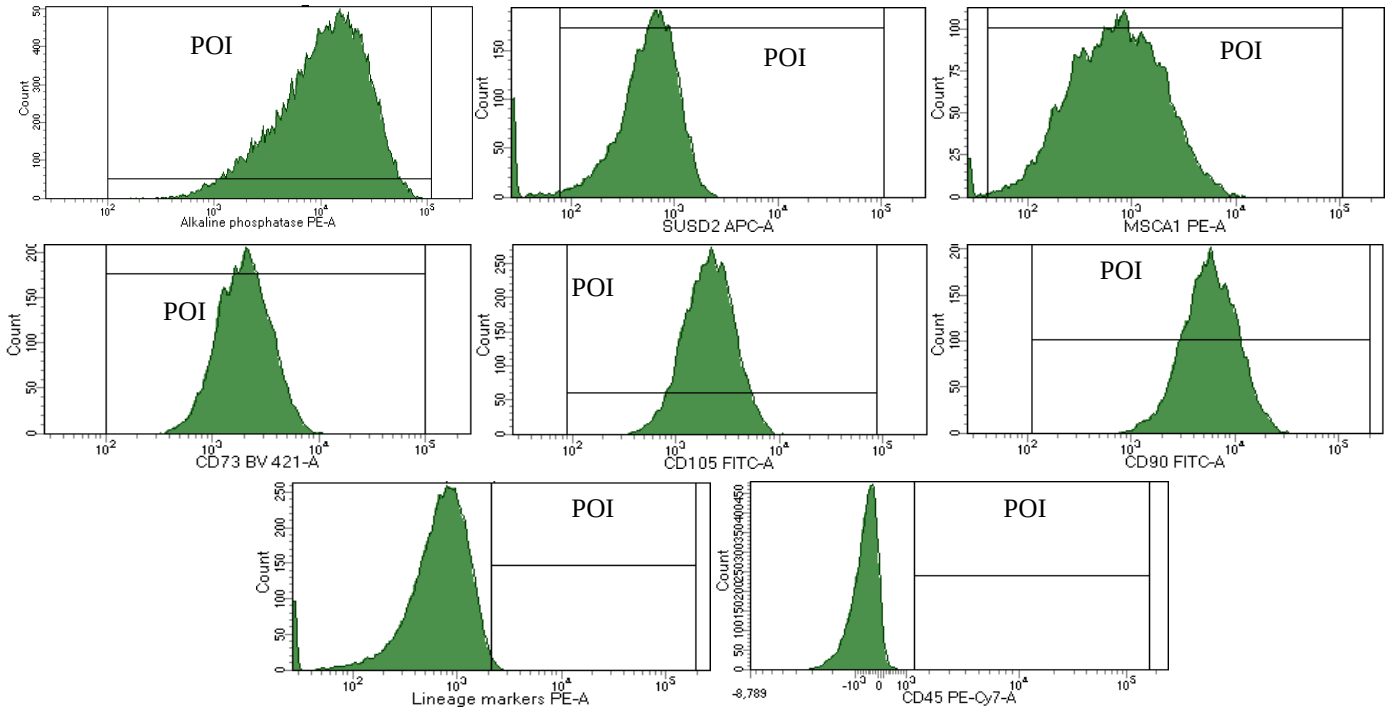

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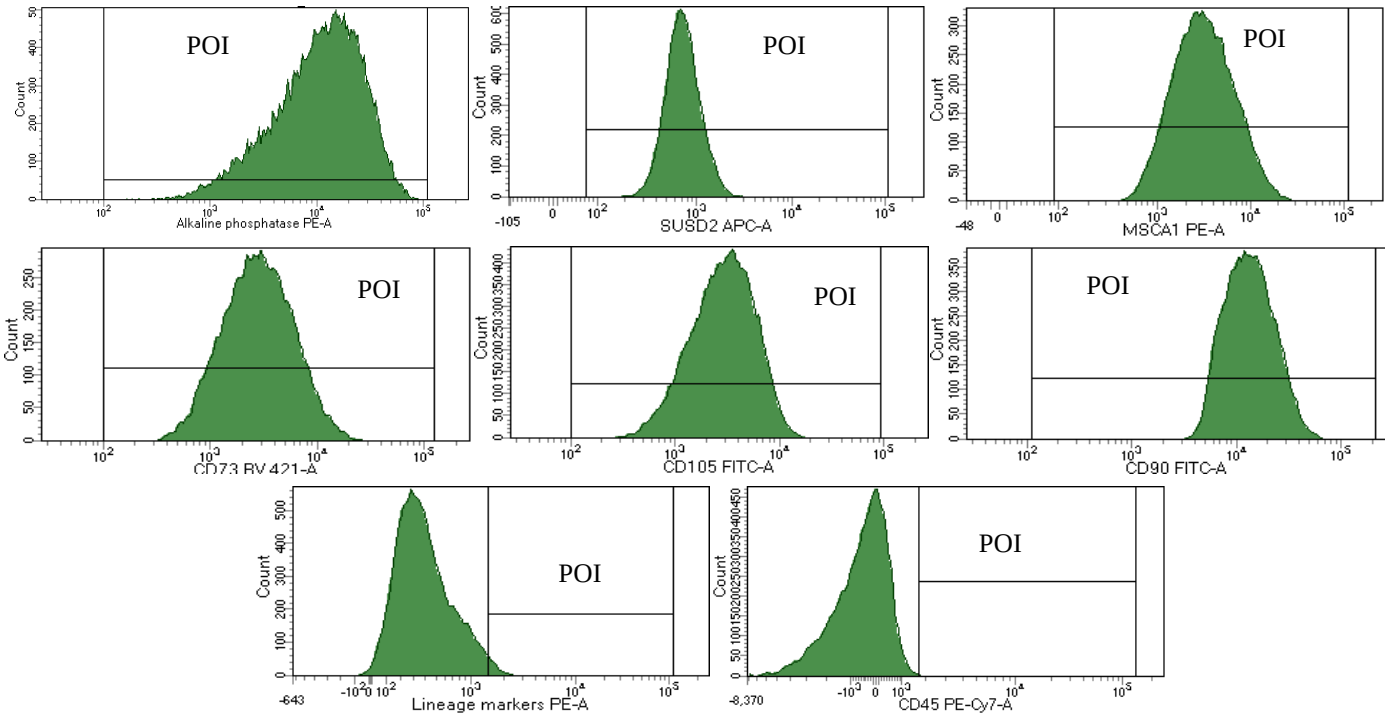

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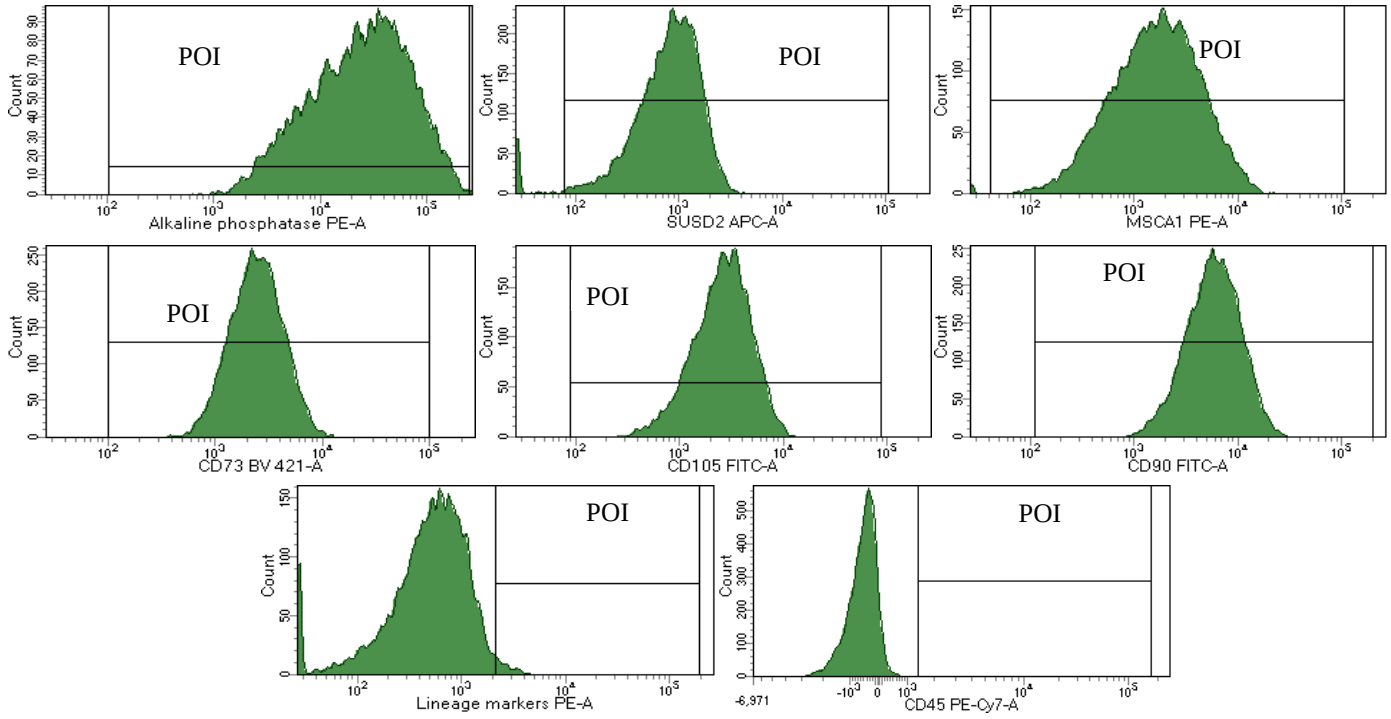

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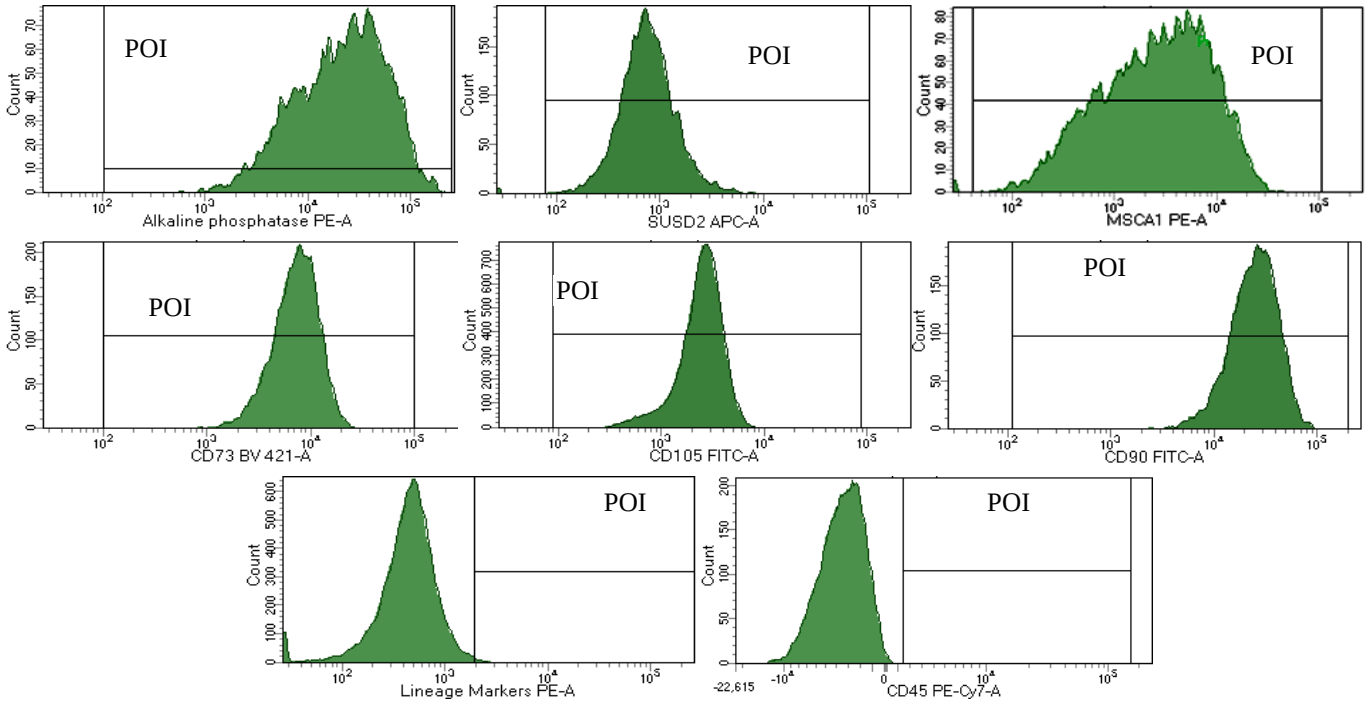

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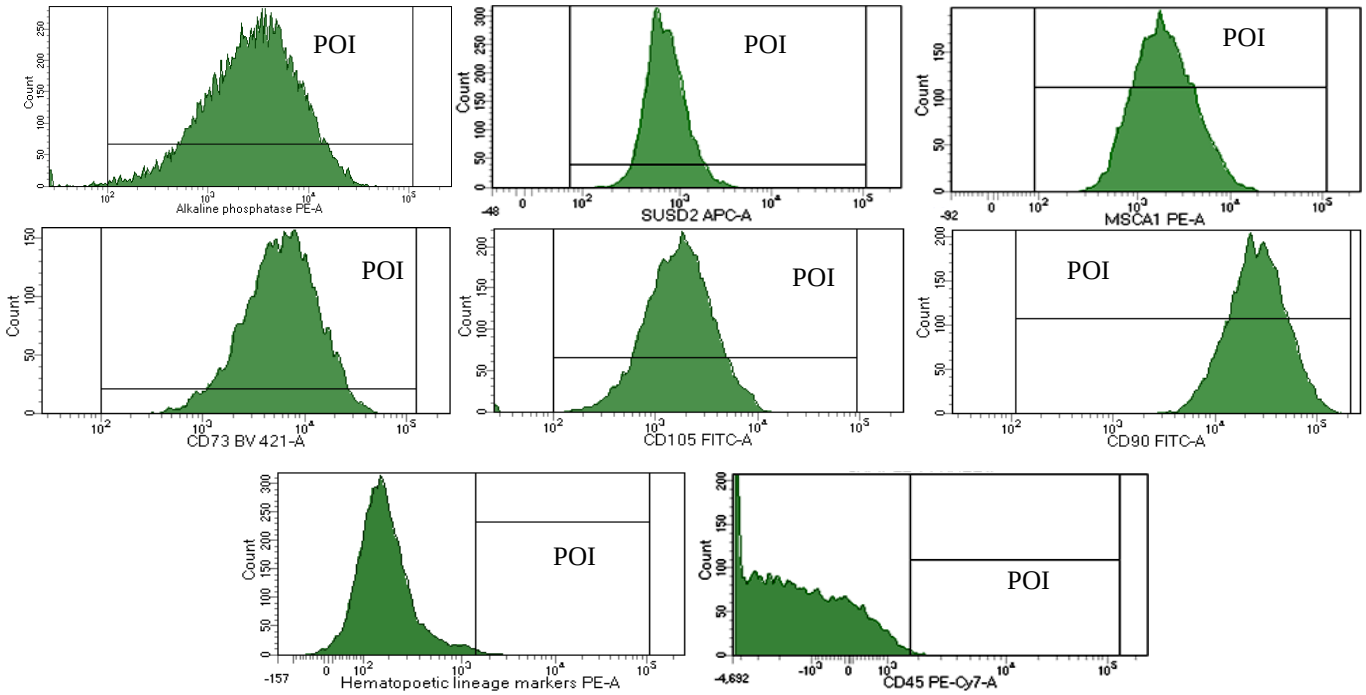

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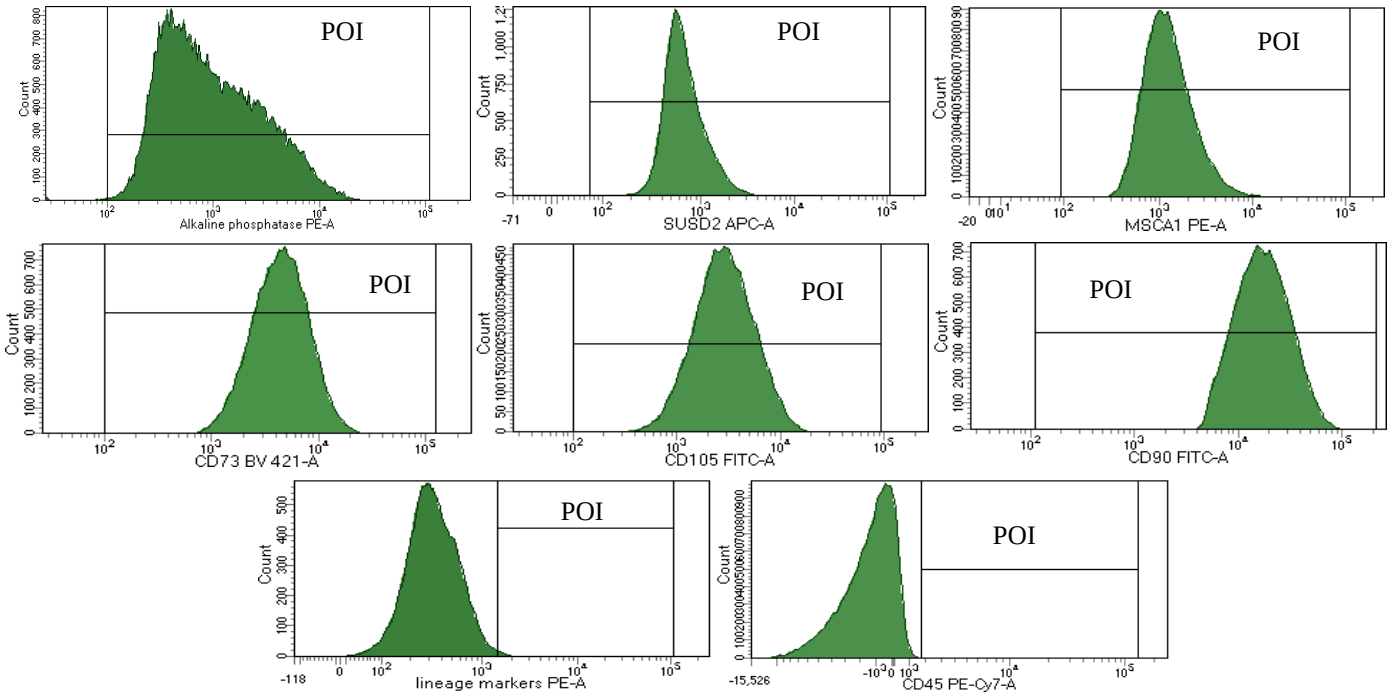

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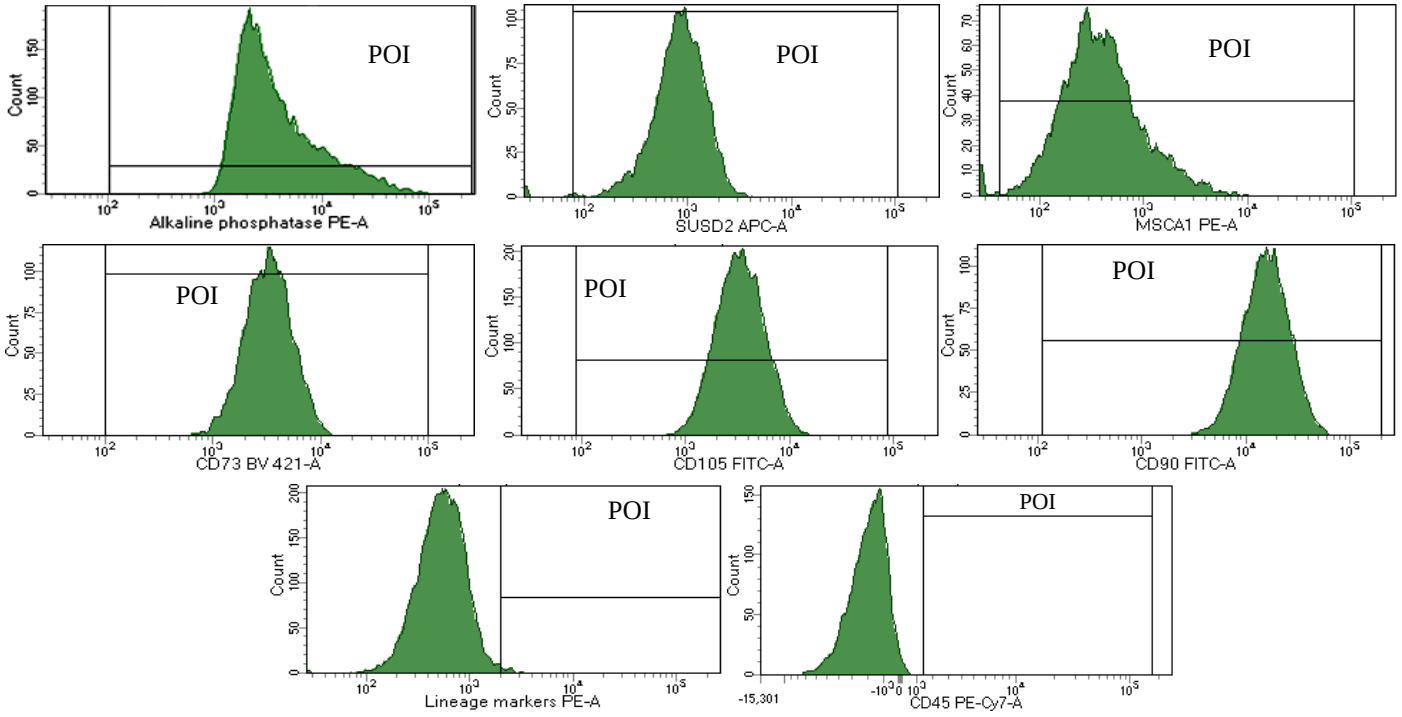

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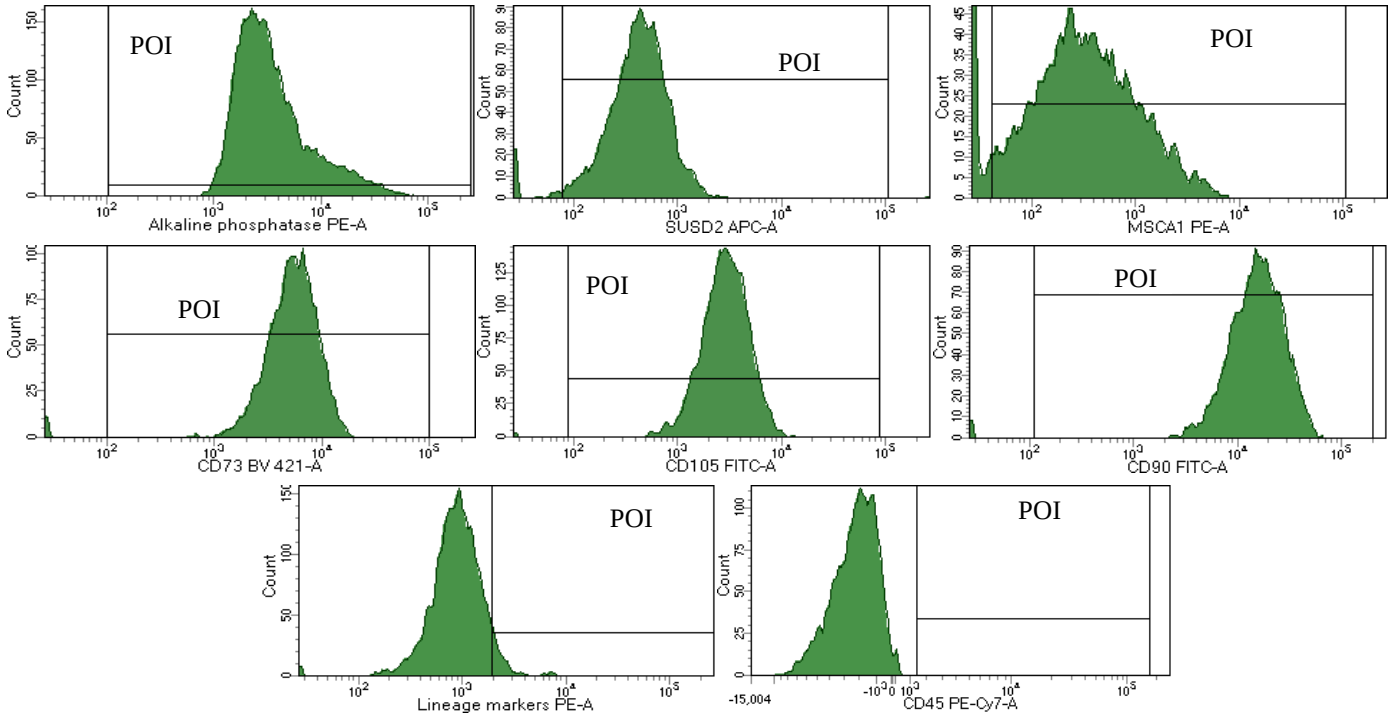

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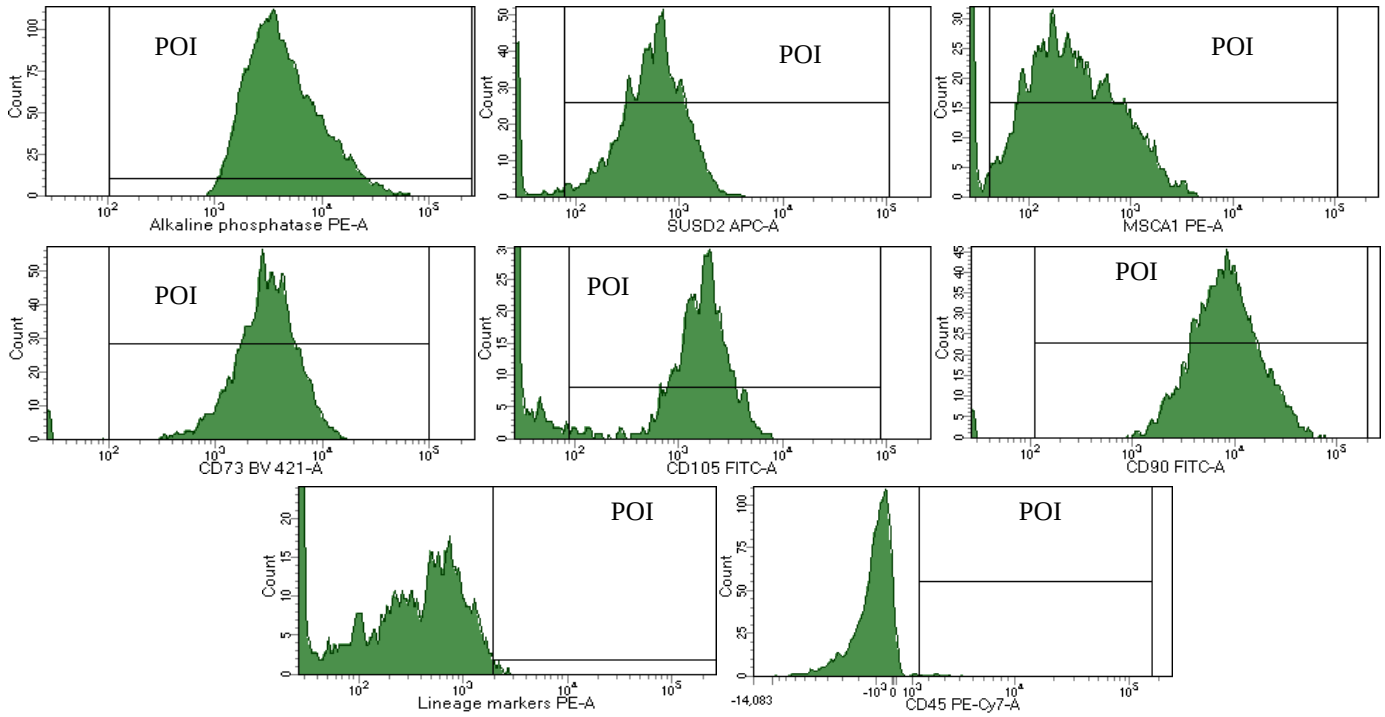

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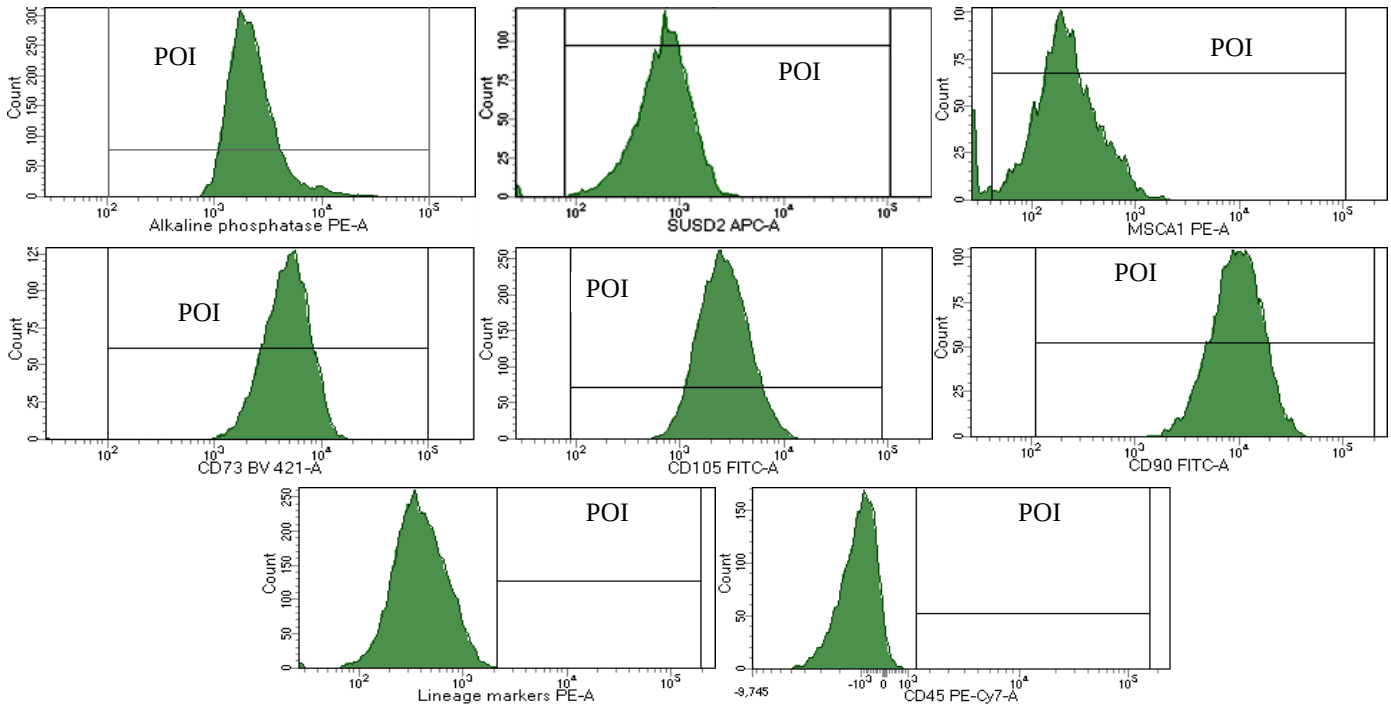

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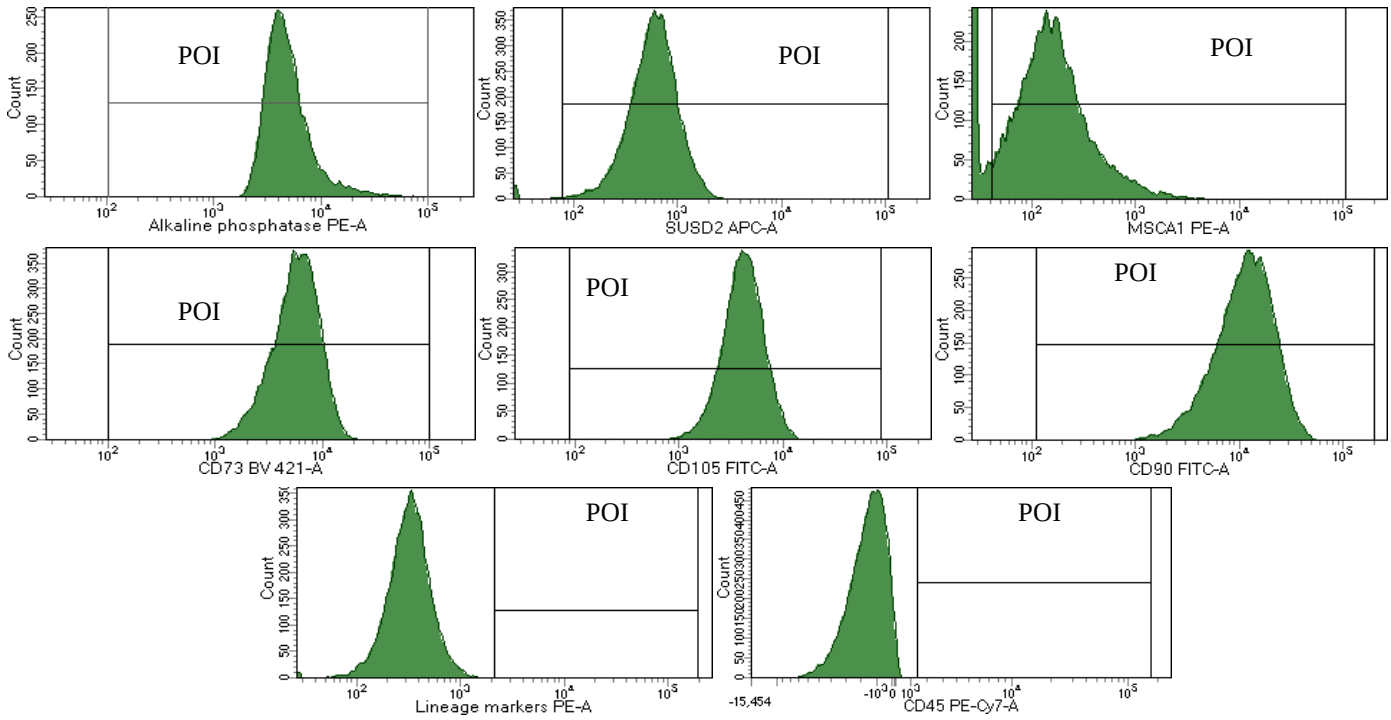

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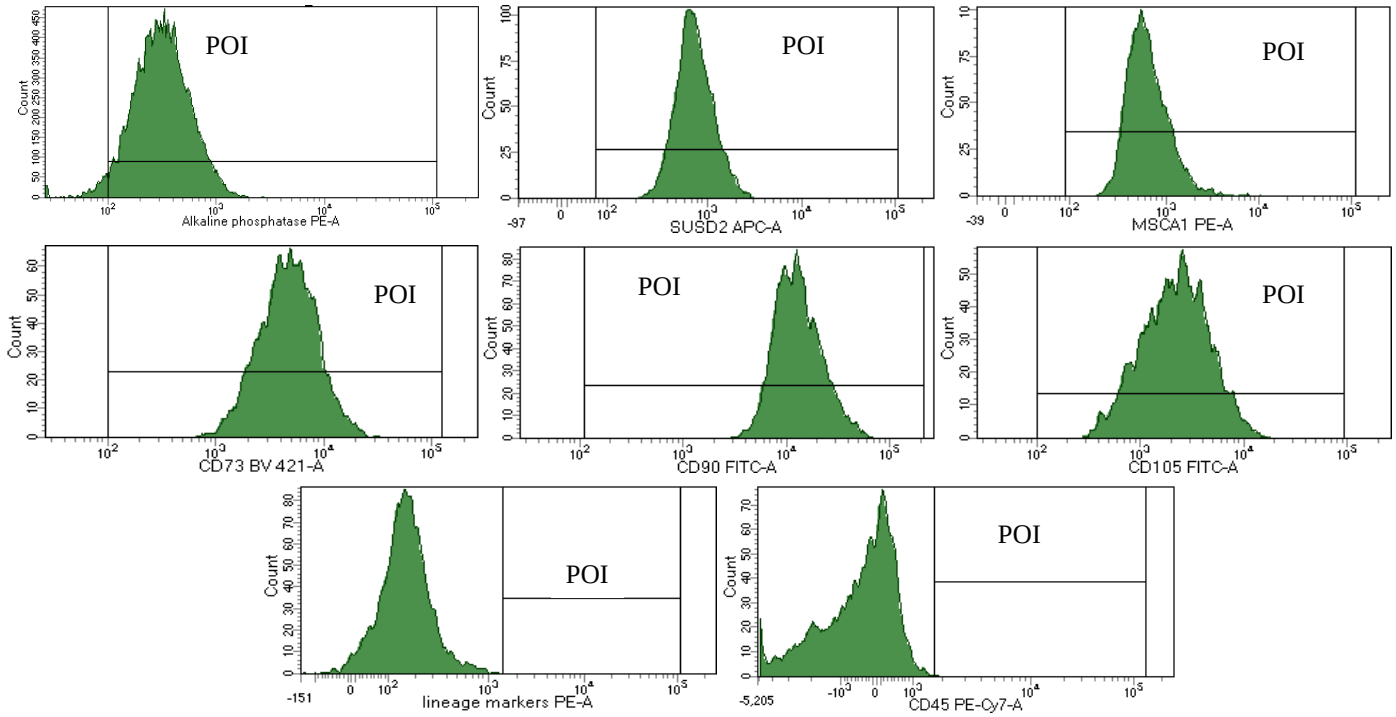

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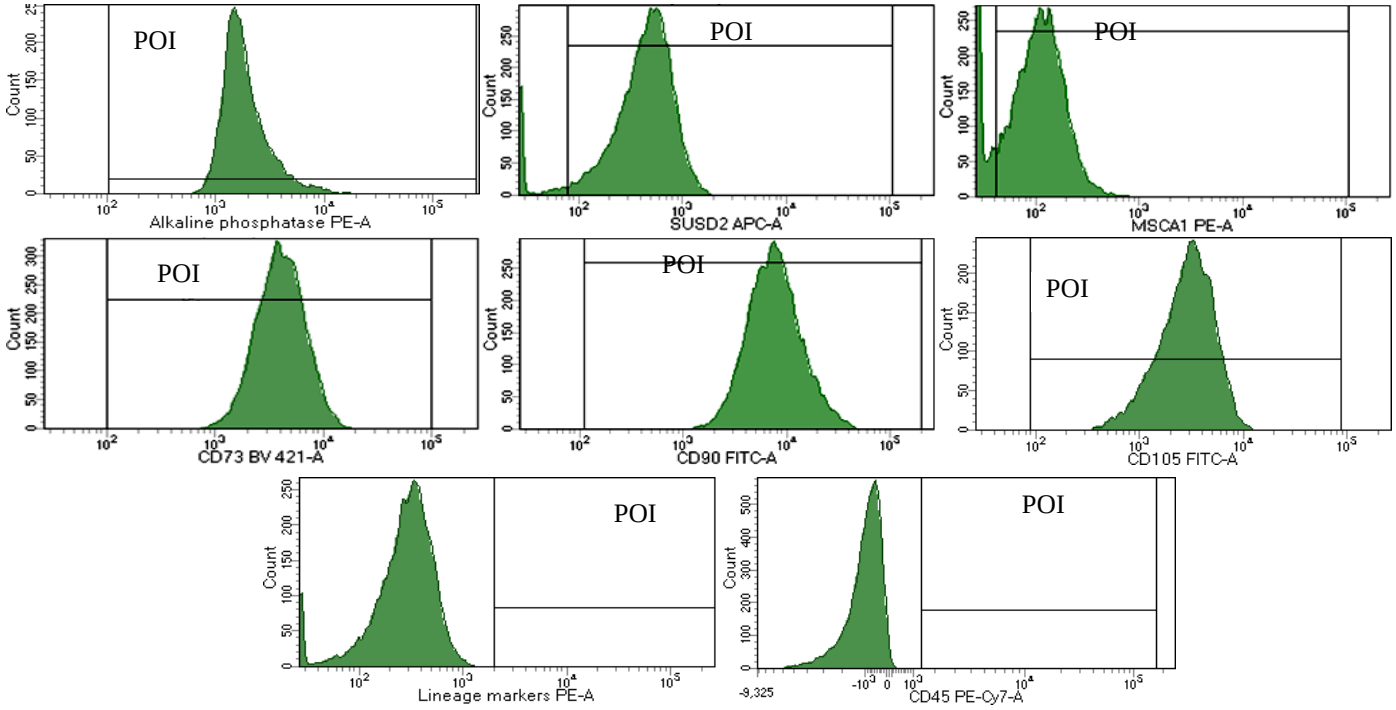

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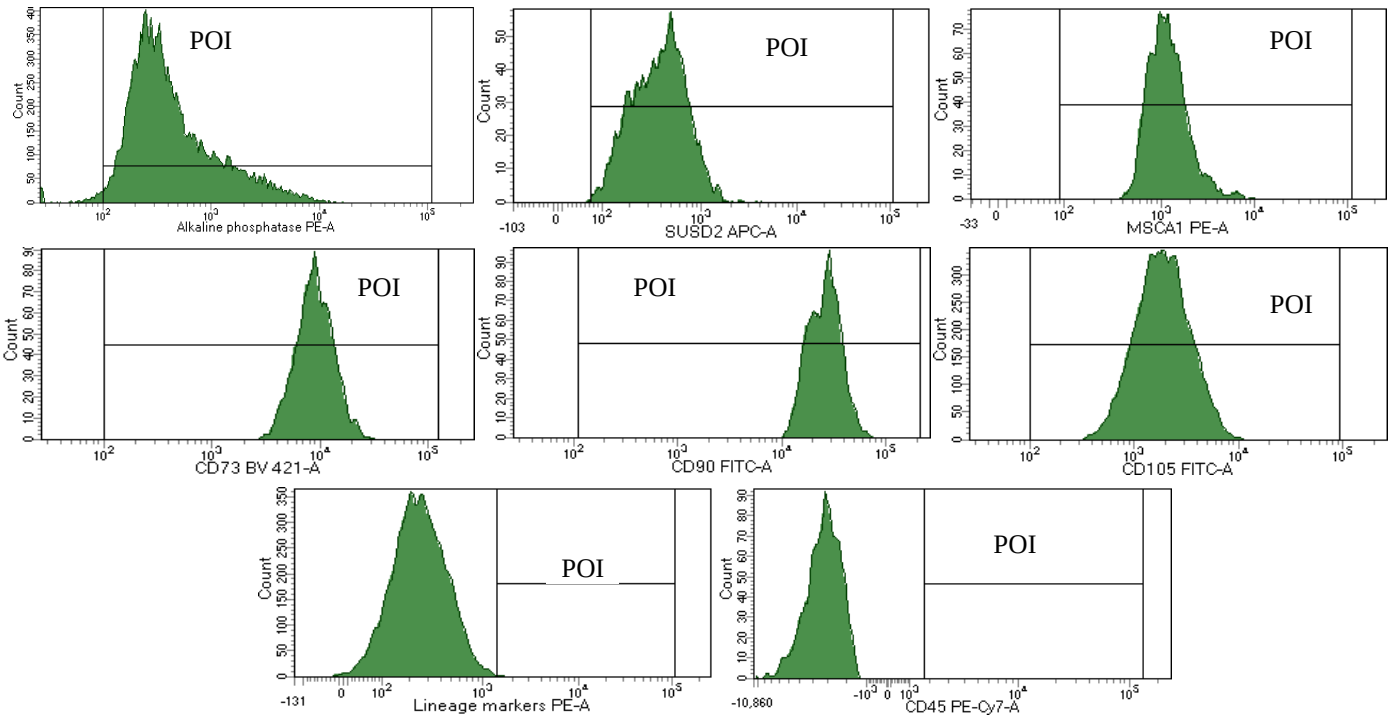

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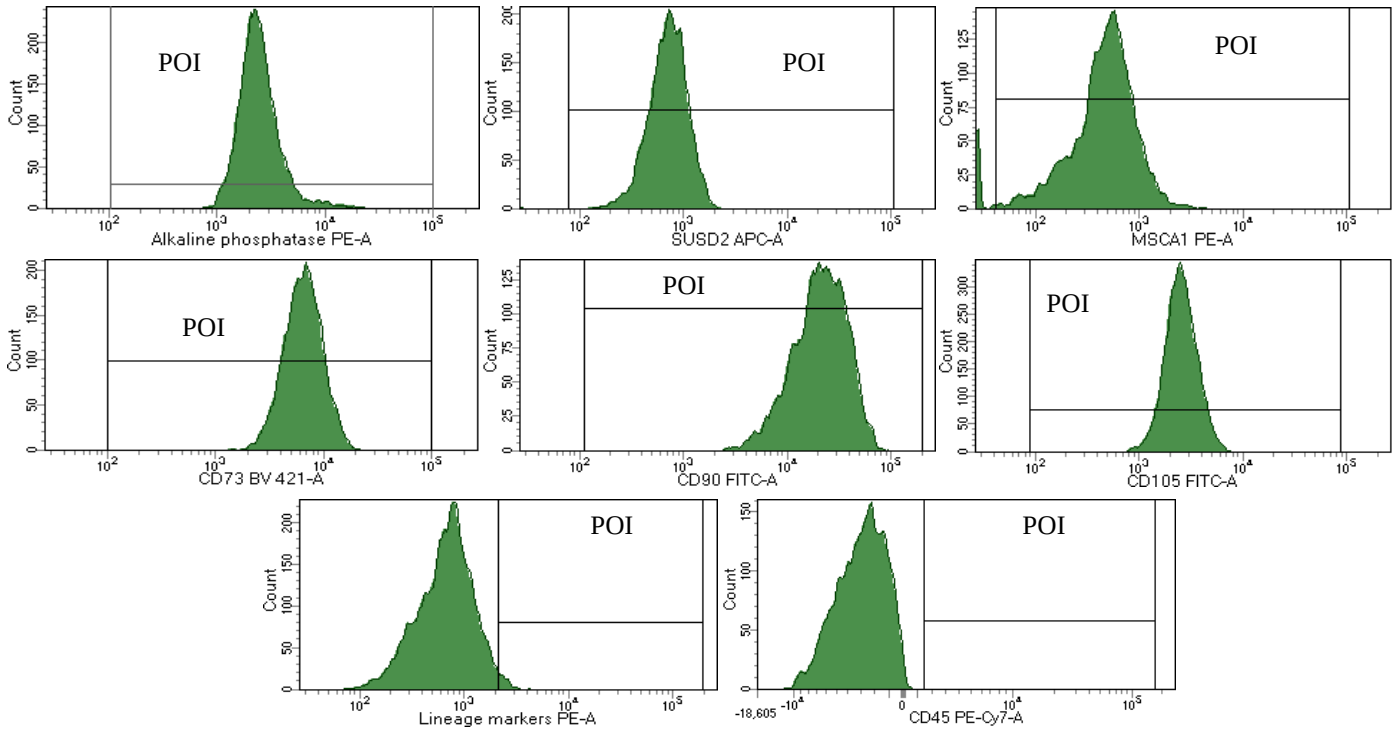


8.5 Children information sheet and Assent form (13-16 years old)

Patient information sheet and Consent form (16-17 years old)

Parental information sheet and Parental agreement / Consent form

CHILDREN INFORMATION SHEET

Study title: The role of Stem Cells in Fracture Healing

"Collection of Bone Marrow and Blood"

PART 1

(Please read part 1 and if you agree continue on reading part 2)

You are being invited to take part in a RESEARCH study!

Please take the time to read the following information carefully and discuss it with your family, friends, and your General Practitioner if you wish.

- What research means?

Research is when doctors try to understand new things about a disease or about something special!

- Why are we doing this research?

We would like to study the behaviour of some special cells of our body (stem cells), under specific conditions such as scoliosis surgery.
We believe that those cells have a different behaviour in young people, compared to adults, and for this reason we need your help!

- What is the procedure that is being tested?

In this research we need to study the behaviour of those special cells during your scoliosis operation. You will not be required to take any additional medicine, and we will not be testing any device on you!

- Why have I been invited to take part in this research?

We are looking for children-young people (above the age of 13years) that are having surgery like yours (scoliosis correction). In this project we need to study 30 children.

- Do I have to take part in your research?

Your parents or guardians have to say it's OK for you to be in the study. After they decide that, you get to choose if you want it as well. If you don't want to be in the study, there is no problem, no one will mind. If you want to be in the study now and change your mind later, that's also OK. You can stop at any time. This will not affect the standard of care that you receive.

- What will happen to me if I take part?

You will be involved only during the first week of your stay in the hospital.
You will not lose any of your time, for us.
The total research program will last one year, but this time is necessary only for us doctors to study all the collected information. The only thing that we will ask you to do is, to allow us to take and analyse a small sample of your blood that will be collected during the standard blood tests that you would have anyhow during your stay in the hospital.

- What will I be asked to do?

If you decide to be a part of this study, you will have to write your name to a special paper called Assent form. Then nothing of the standard things that would happen anyhow during your stay with us will change. We will collect the small sample of blood that we need, during the time that we will collect blood for our routine tests. There will be no more needles. You will not lose any additional time from school.

- What other medicines could I have instead?

In this research we don't use medicines. Our study is related only to the blood tests.

- Is there anything else to be worried about if I take part?

No, don't worry, none of the aspects of this research can give you trouble/problems.

- What are the possible benefits of taking part?

We cannot promise the study will help you, but the information we get will help other children and in the future. You will have the opportunity to help us out in our work and be an important member of our research team.

- Contact details

If something is not clear or if you would like more information please ask us.

Take your time to decide whether you wish or do not wish to take part.

Contact Prof. Peter V. Giannoudis on 0113 392 2750, for any further information.

Thank you for reading this.

If you agree please read now Part 2

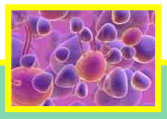
PART 2

What is the study about?

In our body there are special cells everywhere, which help to make different parts of our body such as, bone, cartilage, muscle, tendon or ligament.

We are doing this study on children, and we would like to ask for your help, because we want to know if there are any differences between adults and children, in surgery, such as a scoliosis operation.

We are need to find how these cells influence the process of fixing problems.
Our research aims to understand how these cells work.



Why is the study being done?

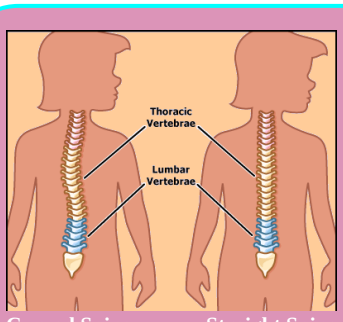
These cells help to heal different parts of the body, but just how they work is still not understood.

So, with your help we would like to understand how these cells think and work, in a young person like yourself !!!

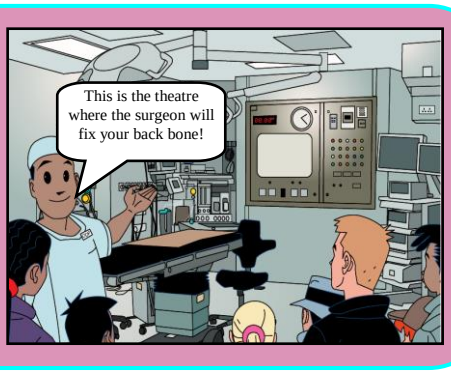
Why have I been chosen?

You suffer from a condition called ***scoliosis*** (means that the back is curved to the side), which may need surgery, like the operation you are having.

We are doing this study on children with the same age as yourself, and we would need to involve around 30 different young people with scoliosis.



Curved Spine Straight Spine



What will happen to me in the study?

You will be asked to allow us to take an extra small amount of blood (few extra drops) during the normal tests, which are routinely made before, during, and after your operation.

During the operation, the surgeon will take a very small piece of bone marrow from the area of the surgery, like a little a bread crumb that will be also analysed.
This piece is not going to give you any pain or problems.

You will not be asked to have any more needles.
We will collect the few extra drops of blood just before your surgery, during the surgery and at the first day, the third, the fifth and the seventh day after your surgery while you are still in the hospital.

Do I have to take part?

It is up to you to decide whether or not to take part in this study, but we do hope you will. Take your time to think and discuss with any body you want, and feel free to decide. Whatever is your decision, will not change the way we take care of you!

What do I have to do?

Apart from agreeing to take part to this study, nothing else.
What we need to collect for the study (the blood samples), will be collected during the time we would have to take blood as in all people that have surgery like yours.

What are the possible benefits and risks of taking part?

There are no side effects or risks. Asking you to give us a very small extra quantity of your blood, (just a few drops), will not put you at risk.

You will not benefit directly from taking part in this research and all other aspects of your care will be the same as if you did not take part.

Helping us, we could help other children like you, in the future!

Will I need to take time out off school, and if so how much time?

This research will not make you miss any additional time from school! It is related only with the minimum necessary time spent in hospital for your good recovery after the surgery.

Would this affect my social life?

The goal of this surgery is to help you to get a better social life and, the aim of this research is to help other people like you in the future!

When will the study begin and possibly finish?

This is a pilot study and will last one year, starting on June 2012.
After the evaluation of all the data collected we will think about the possibility to expand the study in more people and hospitals.

What will happen to the results of the study?

At the end of the study, the results will be published in a clinical scientific journal. They will be also discussed in big meetings of doctors that are very interested to learn such things.

What happens when the research project stops?

As the study is not related with any type of drugs, and you are not under a special treatment, you must not worry if the study is going to end.

What happens if new information from this research comes along?

Sometimes during a research study, new things are found. If this happens we will inform yourself and your family or guardians.

What if something goes wrong?

This research can not cause you damage, for this reason there are no special compensation arrangements if you are harmed by taking part. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it.
Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal NHS complaints procedure will be available to you.

Will my taking part in this study be kept confidential?

Other people will not know if you are in our study. We will put all the information we learn about you, together with information regarding other children-young people, so no one will know what details came from you. When we tell other people about our research, we will not use your name, so no one can tell who we are talking about.

What will happen to any samples I give you?

The collection and storing of all samples is done in such a way as to ensure maximum safety. After their examination in the laboratory, are neutralised and discharged under special conditions, respecting particular rules.

Where will the study take place?

This project is being organised by Doctors of this hospital (Leeds General Infirmary) and Leeds Institute Molecular Medicine under the supervision and support of Leeds University.

Who has reviewed this study?

This study has been reviewed by the independent ethics committee called the Leeds (East) Research Ethics Committee. This committee control if this research is ethical and correct respecting the rights and the well-being of patients. We have received approval from this committee to do this study.

Who will lead the study?

Prof. Peter V. Giannoudis is the principal investigator. He is the director of the Department of Trauma and Orthopaedic Surgery, Leeds General Infirmary.

If you need to ask more information about this study or if you decide you don't want to be in the study any more, please feel free to contact Prof. Peter V. Giannoudis on 0113 392 2750.

NOTES

The Leeds Teaching Hospitals NHS
NHS Trust

CHILDREN ASSENT FORM

Title of Study: The role of Stem Cells in Fracture Healing

"Collection of Bone Marrow and Blood"

Patient Name: ……
Patient Identification Number: ……

Please circle all you agree (or if unable, to be completed by parent / guardian on their behalf)		
1. Have you read all (or had read to you) the information about this study?	**Yes**	**No**
2. Has somebody else explained this project to you?	**Yes**	**No**
3. I have had the opportunity to ask questions about the study and to discuss it with my family and friends if I so wish to.	**Yes**	**No**
4. Do you understand what this study is about?	**Yes**	**No**
5. I understand that all information collected in the study will be held in confidence and that, if it is presented or published as scientific paper, all personal details will be removed.	**Yes**	**No**
6. I agree for the data collected to be used for teaching, study and research in order to help more patients in the future.	**Yes**	**No**
7. Have you asked all the questions you want?	**Yes**	**No**
8. Have you had your questions answered in a way you understand?	**Yes**	**No**
9. Do you understand that it's ok to stop taking part, in the study, at any time?	**Yes**	**No**
10. Are you happy to take part?	**Yes**	**No**

If any answers are 'no' or you don't want to take part, don't sign your name!
If you do want to take part, you can write your name below

Child's agreement
I agree to participate in the study I have been told about.

Name:…… **Date**: ……

Investigator/Sub-investigator

I have explained the study to the above named participant and he/she has indicated his/her willingness to participate

Name (BLOCK CAPITALS): ……

Signed: …… **Date:**……

Thank you for your help.

The Leeds Teaching Hospitals NHS
NHS Trust

PATIENT INFORMATION SHEET

Regarding Investigation for a Young Person over 16 to 17 years old

Study title: Biological properties of Mesenchymal Stem Cells in Fracture Healing
"Collection of Bone Marrow and Blood"

PART 1

(Please read part 1 and if you agree continue on reading part 2)

You are invited to participate in a RESEARCH study.

You are selected as a possible participant, because you are within the age range that we are interested to study. Before reaching any decision, it is important to understand why the research is being done, and what it will involve.
Please read the following information carefully, and discuss it with your relatives, partner, friends, and if you wish your GP.

Take your time to decide whether or not you wish to take part in our study.

- ***What is the purpose of the study?***

Special cells of the human body known as Mesenchymal Stem Cells (MSCs) are involved in the production of bone, cartilage, muscle, tendon and ligaments. These cells have been found in all tissues to date, but exactly how they work is still not well understood. The purpose of this research is to find if there are any differences between the behaviour of young people's and adult's MSCs under specific conditions such as surgery.

- ***What is the medicine, device or procedure that is being tested?***

In this research we need to study only the behaviour of Mesenchymal Stem Cells (MSCs). Will not be required to take any kind of medicine for the purposes of the study, and we will not be testing any device or procedure!

- ***Why have I been invited to take part in the study?***

You have been selected as a possible participant because you suffer from Idiopathic Scoliosis which after a certain point of its evolution is treated surgically.
Secondly you are in the age range that we are interested to study (between 13 to 17 years old).
In order to achieve statistically valid results we need to study about 30 children/young people of the same age.

- ***Do I have to take part?***

Of course, the decision to participate or not, is absolutely yours. Should you decide to participate in the study and change opinion afterwards, this will not represent a problem. You can withdrawal from the study at any time, and this will not affect the standards of care that you receive.

- ***What will happen to me if I take part in the study?***

You will be involved only during the first week of your stay into the hospital and,you will not lose any more of your time, for the purposes of the study.
The whole study will last for one year, in order to recruit the necessary number of cases and to analyse the collected data.
The only thing that we need is your permission/consent to take a small sample of your blood during the normal blood tests, with no additional punctures or procedures than the standard ones, as well as a small sample of bone marrow during the surgery from the area of the operation.

- ***What will be asked to do?***

Apart from donating a small quantity of blood and bone marrow for the research purposes, there are no other requirements or tests that will be needed.
You will not lose any additional time from school/college due to this research.
Before you will be recruited in the study, we will give you a copy of this information sheet and you will be asked to authorise us by signing a special consent form.

- ***What other medicines could I have instead?***

In this research we don't use medicines, thus we do not anticipate any side effects or complications. Our study is related only to the analysis of small samples of bone marrow and blood from your organism.

- ***Is there anything else to be worried about, if I take part in the study?***

No, none of the aspects of this research can create any problem to you.

- ***What are the possible benefits of taking part in the study?***

We cannot promise that the study will help you directly. However, the information we will get after our analysis, might help other children and people in the future. It will also be an opportunity for you to participate to a scientific research study approved by the Leeds Research Ethics Committee and the Research and Development department of this NHS Trust.

- ***Contact details***

If something is not clear or if you would like more information, please don't hesitate to ask us.
Take your time to decide whether you wish or do not wish to take part in the study.
Contact Prof. Peter V. Giannoudis on 0113 392 2750.

Thank you for reading this.

PART 2

What is the purpose of the study?

Special cells of the human body known as Mesenchymal Stem Cells (MSCs) are involved in the production of bone, cartilage, muscle, tendon and ligaments. These cells have been found in all tissues, but exactly how they work is still not well understood.
There is great interest in the use of MSCs as a way of repairing damaged tissues.
Our research aims to improve our understanding of how these cells work in health and in disease.
More specifically, we would like to study if these cells have a different behaviour between adults and children/young people, in case of major surgery, such as a scoliosis operation.

Why have I been chosen?

You suffer from idiopathic scoliosis, which after a certain point of its evolution is treated surgically.
Also, we are conducting this study on children/young people within the age range between 13 to 17 years old.
To undergo this sort of lengthy and complex surgery, albeit quite safe, does cause these stem cells to come into action so as to protect patients during surgery and mobilise natural healing.
We want to know what happens to these stem cells during surgery and what triggers them into action.
In order to achieve a statistically valid result of our research program we need to study approximately 30 children.

What will happen to me if I take part in the study?

You will be asked to donate a small extra amount of blood (few extra grams for every withdrawal) which is routinely taken before, during and after the operation.
The plan is to take small samples of peripheral blood (just 3 ml more, than the standard quantity of blood collection) before the start of the operation, intra-operatively, at the end of the surgery and four more times later (1st, 3rd, 5th and 7th day after the surgery).
Blood samples are taken before and after surgery routinely, as standard of care, in this type of operation. We would like to obtain smalls additional amounts of blood in the same samplings for our research. Withdrawing this extra small amount of blood does not influence the final result of the operation of your child or his/her recovery.
During surgery similar blood samples are often taken as part of intra-operative monitoring. At that setting we would like also to obtain a small additional amount for our research. This additional sample will not cause you any further discomfort, as it is already part of the normal procedure and will not delay healing in any way.
Also, during surgery (from the field of the surgical area) in the bony part of the operation, when the metalwork is inserted we shall take a very small piece of bone marrow aspirate, so in this way we are able to study the biology of these stem cells. This will not in any way harm your child who will feel absolutely no discomfort whatsoever over and above a routine scoliosis operation. This bone marrow normally leaks out and is washed out and discarded during the operation.

Do I have to take part?

It is up to you to decide whether or not you would like to participate. There is absolutely no obligation at all to take part in this study although being quite harmless to you; we do hope you will agree to join our research team. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time without giving a reason. This will not affect the way we take care of you..

What do I have to do?

Apart from donating a small quantity of blood, and bone marrow (only once), for research purposes, there are no other requirements/tests.
It is very important, when you are giving your medical history to the doctor, to inform if you are regularly taking any medicine. Taking certain types of medication, may mean that we will have to exclude you from this study.

What are the possible benefits and risks of taking part?

You will not benefit directly from taking part in this research and all other aspects of your care will be the same as if you did not take part.
Although there may be no direct benefit to you, this will be of great value to future scoliosis patients and other people of any age that undergo major surgery.

What are the side effects of taking part?

There are no side effects. Asking to give us a very small amount of your blood, (just a few drops), will not put yourself at any type of risk.

What other medicines could I have instead?

We do not require you to take any kind of research related medications. Our study is related only with the bone marrow and blood samples and the specific tests that we will run for analysis.

Is there anything else to be worried about if I take part?

No, none of the aspects of this research can cause problems to you.

Will I needs to take time out of school/college, and if so how much time?

This research will not require taking any time out of school/college! It is related only with the first week of the total period of staying in the hospital at the time of the scoliosis surgery. The whole period of time spent in the hospital is the minimum necessary for your good recovery after such major surgery, and is not related with the research study.

Would this affect my social life?

Not at all, as it will not affect your recovery and will not cause any additional problems or discomfort. The aim of this research is to help other people and children/young people like you in the future. The goal of scoliosis surgery is to improve the quality of life and your social aspect..

When will the study begin and possibly finish?

This is a pilot study, and will last one year, starting in June 2012.
The duration of the study depends principally on the evaluation of all the data collected after acquiring samples from 30 patients, and not on the future availability of any of these patients.

What will happen to the results of the study?

They will be analysed very carefully, published in a major scientific journal so that the rest of the world will appreciate its significance not only in scoliosis surgery but other comparable major operations.

Will my taking part in this study be kept confidential?

Although you will be an important part of the study, as soon as the samples are withdrawn from you, all information that may identify you will be removed so that you cannot be recognised or linked to the research material.

What will happen to any samples you give?

The collection and storing of all samples is done in a way that ensures maximum safety. After examination in the laboratory they are neutralised and discarded under special conditions, respecting particular rules, within the trust guide lines.

What if something goes wrong?

If you are harmed by taking part in this research (again this is not expected to be the case by any means), there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal NHS complaints procedure is available to you.

What happens when the research project stops?

Considering that this study is not related to any type of drugs, and you are not under any type of special treatment, you must not worry if the study is going to end.

What happens if new information about the research medicine comes along?

Sometimes during a research study, new things can be discovered or new related evidence can become available. If something relevant to this study becomes available which affects the design or the subject of this project we will inform you in accordance to the changes occurred.

Where will the study take place?

This project is being organized by Doctors of this hospital (Leeds General Infirmary) and researchers of the Leeds Institute of Molecular Medicine, with supervision and support from the University of Leeds.

Who has reviewed this study?

This study has been reviewed by the independent ethics committee called the Leeds (East) Research Ethics Committee. This committee is appointed to determine that research studies are ethical and do not impair the rights or well-being of patients. Also the Research and Development department of the Leeds Teaching Hospitals NHS Trust has also reviewed and approved this study.

Who will lead the study?

Prof. Peter V. Giannoudis is the principal investigator. He is the chairman of the Academic Department of Trauma and Orthopaedic Surgery, of the School of Medicine of the University of Leeds, and is also a Honorary Consultant at the Leeds Teaching Hospital NHS Trust, based at the Leeds General Infirmary.
If you require further information about this study, or if you decide that you don't want to participate in the study any more, please do not hesitate to contact Professor Peter Giannoudis on 0113-39-22750, 0113-39-23290.

NOTES

The Leeds Teaching Hospitals NHS
NHS Trust

PATIENT CONSENT FORM

Regarding Investigation for a Young Person aged between 16 to 17 years of age.

Study title: Biological properties of Mesenchymal Stem Cells in Fracture Healing

"Collection of Bone Marrow and Blood"

Patient's Name: ………………………………………………………..………………………….
Patient's Identification Number: …..……………………………..……………………………

Please circle as appropriate		
1. I have read (or had read to me) and understand the patient information sheet for the above study.	**Yes**	**No**
2. I have had the opportunity to ask questions about the study and to discuss it with family and friends, if I so wish to.	**Yes**	**No**
3. I understand the purpose of the study, and how I will be involved.	**Yes**	**No**
4. I understand, and accept, that if I take part in the study I may not gain direct personal benefit, but many more people will benefit from it.	**Yes**	**No**
5. I understand that all information collected in the study will be held in confidence and if the results will be written into a scientific paper, all personal details will be removed.	**Yes**	**No**
6. I give permission for those responsible doctors, to have access to my medical notes, where it is relevant to my taking part in the research. This is on the understanding that no personal details which might identify me, will be presented or published without my permission.	**Yes**	**No**
7. I agree for the data collected to be used in future for teaching, study and research for the benefit of all patients.	**Yes**	**No**
8. I confirm that I will be taking part in this study of my own free will, and I understand that I am free to withdraw from the study at any time without giving a reason and without affecting future care or legal rights.	**Yes**	**No**
9. I have spoken to Mr. Fragkakis Evangelos	**Yes**	**No**
10. I agree to take part in this research study.	**Yes**	**No**

Patient or Legal Representative (BLOCK CAPITALS):

Name: …………………………………………………………........…………………….……….....

Signed: ………………..…………………………………..........………..…….. **Date:** ………………….

Investigator/Sub-investigator (BLOCK CAPITALS):

I have explained the study to the above named participant and he/she has indicated his/her willingness to participate

Name: ……………………….……………………………………………….…………………........……..

Signed: ……….........…………………………………………..…………………… **Date:** ………..……….

Thank you for the help

The Leeds Teaching Hospitals NHS
NHS Trust

PARENTAL INFORMATION SHEET

Regarding Investigation or Treatment for a Child or Young Person

<u>Study title</u>: Biological properties of Mesenchymal Stem Cells in Fracture Healing
"Collection of Bone Marrow and Blood"

PART 1

(Please read part 1 and if you agree continue on reading part 2)

Your child has been invited to take part in a RESEARCH study.

She/he was selected as a possible participant, because she/he is within the age range that we are interested to study.
Before reaching any decision, it is important to understand why the research is being done, and what it will involve.
Please read the following information carefully, and to discuss it with your partner, friends, relatives, and if you wish your GP.

Take your time to decide whether or not you wish your child to take part in our study.

- ***What is the purpose of the study?***

Special cells of the human body known as Mesenchymal Stem Cells (MSCs) are involved in the production of bone, cartilage, muscle, tendon and ligaments. These cells have been found in all tissues to date, but exactly how they work is still not well understood. The purpose of this research is to find if there are any differences between the behaviour of children's and adult's MSCs under specific conditions such as surgery.

- ***What is the medicine, device or procedure that is being tested?***

In this research we need to study only the behaviour of Mesenchymal Stem Cells (MSCs). Your child will not be required to take any kind of medicine for the purposes of the study, and we will not be testing any device or procedure!

- ***Why has your child been invited to take part in the study?***

Your child was selected as a possible participant because she/he suffers from Idiopathic Scoliosis which after a certain point of its evolution is treated surgically.
Secondly your child is in the age range that we are interested to study (above the age of 13 years).
In order to achieve statistically valid results we need to study about 30 children of the same age.

- ***Does she/he have to take part?***

Of course, the decision to allow your child to participate or not, is absolutely yours. Should you decide to let your child participate in the study and change opinion afterwards, this will not represent a problem. Your child can withdrawal from the study at any time, and this will not affect the standards of care that your child receives.

- ***What will happen to my child if she/he takes part in the study?***

She/he will be involved only during the first week of her/his stay into the hospital and, will not lose any more of her/his time, for the purposes of the study.
The whole study will last for one year, in order to recruit the necessary number of cases and to analyse the collected data.
The only thing that we need is your permission/consent to take a small sample of your child's blood during the normal blood tests, with no additional punctures or procedures than the standard ones, as well as a small sample of bone marrow during the surgery from the area of the surgery.

- ***What does she/he have to do?***

Apart from donating a small quantity of blood and bone marrow for the research purposes, there are no other requirements or tests that will be needed.
She/he will not lose any additional time from school due to this research.
Before your child will be recruited in the study, we will give you a copy of this information sheet and you will be asked to authorise us by signing a special consent form.
If you agree for your child to participate in this study, will be given to your child a specially prepared document with a brief description of the study in simple terms. She/he will have to agree with the overall plan and write her/his name in a special form called Assent form.

- ***What other medicines could she/he has instead?***

In this research we don't use medicines, thus we do not anticipate any side effects or complications. Our study is related only to the analysis of small samples of bone marrow and blood from your child.

- ***Is there anything else to be worried about, if she/he takes part in the study?***

No, none of the aspects of this research can create any problem to your child.

- ***What are the possible benefits of taking part in the study?***

We cannot promise that the study will help your child directly. However, the information we will get after our analysis, might help other children and people in the future. It will also be an opportunity for yourself and your child to participate to a scientific research study approved by the Leeds Research Ethics Committee and the Research and Development department of this NHS Trust.

- ***Contact details***

If something is not clear or if you would like more information, please don't hesitate to ask us.
Take your time to decide whether you wish or do not wish to take part in the study.
Contact Prof. Peter V. Giannoudis on 0113 392 2750.

Thank you for reading this.

PART 2

What is the purpose of the study?

Special cells of the human body known as Mesenchymal Stem Cells (MSCs) are involved in the production of bone, cartilage, muscle, tendon and ligaments. These cells have been found in all tissues, but exactly how they work is still not well understood.
There is great interest in the use of MSCs as a way of repairing damaged tissues.
Our research aims to improve our understanding of how these cells work in health and in disease.
More specifically, we would like to study if these cells have a different behaviour between adults and children, in case of major surgery, such as a scoliosis operation.

Why has your child been chosen?

Your child suffers from idiopathic scoliosis, which after a certain point of its evolution is treated surgically.
Also, we are conducting this study on children with the same age range as your child's.
To undergo this sort of lengthy and complex surgery, albeit quite safe, does cause these stem cells to come into action so as to protect patients during surgery and mobilise natural healing.
We want to know what happens to these stem cells during surgery and what triggers them into action.
In order to achieve a statistically valid result of our research program we need to study approximately 30 children.

What will happen to my child if she/he takes part?

Your child will be asked to donate a small extra amount of blood (few extra grams for every withdrawal) which is routinely taken before, during and after the operation.
The plan is to take small samples of peripheral blood (just 3 ml more, than the standard quantity of blood collection) before the start of the operation, intra-operatively, at the end of the surgery and four more times later (1st, 3rd, 5th and 7th day after the surgery).
Blood samples are taken before and after surgery routinely, as standard of care, in this type of operation. We would like to obtain smalls additional amounts of blood in the same samplings for our research. Withdrawing this extra small amount of blood does not influence the final result of the operation of your child or his/her recovery.
During surgery similar blood samples are often taken as part of intra-operative monitoring. At that setting we would like also to obtain a small additional amount for our research. This additional sample will not cause to your child any further discomfort, as it is already part of the normal procedure and will not delay healing in any way.
Also, during surgery (from the field of the surgical area) in the bony part of the operation, when the metalwork is inserted we shall take a very small piece of bone marrow aspirate, so in this way we are able to study the biology of these stem cells. This will not in any way harm your child who will feel absolutely no discomfort whatsoever over and above a routine scoliosis operation. This bone marrow normally leaks out and is washed out and discarded during the operation.

Does she/he have to take part?

It is up to you to decide whether or not you would like your child to participate. There is absolutely no obligation at all for your child to take part in this study although being quite harmless to her/him; we do hope you will agree to join our research team. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to request for your child to withdraw at any time without giving a reason. This will not affect the standard of care that your child receives.

What does she/he has to do?

Apart from donating a small quantity of blood, and bone marrow (only once), for research purposes, there are no other requirements/tests.
It is very important, when you are giving her/his medical history to the doctor, to inform if she/he is regularly taking any medicine. Taking certain types of medication, may mean that we will have to exclude your child from this study.

What are the possible benefits and risks of taking part?

Your child will not benefit directly from taking part in this research and all other aspects of her/his care will be the same as if she/he did not take part.
Although there may be no direct benefit to your child, this will be of great value to future scoliosis patients and other people of any age that undergo major surgery.

What are the side effects of taking part?

There are no side effects. Asking to give us a very small amount of your blood, (just a few drops), will not put your child at any type of risk.

What other medicines could she/he have instead?

We do not require your child to take any kind of research related medications. Our study is related only with the bone marrow and blood samples and the specific tests that we will run for analysis.

Is there anything else to be worried about if she/he takes part?

No, none of the aspects of this research can cause problems to your child.

Will she/he needs to take time out of school, and if so how much time?

This research will not require taking any time out of school! It is related only with the first week of the total period of staying in the hospital at the time of the scoliosis surgery. The whole period of time spent in the hospital is the minimum necessary for her/his good recovery after such major surgery, and is not related with the research study.

Would this affect her/his social life?

Not at all, as it will not affect her/his recovery and will not cause any additional problems or discomfort. The aim of this research is to help other people and children like yours in the future. The goal of scoliosis surgery is to improve the quality of life and social aspect of your child.

When will the study begin and possibly finish?

This is a pilot study, and will last one year, starting in June 2012.
The duration of the study depends principally on the evaluation of all the data collected after acquiring samples from 30 patients, and not on the future availability of any of these patients.

What will happen to the results of the study?

They will be analysed very carefully, published in a major scientific journal so that the rest of the world will appreciate its significance not only in scoliosis surgery but other comparable major operations.

Will her/his taking part in this study be kept confidential?

Although your child will be an important part of the study, as soon as the samples are withdrawn from her/him, all information that may identify your child will be removed so that she/he cannot be linked or recognised to the research material.

What will happen to any samples that she/he gives you?

The collection and storing of all samples is done in a way that ensures maximum safety. After examination in the laboratory they are neutralised and discarded under special conditions, respecting particular rules, within the trust guide lines.

What if something goes wrong?

If your child is harmed by taking part in this research (again this is not expected to be the case by any means), there are no special compensation arrangements. If she/he is harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way your child has been approached or treated during the course of this study, the normal NHS complaints procedure is available to you.

What happens when the research project stops?

Considering that this study is not related to any type of drugs, and your child is not under any type of special treatment, you must not worry if the study is going to end.

What happens if new information about the research medicine comes along?

Sometimes during a research study, new things can be discovered or new related evidence can become available. If something relevant to this study becomes available which affects the design or the subject of this project we will inform you in accordance to the changes occurred.

Where will the study take place?

This project is being organized by Doctors of this hospital (Leeds General Infirmary) and researchers of the Leeds Institute of Molecular Medicine, with supervision and support from the University of Leeds.

Who has reviewed this study?

This study has been reviewed by the independent ethics committee called the Leeds (East) Research Ethics Committee. This committee is appointed to determine that research studies are ethical and do not impair the rights or well-being of patients. Also the Research and Development department of the Leeds Teaching Hospitals NHS Trust has also reviewed and approved this study.

Who will lead the study?

Prof. Peter V. Giannoudis is the principal investigator. He is the chairman of the Academic Department of Trauma and Orthopaedic Surgery, of the School of Medicine of the University of Leeds, and is also a Honorary Consultant at the Leeds Teaching Hospital NHS Trust, based at the Leeds General Infirmary.
If you require further information about this study, or if you decide that you don't want to participate in the study any more, please do not hesitate to contact Professor Peter Giannoudis on 0113-39-22750, 0113-39-23290.

NOTES

The Leeds Teaching Hospitals NHS
NHS Trust

PARENTAL AGREEMENT - CONSENT FORM

to Investigation for a Child or Young Person

Study title: Biological properties of Mesenchymal Stem Cells in Fracture Healing

"Collection of Bone Marrow and Blood"

Patient's Name: ……..…….

Patient's Identification Number: …..……..…….……

Please circle as appropriate		
1. I have read the parental information sheet for the above study, and confirm that I have parental responsibility for this child.	**Yes**	**No**
2. I have had the opportunity to ask questions about the study and to discuss it with family and friends, if I so wish to.	**Yes**	**No**
3. I understand the purpose of the study, and how my child will be involved.	**Yes**	**No**
4. I understand, and accept, that if my child takes part in the study he/she may not gain direct personal benefit, but many more children will benefit from it.	**Yes**	**No**
5. I understand that all information collected in the study will be held in confidence and if the results will be written into a scientific paper, all personal details will be removed.	**Yes**	**No**
6. I give permission for those responsible doctors, to have access to my child's medical notes, where it is relevant to her/his taking part in the research. This is on the understanding that no personal details which might identify him/her, will be presented or published without my permission.	**Yes**	**No**
7. I agree for the data collected to be used in future for teaching, study and research for the benefit of all patients.	**Yes**	**No**
8. I confirm that my child will be taking part in this study of his/her own free will, and I understand that we are free to withdraw from the study at any time without giving a reason and without affecting future care or legal rights.	**Yes**	**No**
9. I have spoken to Mr Fragkakis Evangelos	**Yes**	**No**
10. I agree to take part in this research study.	**Yes**	**No**

Parents or Legal Representative (BLOCK CAPITALS):

Name: ……..……..……....

Relationship to the child: ……..……...

Signed: ……...……...…….. **Date:** …….

Investigator/Sub-investigator (BLOCK CAPITALS):

I have explained the study to the above named participant and he/she has indicated his/her willingness to participate

Name: ……..……..……..

Signed: ……..…… **Date:** ….....……..……

Thank you for the help

8.4 Ethical approvals

NHS
Health Research Authority

NRES Committee Yorkshire & The Humber - Leeds East
Yorkshire and Humber REC Office
First Floor, Millside
Mill Pond Lane
Meanwood
Leeds
LS6 4RA

Tel: 0113 3050108
Fax:

12 April 2012

Prof Peter Giannoudis
Consultant
Department of Trauma and Orthopaedics
St James's University Hospital
LS9 7TF

Dear Prof Giannoudis

Study title:	**Biological properties of Mesenchymal Stem Cells in Fracture Healing**
REC reference:	**06/Q1206/127**
Amendment number:	**3/1**
Amendment date:	**11 April 2012**

Thank you for submitting the above amendment, which was received on 12 April 2012. It is noted that this is a modification of an amendment previously rejected by the Committee (our letter of 22nd February 2012 refers).

The modified amendment has been considered on behalf of the Committee by the Vice-Chair.

Ethical opinion

I am pleased to confirm that the Committee has given a favourable ethical opinion of the modified amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved are:

Document	Version	Date
Parental Agreement - Consent Form	1.1	23 March 2012
Participant Consent Form: Patient Consent Form 16 - 17 years old	1.0	23 March 2012
Participant Consent Form: Children Assent Form	1.0	01 February 2012
Participant Information Sheet: Patient Information Sheet 16 - 17 years old	1.0	23 March 2012
Participant Information Sheet: Parental Information Sheet	1.1	23 March 2012
Participant Information Sheet: Children Information Sheet	1.1	23 March 2012
Protocol	6.0	19 December 2011
Modified Amendment		11 April 2012

Covering Letter		

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q1206/127:	Please quote this number on all correspondence

Yours sincerely

Alan Ebbutt
Vice Chair

E-mail: jade.thorpe@nhs.net

NHS
Health Research Authority
NRES Committee Yorkshire & The Humber - Leeds East

North East REC Centre
Room 002
TEDCO Business Centre
Viking Industrial Park
Rolling Mill Road
Jarrow
NE32 3DT

Tel: 0191 4283545

12 December 2013

Prof Peter Giannoudis
Consultant
Univeristy of Leeds
Department of Trauma and Orthopedics
St James's University Hospital
LS9 7TF

Dear Prof Giannoudis

Study title: **Biological properties of Mesenchymal Stem Cells in Fracture Healing**
REC reference: **06/Q1206/127**
Amendment number: **1**
Amendment date: **15 June 2010**
IRAS project ID:

Thank you for your letter of 15 June 2010, notifying the Committee of the above amendment.

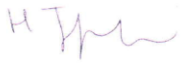
The Committee does not consider this to be a "substantial amendment" as defined in the Standard Operating Procedures for Research Ethics Committees. The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

Documents received
The documents received were as follows:

Document	Version	Date
Notification of a Minor Amendment – Extension to study (January 2017)	1	15 June 2010

Statement of compliance
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

Yours sincerely



Hayley Jeffries
REC Manager

E-mail: nrescommittee.yorkandhumber-leedseast@nhs.net