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University of Siena – Department of Medical Biotechnology Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC)

XXXIV Cycle (2019-2022)

Coordinator: Prof. Francesca Ariani

In vitro hBM-MSCs characterization and differentiation on the scaffold for bone regenerative medicine

Scientific disciplinary sector: BIO/11-Molecular Biology

Tutor:

Prof. Stefano Giannotti

PhD Candidate:

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Prof. Riccardo Ferracini
Specialista in Ortopedia e Traumatologia
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Università di Genova

Torino, 24 Ottobre 2022

Upon request of the evaluation of the Doctorate Thesis at the University of Siena – Department of Medical Biotechnology Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC) XXXIV Cycle (2019-2022)

Coordinator: Prof. Francesca Ariani.

Scientific disciplinary sector: BIO/11-Molecular Biology

Thesis Title: "***In vitro* hBM-MSCs characterization and differentiation on the scaffold for bone regenerative medicine".**

PhD Candidate: Laura Di Sarno

Tutor: Prof. Stefano Giannotti

Thesis Evaluation:

The Thesis topic holds a solid scientific and especially a bio-industrial relevance.

The state of the art is properly focused and properly discussed.

The main objective of the PhD work is clearly stated and valuable for this research field.

The methods used in this Thesis, albeit commonly used in this research field, were appropriately employed, and validated.

The achieved results solve the Thesis objective and altogether provide a significant advantage over existing solutions.

The written form of the manuscript is excellent. Structure, grammar and sentencing are flawless and easily readable.

Overall, I recommend the PhD candidate should be admitted to the public defense of the Thesis. No additional review is required.

Truly,

Professor Riccardo Ferracini

regenlab
PRP & CELL THERAPY SPECIALISTS

Lausanne, 23 October 2022

Subject : Evaluetion Thesis

Regen Lab SA and CEO Dr. Antoine Turzi are evaluating the Thesis in « ***In vitro hBM-MSCs characterization and differentiation on the scaffold for bone regenerative medicine*** » drawn up by candidate Dottoranda Laura di Sandro under the supervision of Tutor Prof. Stefano Giannotti.

Following a careful analysis of the text and the results obtained, it is possible to affirm that the Thesis under evaluation has exceeded our expectations, both in terms of deepening the topics covered and in terms of overall commitment to the conduct of the research for its entire duration.

Particular appreciation is to be attributed to the critical ability of the researchers, to the completeness of the well-described content, as well as to the biotechnological value of the topic under study.

Sincerely.

Antoine Turzi
(Ceo Regen Lab SA)

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Abstract

New methods have been developed to achieve tissue regeneration of complex bone defects and restore the healing process, which is impaired by several factors; in this contest bone tissue engineering (BTE) is an alternative to autologous gold-standard treatment. BTE combines biocompatible scaffolds with morphogenic signals and stem cells, to create a biomimetic microenvironment that provides mechanical and chemical cues.

In this project, we studied all key elements of BTE and evaluated Mesenchymal stem cells (MSC) with xenograft-derived bone scaffold (SmartBone® SBN) and rhBMP-2 as growth factor. MSCs are an attractive source of stem cells because of their ability to undergo self-renewal, multi-lineage differentiation (including into osteoblast lineage) and paracrine actions. Here, we reported our experience in MSCs expansion protocols and proposed human Platelet-Rich Plasma (PRP) as a substitute for Fetal Bovine Serum (FBS) in media supplementation. After MSCs isolation from patients bone marrow, we expanded the cells in media supplemented with FBS or PRP, obtained from a venous blood sample of the same patients. Our final outcome indicated that PRP is a good cell culture supplement, since it did not impact MSCs marker expression and differentiation potential.

In the second part of the project, we seeded hBM-MSC on xenograft-derived bone scaffold and cultured them in osteogenic and MSC expansion media to investigate the effects of the composition of support on cell metabolic activity and osteogenic differentiation. The scaffold retains biological properties and resembles the human bone structure. We demonstrated its biocompatibility supporting both BM-MSCs proliferation and differentiation. Moreover, new collagen deposition was revealed in both analyzed conditions, suggesting a good osteoconductivity of the scaffold.

Finally, we aimed to modify the scaffold by the addition of rhBMP-2 to improve its osteogenic abilities and enhance new bone formation. Future experiments will assess the impact of BMP2 modification on cellular differentiation.

1. Introduction

1.1 Structure of bone

Bone is a highly specialized connective tissue, which exerts a wide range of functions in human physiology. It provides support to body structure and protection to internal organs, allows body movements and finally is involved in metabolic and haemostatic processes. It represents a natural reservoir for several substances and contributes to the significant supply of calcium, phosphate and magnesium, finally, it harbours bone marrow (Maffioli et al., 2015).

At the macroscopic level, bone tissue is composed of two subtypes: an external layer known as the cortical bone and an internal layer called the cancellous structure. The former corresponds to 80% of the total amount of bone, while the other component represents the remaining 20%. Cortical and cancellous structures have different tissue architectures and properties which also reflect their functions (Battafarano et al., 2021). Cortical bone, also referred to as compact bone, is a solid bone component which contributes more to the tissue's mechanical strength and provides support and protection against external forces. It is organized into functional units called osteons, which consist of concentric lamellae surrounding a central Haversian canal containing blood vessels and nerves (Ascenzi et al., 2012; Chang et al., 2021). In contrast, cancellous tissue, also known as trabecular or spongy bone, is a highly porous material. It has a complex microstructure composed of a network of struts called trabeculae, which are surrounded by red bone marrow, where haematopoiesis occurs. The outer surface of almost all bones is covered by the periosteum, a connective tissue that acts as a niche of mesenchymal stem cells, osteoprogenitors and pre-osteoblasts and promotes the ossification process during prenatal development and postnatal fracture healing (Ueno et al., 2001; Arnsdorf et al., 2009).

At the molecular level, bone is a natural composite tissue characterized by a mineralized extracellular matrix made of water, organic and inorganic phases. The main component of the organic phase is type I collagen (approximately 90%) that plays several roles; it is involved in force transmission and preserves bone structural integrity and architecture. Impaired collagen structure significantly affects bone mechanical properties and has broad implications in several diseases including osteogenesis imperfecta, osteoarthritis and osteoporosis (Rubin et al., 2003; Tzaphlidou et al., 2005). The remaining part of the organic matrix is characterized by non-collagenous proteins

such as alkaline phosphatase, osteocalcin, and osteopontin which regulate matrix mineralization and bone remodeling processes. The collagen fibrils are surrounded by an inorganic component made of calcium phosphate called hydroxyapatite (HA) crystals: $Ca_{10}(PO_4)_6(OH)_2$. HA crystals are distributed along the long axis of collagen fibrils, and they not only represent a reservoir of calcium and phosphate ions, but also provide stiffness.

Bone is a dynamic and living organ that responds to internal and external stimuli, adjusts to mechanical changes and is continuously renewed. The remodeling process involves two phases: resorption of old bone mediated by osteoclasts and deposition and formation of new bone achieved by osteoblasts. The entire process takes place under the control of three bone cell types: osteoblasts, osteocytes and osteoclasts. Osteoblasts are cuboidal cells derived from mesenchymal stem cells. Functionally, they produce an organic matrix and synthesize a range of macromolecules such as type I collagen and other non-collagenous proteins, including alkaline phosphatase, osteopontin and osteonectin which are involved in extracellular mineralization. After matrix deposition, osteoblasts are buried within bone matrix, they change their morphology, acquire a stellar shape and become osteocytes. Osteocytes are mature osteoblasts, which act as mechanosensory cells and create an extensive network of cell communication. Since they direct both osteoblast and osteoclast activities, they control bone remodeling and homeostasis (Bonewald et al., 2008). Finally, osteoclasts are multinucleated cells which originate from the monocyte-macrophage lineage cells. They resorb bone matrix, by the release of H^+ ions which reduces the pH of the microenvironment and degrades the mineralized matrix. The activities of bone-resorbing osteoclasts and bone-forming osteoblasts are related; many studies have demonstrated that both cell types produce and secrete extracellular signaling molecules which reciprocally stimulate or inhibit the differentiation process. Osteoclasts recruit osteoblast progenitors and stimulate MSC differentiation towards the osteoblast lineage. At the same time osteoblasts have an active effect on osteoclastogenesis producing molecules which stimulate or inhibit osteoclast differentiation (Glass et al., 2005).

1.2 Bone fracture and healing mechanism.

One of the main characteristics of bone is its ability to self-repair; after damage, tissue injury causes a series of sequential events which lead to the renewal of the structures without the formation of fibrous scar tissue (Tosounidis et al., 2009).

Healing is a complex process strictly regulated by various factors. Based on the injured area, tissue repair is achieved through different ossification processes that recapitulate the development of bone during embryonic stage: intramembranous and endochondral ossification (Ferguson et al., 1999). Both mechanisms are similar, however, intramembranous ossification occurs primarily in the periosteum, where undifferentiated mesenchymal stem cells (MSCs) differentiate into osteoprogenitor cells and osteoblasts which produce collagen and woven bone, bypassing the formation of soft callus (Dimitriou et al., 2005). Whereas during endochondral ossification, MSCs produce a cartilage template, which is gradually replaced by bone. Two types of healing process could be distinguished: primary (or direct) and secondary (or indirect) mechanisms. When the fractured bone-ends are close with a stable fixation, the primary process occurs, in which MSCs directly differentiate into osteoblasts. In the other cases, when the gap size is moderate, the secondary healing process takes place that involves a combination of both intramembranous and endochondral ossification.

In this case, the healing process consists of the overlapping of three stages: inflammatory, repair and remodeling. After bone injury and the destruction of blood vessels, the first phase of the repair mechanism is the formation of a hematoma, which is a fibrin clot that prevents excess blood loss, and simultaneously represents the template for callus formation. The hypoxic microenvironment of the hematoma niche supports the healing process and stimulates the release of different angiogenic factors (Shiu et al., 2018; Schell et al., 2017). Many studies have established the crucial role of the inflammatory phase, as a balanced immune response loads a successful bone healing process. After trauma, macrophages, monocytes, lymphocytes, dendritic and endothelial cells migrate to the injured sites and release cytokines, chemokines and growth factors. Proinflammatory mediators have different roles, for example macrophages are polarized to the M1 phenotype and actively contribute to host defense, they clean the area by removing microorganisms, debris and necrotic tissue and they recruit to injured sites more inflammatory cells and MSCs. In this contest, MSCs release transforming growth factor β (TGFβ), bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2) which stimulate and promote osteogenesis and angiogenesis.

In the repair phase, MSCs differentiate into several cell types and generate extracellular matrices. During endochondral ossification, MSCs become chondrocytes and form a cartilaginous template called soft callus which will be gradually replaced by bone formation. Chondrocytes proliferate, produce collagen and extracellular matrix; after the hypertrophic phases the cells get embedded in

the matrix and undergo apoptosis. Meanwhile MSCs migrate to the site and differentiate into osteoblasts that produce bone matrix, transforming the soft callus into a hard callus. Finally, during the last phases of the healing process, bone gradually restores its biomechanical functions; the hard callus grows and undergoes continuous remodeling characterized by bone resorption and deposition played by osteoclasts and osteoblasts respectively **(figure 1).**

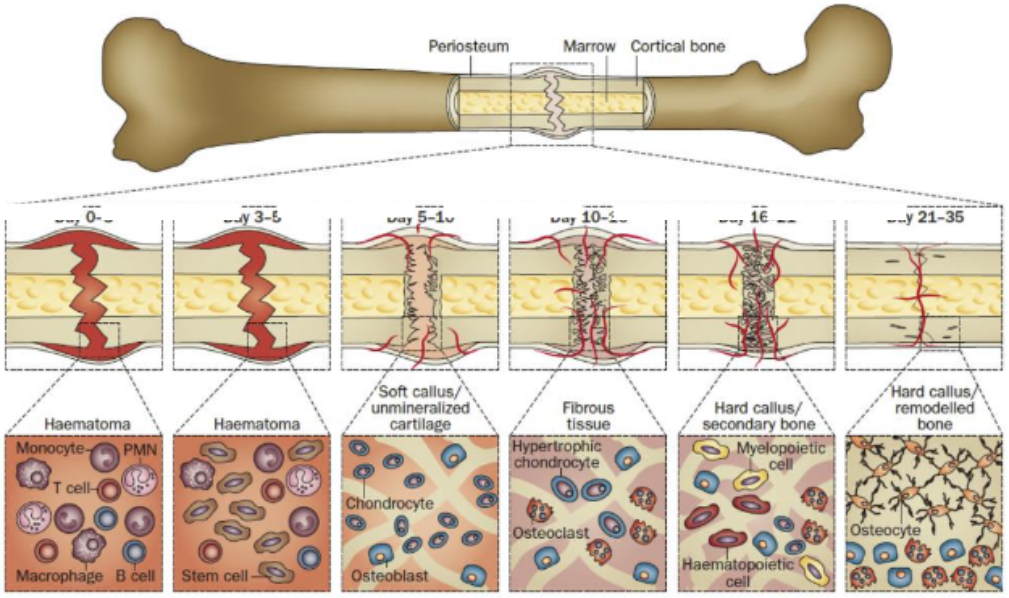

Figure 1: *Illustration of phases of healing process, including the cells and growth factors involved, which influence tissue repair mechanisms. Duan, R. (2019). Manipulating calcium phosphate materials with surface topography for bone regeneration. University of Twente. https://doi.org/10.3990/1.9789036547734*

Notwithstanding the well-regulated process, bone repair could fail, and the injured tissue does not heal and restore its original structure, functions and mechanical stability. Several factors influence and interrupt the physiologic process. Moreover, bone regenerative potential decreases with age, which also affects the immune response (Gruver et al., 2007). In addition, large size defects, inadequate vascularization, reduced mechanical stability, and some medical conditions, including diabetes mellitus, cancer and vascular diseases, negatively impact osseous tissue healing. For example, diabetes reduces the levels of some growth factors such as PDGF, VEGF and TEGF-β while hypothyroidism inhibits endochondral ossification and soft callus formation, resulting in failure of healing (Gaston et al., 2007). Immune cells participate in different healing phases and immune modulation has been well recognized as a crucial factor. A failed resolution of inflammation causes chronic inflammation, in which impaired production of pro-inflammatory

cytokines hinders the achievement of complete healing. In particular, the excessive release of tumor necrosis factor (TNFα) inhibits osteoblast and bone formation, while it stimulates osteoclastogenesis (Osta et al., 2014; Maruyama et al., 2020). All of these factors affect the cellular environment and predispose to the development of non-unions. According to the Food and Drug Administration (FDA), non-unions conditions occur when a fracture does not heal within 9 months after a traumatic event and has not shown any signs of healing for 3 consecutive months (Calori et al., 2017). It represents around 5-10% of all cases (Mills et al. 2013).

1.3 Current State-of-the-Art Therapy for Impaired Fracture Healing.

The treatment of non-unions represents a significant healthcare cost because of its complex management and complications which require a long time for resolution (Bradley et al., 2004; Bonafede et al., 2013), and result in patient reduced quality of life and disability (Hak et al., 2014). For these reasons, the treatment of impaired fractures is a dynamic field of research, in which all therapies ideally aim to achieve full bone restoration without interfering with the natural regenerative processes, but rather supplementing them.

Current approaches consist of transplanting a bone graft into the defect and stimulating bone healing. An ideal graft promotes the formation of bone, which is indistinguishable from the pre-injured one and exhibits osteoconductivity, osteoinductivity and osteogenicity properties. Osteoconductivity is the ability of a graft to support cell attachment and bone growth on its surface or down into the pores (Khan et al., 2005). Further, osteoinductivity is the capacity to recruit undifferentiated or osteoprogenitor cells and stimulate their differentiation towards the osteoblast lineage (Burchardt et al., 1983). The last parameter refers to the process by which osteoblasts differentiate and mineralize the tissue. Based on their nature and origin, bone grafts are classified in autologous, allogeneic and xenogeneic.

Autologous implants (autografts) still represent the gold standard because they are harvested from the patient and satisfy all already mentioned properties. Indeed, autografts provide both growth factors and cells (including osteoblasts and osteoprogenitor cells) supplying all indispensable factors for the integration of implants and bone formation. However, this procedure requires an additional surgery and increases patient morbidity. Moreover its availability is limited in quantity and clinical procedures are expensive, and are associated with complications including bleeding, inflammation and infection (Haugen et al., 2019). In comparison, allografts are obtained from human donors thus they overcome the requirement of patient additional surgical procedures for the

graft harvest. They are relatively abundant, but the sterilization processes, required to decrease the risk of immunological response and disease transmission, reduce their osteogenic potential (Baldwin et al., 2019; Arun et al., 2014). The last alternatives are xenografts which originate from non-human species. These grafts have structure and properties similar to those of human bone. The main advantages are the lower price and abundance of donors, but at the same time they are associated with ethical issues. Moreover, sterile processes, which are required for the risk of immune rejection and disease transmission, affect osteoinductive properties (Shibuya et al., 2015).

Each of these approaches has its limitations and the development of new strategies to enhance bone tissue healing remains a hot topic. Current studies aim to improve the understanding of bone biology by identifying key molecules that can be used to promote bone regeneration. In this contest, several proteins have been studied including parathyroid hormone (PTH) and Fibroblast Growth Factor 2 (FGF2) whose local application could support and accelerate the healing of complicated fractures (Peichl et al., 2011). Considering the tight interaction between the immune system and bone healing, the inflammatory system represents a promising therapeutic target for enhancing tissue repair (Xing et al., 2010; Schmidt-Bleek et al., 2012). Adequate inflammatory phase after injury is a crucial prerequisite for the regeneration process. In contrast, a prolonged or chronic inflammatory response has been shown to negatively influence healing and is a causative factor of non-unions. Hence, immune modulatory strategy could promote bone repair. Recent studies have demonstrated a potential immunosuppressive role of Iloprost. It is a prostacyclin (PGI2) analogue, already used in the treatment of disorders of the vascular system like pulmonary arterial hypertension, scleroderma disease and bone marrow oedema (Roman et al., 2012; Colaci et al.,2017; Disch et al., 2005). During the healing process, Iloprost modulates both the innate and adaptive immune systems and inhibits the secretion of proinflammatory cytokines, stimulating macrophage polarization toward the M2 pro-regenerative phenotype (Wendler et al., 2019).

Other treatments include Bone Tissue Engineering (BTE), which aims to develop bone substitutes and support new tissue formation allowing the recovery of affected bones. BTE is a cross-disciplinary science that involves the use of stem cells combined with scaffolds and growth factors to create an artificial environment that promotes tissue regeneration and restores function and lost architecture of tissue. The combination of these factors, including cell properties and the intrinsic characteristics of the scaffold, represents an alternative strategy to overcome current treatments limitations. For this purpose, the design of synthetic or natural implantable biomaterials is crucial, since it should mimic the physical and mechanical properties and meet bone graft

requirements **(figure 2)**. Cells can be harvested from various tissues. Over the last decade human Mesenchymal Stem Cells (hMSCs) have been widely studied and used as osteoblastic progenitor cells, since they can differentiate into osteoblasts and secrete different growth factors which support bone regeneration.

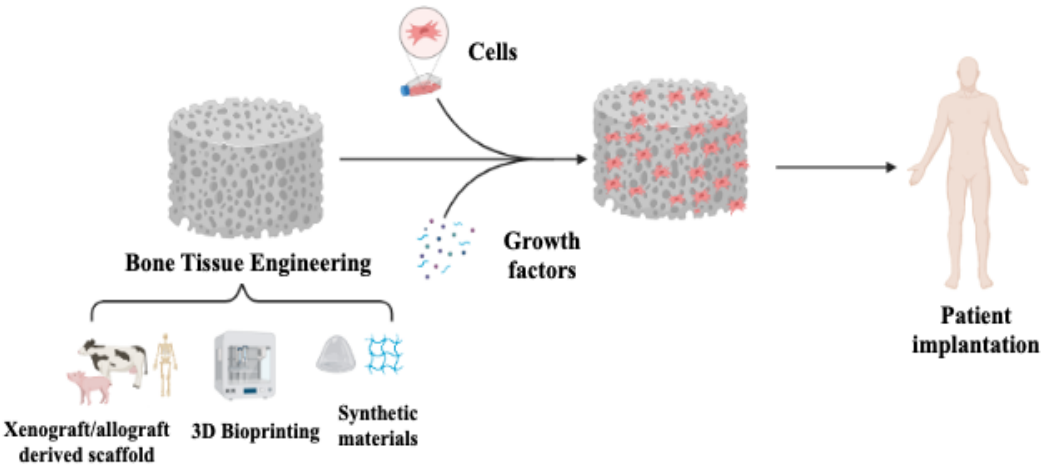

Figure 2: *Indicative representation of bone tissue engineering (BTE), that combines different types of scaffold with cells and growth factors.*

1.4 Mesenchymal stem cells (MSCs).

Mesenchymal Stem Cells (MSCs) represent an attractive source for tissue engineering. They were first described in 1967 by Friedenstein, who discovered and isolated a cellular population in the bone marrow characterized by *in vitro* replication and differentiation ability (Friedenstein et al., 1968; Friedenstein et al., 1970). Moreover he observed that bone marrow transplantation into kidney induced ectopic bone formation, suggesting that a cellular population in the bone marrow was capable of forming a bone structure (Friedenstein et al., 1974). Later, in 1991, Caplan coined the term Mesenchymal Stem Cells to define these cellular components and describe their ability to differentiate into several cellular lineages (Caplan et al., 1991).

Although MSCs have been extensively studied, numerous questions on their biology, functions and characteristics remain still unknown. Moreover, MSC nomenclature itself still presents some

controversies. Based on their cellular properties, the abbreviation has different meanings, including Multipotent Stromal Cells, Marrow Stromal Cells, Mesodermal Stem Cells, Mesenchymal Stromal Cells, and many more. Caplan himself, in his latest publication, described MSCs as Medicinal Signaling Cells (Caplan et al., 2017). In 2006 the International Society for Cellular Therapy (ISCT) defined MSCs as multipotent Mesenchymal Stromal Cells and established three criteria for their identification (Dominici et al., 2006): (1) MSCs must be plastic-adherent when they grow under *in vitro* conditions, (2) they are characterized by the presence of a cluster of surface molecules (CD105, CD90 and CD73), but they must not express other markers (CD45, CD34, CD14, CD19 and CD11b); (3) they show the ability to differentiate towards osteoblasts, adipocytes and chondroblasts.

Because of their beneficial properties, MSCs have attracted a lot of attention. They are a heterogeneous population which displays self-renewal and multipotent differentiation abilities. The capacity to proliferate and give rise to mesodermal lineage cells suggests them as a new tool to replace local cells after tissue injury and harness their regenerative potential for the treatment of some diseases such as osteoarthritis, bone defects, neurodegenerative disorders and myocardial infarction.

However, the most interesting functions of MSCs are their migratory, paracrine and immunomodulatory properties. In response to various types of injury, the level of endogenous MSCs increases, since they migrate toward damaged sites by chemoattraction, and secrete a range of growth factors and extracellular vesicles (EVs) referred to as the secretome. Most studies have shown that the secretome is responsible for the regenerative effect of MSCs, since it actively participates in tissue repair by exerting several effects on the process (Andrzejewska et al., 2019). For example, some cytokines, such as IGFI, BCL-2 and STC1, inhibit apoptosis and promote tissue regeneration (Galderisi et al., 2022). Moreover, it promotes angiogenesis by VEGF, FGF, SDF1 releasing which stimulates endothelial cell growth and vascularization (Rhee et al., 2015).

Finally, MSCs exert immunomodulatory properties and based on the microenvironment, they produce some chemokines and growth factors which modify immune reactions, by direct or indirect mechanisms. For example, they secrete programmed death‑1 and 2 ligands (PD-L1 and PD-L2) which interact with PD-1 and PD-2 receptors, that are expressed on the activated T and B cell surface, and inhibit cellular activation and proliferation (Davies et al., 2017). Immunomodulatory properties affect both primary and acquired immune responses, via the production of soluble

factors, including Transforming Growth factor-β1 (TGF-β1) and Prostaglandin E2 (PGE2). MSCs suppress the proliferation of NK cells and the differentiation of dendritic cells and B lymphocytes (Liu et al., 2015; Thiemermann et al., 2019). This anti-inflammatory effect also involves macrophage activity. Different studies have indicated that MSCs turn macrophage polarization from M1 (pro-inflammatory) into M2 state which plays a crucial role in tissue regeneration (Le Blanc et al.,2012; Medhat et al., 2019).

Despite MSCs great potential, the outcomes in the clinical application and industrial development are still far from satisfactory; several aspects limit their curative effect and benefits. MSCs consist of cellular subpopulations characterized by different morphology, proliferation and differentiation potential. Various studies have demonstrated that donors, source tissue, isolation and expansion protocols influence the heterogeneity of MSCs and contribute to different research outcomes (Liu et al., 2022). Specifically, MSCs harvested from several individuals have different properties and both donor's age and health status are responsible for cellular heterogeneity and favor stem cells senescence (Costa et al., 2021). MSC capabilities, including multilineage differentiation and immunomodulatory ones, depend on telomere length which decreases with age and obviously older donors have an increased percentage of apoptotic cells characterized by slower proliferation and differentiation rates (Zhou et al, 2008).

MSCs can be isolated from various human tissues. The most common sources are bone marrow (BM), adipose tissue (AT), umbilical cord (UC), skin, placenta (PL) Wharton's jelly (WJ) and dental pulps (DP) (Timper et al., 2006; Romanov et al., 2011; Estrela et al., 2011). Independently from the donor, cells harvested from different tissues show some differences in terms of proliferation, multipotency, immunophenotype, and secretome activities (Markov et al., 2021). For example, cells isolated from AT and BM share the same surface marker expression, but AT-MSC have a greater proliferative capacity, minor differentiation ability toward osteoblasts and different secretome properties compared to BM-MSC (Li et al., 2015). Specifically, some studies have demonstrated that AT-MSCs secrete higher amounts of pro-angiogenic molecules, including vascular endothelial growth factor (VEGF), than BM-MSC.

Isolation and expansion protocols are other topics widely discussed in the scientific community because both techniques can affect the quantity and quality of MSCs. After isolation, MSCs must be expanded *in vitro* to get a sufficient number of cells required for clinical treatments. However, the clonogenicity of MSC decreases and proliferation and differentiation rates slow down from passage

to passage. Many approaches have been described to maximise cell yield, but it is also important to preserve the initial MSC properties. The choice of culture conditions also depends on MSC clinical application. For example, a strategy includes cellular expansion in hypoxic conditions (O2 concentration of 1-7%), which mimic the original MSC niche (Holzwarth et al., 2010). Data highlighted that normoxic culture conditions increase MSC proliferation compared to hypoxic conditions, which stimulate MSC secretome activity (Fehrer et al., 2007; Pezzi et al., 2017; Kuan et al., 2021). In addition, most culture media are supplemented with Fetal Bovine Serum (FBS), which supports cellular proliferation, and acts as the major source of proteins, vitamins, growth factors and hormones. Nevertheless, FBS influences cell properties and there are also some issues concerning the safety of FBS use for MSC culture, because it is a potential source of xenogeneic antigens and zoonotic infections, which could cause a host immunological response (Vanda et al., 2018). For this purpose, the European Medicines Agency (EMA) and ISCT recommend FBS replacement, and a valid alternative is Platelet-Rich Plasma (PRP), a highly concentrated platelets suspension isolated from whole autologous blood. Additional studies have shown that PRP exerts beneficial effects on cellular proliferation, differentiation and extracellular matrix synthesis. In addition it exhibits a crucial role in bone formation and healing (Kitoh et al., 2007).

1.5 Scaffold

Tissue engineering aims to achieve functional and structural restoration of damaged tissue by the application of an implant or a scaffold that must reproduce, as much as possible, the natural microenvironment in which the cells interact with extracellular matrix and other cells leading to tissue healing. For this purpose, the scaffold must act as the extracellular matrix and support cell proliferation, differentiation and communication providing an appropriate environment. As bone grafts, the scaffold must exhibit several features: (i) being incorporated into damaged/host tissue without causing any immune response and supporting cell adhesion and proliferation (biocompatibility); (ii) degrading with time to allow new bone deposition releasing substances which should not be toxic for the body (biodegradability); (iii) inducing the osteoblastic differentiation of progenitor cells (osteoinduction); (iv) stimulating new bone deposition on scaffold surface (osteoconduction). In addition, the implant should provide mechanical strength to restore bone functional properties. Porosity, pore size and shape are decisive aspects of the scaffold, because they affect cellular attachment and migration and they also influence diffusion of nutrients and growth factors. For example, the size of pores should be big enough to allow cell distribution but at the same time sufficiently small for an efficient cellular adhesion (Amirazad et al., 2022; Yu

et al., 2018). Due to new bioprinting technologies, all these factors could be modified and modulated to favor cell growth and differentiation on the scaffold (Karageorgiou et al., 2005). A wide range of biomaterials, bone substitutes and fabrication techniques have been reported and used to produce a scaffold resembling native bone. However, no material can be considered as a gold standard that meets all the requirements, since each scaffold has specific advantages and disadvantages. The biomaterials could be classified in the following main groups: bioceramics, polymers, composite and decellularized extracellular matrix (dECM) (Stamnitz et al., 2021; Ghassemi et al., 2018). The bioceramics consist of calcium phosphate (Ca-P), including hydroxyapatite (HA) and tricalcium phosphate (TCP). Both materials share a similar composition to the inorganic bone matrix, therefore they possess osteoconductivity and osteointegration abilities, and they support MSCs osteogenic differentiation (Shih et al., 2014). During the degradation phase, the scaffolds release calcium and phosphate ions which stimulate bone formation; however, because of its brittle nature, bioceramics application is limited to load-free or low-load conditions.

Polymers are extremely versatile materials. They are divided in two categories: natural and synthetic. The former includes collagen, alginate, chitosan, and hyaluronic acid, that possess better biocompatibility, biodegradability and osteogenic properties than synthetic polymers but they could cause host immune response. On the other hand, the most used synthetic polymers are poly(glycolic acid) (PGA), polycaprolactone (PCL), and poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA). They are produced under controlled conditions and they have excellent mechanical properties. Almost all properties, including pore size, porosity and degradation rate, can be modulated to fit the demand of improving polymer mechanical properties and plasticity. Nonetheless, they present a reduced osteoconductivity which limits their application (Donnaloja et al., 2020). To overcome the limits of these materials, new strategies include the combination of ceramics with polymers, which leads to significant enhancement. For example, PLGA and PCL combined with TCP or HA provide excellent mechanical properties and increased osteoconductivity. Similarly, PCL/alginate composite scaffolds offer more benefits compared to pure PCL scaffolds, the composite material shows improved mechanical strength and osteogenic properties. Additional studies have shown that cell viability, calcium deposition and ALP activity were increased in PCL/alginate composite (Kim et al., 2015; Turnbull et al., 2017).

Finally, the decellularized bone scaffold overcomes the problem to replicate native bone tissue microenvironment. It is a natural matrix which undergoes decellularization and sterilization protocols in order to remove genetic material and cellular components and reduce the risk of

transmission of infectious agents and disease. After these processes the matrix still contains all structural components providing a suitable environment that supports cell adhesion and growth. Decellularized bone scaffold exploits natural tissue properties. Several benefits are known, including high biocompatibility and osteogenic capacity; indeed the scaffolds have shown to support bone formation (Kumar et al., 2016; Smith et al., 2015). They could originate from human donors (human decellularized bone allografts) or from other animal species (xenograft-derived bone scaffolds), which show more advantages including low cost and unlimited availability (Amini et al., 2021). In the clinical application, several decellularized bovine bone scaffolds have been used for maxilla-facial or tibial defects. The outcomes are promising, since after six months from implantation, new bone formation was detected, but more trials and studies are required (Karalashvili et al., 2017; Kakabadze et al., 2017).

1.5.1 SmartBone (SBN)

In this study we used a xenograft-derived scaffold called SmartBone® (SBN), manufactured by I.B.I. (SA Mezzovico-Vira, Switzerland) (Pertici et al. 2014; Pertici et al., 2015). The scaffold combines natural and synthetic materials and consists of calcium hydroxyapatite (HA, Ca5(PO4)3(OH)) as the starting material, that is reinforced by poly(l-lactic- co-ε-caprolactone) (PLCL), polysaccharides and RGD (Arg-Gly-Asp), containing collagen fragments extracted from purified bovine gelatin. The implementation of these elements improves the scaffold structure and osteogenic properties that are impaired by decellularization and sterilization processes and it resembles human bone. In particular polymer and polysaccharide coatings reduce the porosity of the scaffold and increase its hydrophilicity, whereas collagen components support cellular attachment and viability. *In vitro* analysis has evaluated and confirmed the capacity of the scaffold to favour viability and proliferation of stromal vascular fraction (SVF), AT-MSC and osteoblasts (Roato et al., 2018; Rahmati et al., 2021).

1.6 Growth factors

The last crucial elements used in BTE are the growth factors (GFs). They are proteins which are involved and strictly orchestrate all phases of the healing process and bone regeneration. To replicate the characteristics of bone microenvironment and achieve tissue repair, providing appropriate molecular signaling, GFs are the last essential element required. The proteins regulate many aspects; they are able to trigger osteoprogenitor proliferation and differentiation, and

stimulate tissue regeneration. For this purpose some GFs such as BMP, VEGF, FGF and PDGF have been employed in numerous preclinical studies as a supplementary therapy. Moreover, the supplementation of exogenous molecules supports scaffold function accelerating MSCs proliferation and differentiation and stimulating new bone formation. Nonetheless these benefits, there are some limitations concerning their clinical application. Indeed, GFs have a short half-life and stability, thus high concentrations and multiple administrations are required to get the needed amount to fulfill their tasks. Moreover, off-target distribution causes some side effects, for example BMPs stimulate bone ectopic formation (James et al., 2016). Different approaches have been developed to stabilize and improve growth factors delivery systems to modulate their release kinetics. The most common strategy includes GFs immobilisation within the scaffold. GFs addition increases osteogenic properties of the scaffold but more studies are needed to define the appropriate GF dose. In this contest, interesting osteoinductive growth factors are Bone Morphogenic Proteins (BMPs), which belong to the transforming growth factor beta (TGFβ) superfamily. BMP signaling pathway includes Smad activation, that occurs through the binding of BMP ligands to their receptors. Phosphorylated Smad migrates into the nucleus and triggers bone-related gene transcription. To date, 12 BMP ligand proteins have been discovered and most play crucial roles in the skeletal system development and tissue homeostasis (Sanchez-Duffhues et al., 2015). Indeed, mutations in the BMP pathway cause a variety of severe skeletal pathologies (Shore et al., 2006). Notably, BMP-2 is reported as a major inducer of cartilage and bone formation, which is also involved in fracture healing (Chen et al. 2019). It regulates MSCs osteogenic differentiation inducing Runx2 and Klf4 transcription (Yu et al., 2021). The application of recombinant human BMP (rhBMP) 2 is approved by Food and Drug Administration (FDA) and it has been used in clinical orthopedic settings including spinal fusions and non-unions treatments (Cecchi et al., 2016). Due to dose-dependent effects and conflicting data, BMPs are currently under investigation for their application in different clinical settings. In particular, BMP-2 osteoconductive properties have been widely studied *in vitro,* indeed the growth factor was loaded on several scaffolds in order to accelerate cellular differentiation. Several strategies have been employed to incorporate BMP-2 into scaffold enhancing GF stability and solubility, including 3D bioprinting which promotes the healing process without ectopic bone formation (Chen X et al., 2021; Lin D et al., 2016; Li Q et al., 2015; Freeman et al., 2020).

2. Research objectives

Bone has an intrinsic self-healing ability that allows it to restore the original anatomic structure of the tissue and recover its mechanical function. Nevertheless, the type of injury and other factors can lead to failure of natural healing. Despite the increasing number of treatments used in the clinic, including improvement in surgical techniques, large segmental bone defects remain still a challenge for orthopedists and tissue engineering represents an attractive field of research to enhance and stimulate bone regeneration. The great potential of this approach depends on the combination of three main components: stem cells that differentiate into desired cells and synthesize new tissue, scaffolds that guide and support the cells and growth factors that provide appropriate inductive signals.

This work attempts to improve the application of bone engineering by focusing on all three elements. We worked on stem cells, scaffolds and growth factors alone or in combination in order to better exploit their intrinsic properties and set up a construct which mimics natural bone tissue.

The specific objectives of this study are as follows:

- To optimize protocols and procedures for the isolation and culture conditions of hBM-MSCs *in vitro*. Specifically, we evaluated PRP as a substitute of FBS to overcome the limitations of traditional MSC expansion media.

- To investigate the properties of a xeno-hybrid bone scaffold, which mimics human bone.

- To improve the regenerative potential of the xeno-hybrid scaffold and enhance its osteogenic properties by application of the recombinant human Bone Morphogenetic Protein 2 (rhBMP-2) to the scaffold. We hypothesized that the combination of rhBMP-2 and xenohybrid bone matrix could provide all cues that the cells require to proliferate and differentiate.

3. Material and methods

3.1 Isolation of hMSC from the bone marrow.

The cells were harvested from bone marrow aspirate, which is obtained by iliac crest needle puncture aspiration from donors during a surgical procedure conducted in the Department of Orthopedic, Azienda Ospedaliero-Universitaria Senese. All the donors gave a written informed consent for the utilization of their samples in research settings.

The samples were collected into tubes containing an anticoagulant solution of sodium citrate and a thixotropic gel. Briefly, after a centrifugation which was performed by RegenCentrigel H-19F (RegenLab), the thixotropic gel acts as mechanical separator, divides blood components and forms a barrier between red blood cells and plasma. The resulting BM-MSC containing plasma was collected and washed twice with 1X PBS to improve cell yield. The cells were plated in culture flasks at three cellular densities: 2×10^6 , 5×10^6 , 1×10^7 cells/cm^2.

The cells were expanded in basic media consisting of Dulbecco's Modified Eagle Medium - Low Glucose (DMEM-LG; Gibco, Life Technologies), 2 mM glutamine (Gibco, Life Technologies), 1% penicillin/streptomycin solution (Gibco, Life Technologies), supplemented with 10% autologous PRP or 10 % Fetal Bovine Serum (FBS; Sigma, Aldrich). When cell cultures reached 90% confluence, the media was discarded and the cell monolayers were washed with 1X PBS, then the cells were detached by Triple Select enzyme (Gibco, Life Technologies) and used for the experiments.

3.1.1 PRP preparation.

PRP was obtained from the venous blood of each patient and collected in a Regen-BCT (Regen Lab) tube containing an anticoagulant and a specific separating gel. By centrifugal force, the separating gel moved between blood sample and PRP containing plasma. The tube was then inverted 20 times in order to homogenize the solution, plasma containing PRP was collected and added to cell culture.

3.1.2 Phenotypic analysis of MSC.

MSCs were analyzed by immunofluorescence for CD105, CD90 and CD73 markers. hBM-MSCs ($6x10^4$) were seeded in 12 well plates. After three 1X PBS washings, the cells were fixed with 4% PFA for 30 min and permeabilized by 0.1% Triton X-100 for 15 min. To reduce nonspecific background staining, the cells were blocked with blocking solution (1% bovine serum albumin (BSA), 10% FBS /PBS solution) for 1h. Primary antibodies were added to the cells and incubated at 4° C overnight. The following primary antibodies were employed: rabbit anti-CD105 (1:50, Thermo-Fisher, MA5-29234), mouse anti-CD73 (1:100, Thermo-Fisher, MA5-15537) and rabbit anti-CD90 (1:100, Bioss, BS-0778R). The following day, the cells were washed three times with 1X PBS incubated for 1h with corresponding secondary antibodies (1:1000, Thermo-Fisher, Anti-Rabbit A11008; 1:1000 Thermo-Fisher, Anti-Mouse A-11004), phalloidin (1:250, Thermo-Fisher, R415) which labels F-actin and 4',6-diamidino- 2-phenylindole (DAPI). The images were taken with a confocal microscope (Leica SP8).

3.1.3 Mesenchymal Stem Cell Lineage Testing.

MSC Adipogenesis and osteogenesis:

Cells treated with different expansion media were plated in 24 well plates at a density of $6x10^4$, and when they reached 80% confluence, the cells were treated with adipogenic or osteogenic induction media.

Adipogenic induction media consists of basic medium (DMEM, Gibco, 61965) supplemented with 100 nM dexamethasone (Sigma, D8893), 50 µM indomethacin (Sigma, I7378) and 0.5 mM 3-isobutyl-1 methyl xanthine (IBMX) (Sigma, I7018). On the other side, the cells were differentiated towards the osteogenic line using a osteogenic induction medium made of basic medium (DMEM, Gibco, 61965) supplemented with 100 nM dexamethasone (Sigma, D8893), 0.05 mM ascorbic acid (Sigma, A8960) and 10 mM β-glycerophosphate (Sigma, G9422). After 14 days of induction the cells were washed twice with 1X PBS and fixed in 4% PFA for 10 minutes and they were ready for the staining process.

The cells were incubated with Oil Red O (ORO) staining, which labels lipid droplet accumulation and assesses adipogenic differentiation. Briefly, fixed cells were washed twice with 1X PBS and stained with ORO solution for 30 minutes, then the stain was discarded and the cells were washed with 1X PBS until background staining of negative control is cleared.

To evaluate osteogenic differentiation, we used Alizarin Red Staining (ARS), which detects calcium deposit; after fixation the cells were washed twice with distilled water and stained with ARS for 10 minutes and finally washed again with distilled water.
In both cases the stainings were assessed using an inverted microscope and negative control groups in which MSCs had been incubated in the expansion medium for the same duration, were used for comparison.

MSC Chondrogenesis

The chondrogenic differentiation was induced using a chemically defined medium (CDM) consisting of basic medium (DMEM, Gibco, 61965), 5% sodium pyruvate (Sigma, S8636), 0,04 mg/ml L-proline (Sigma, P5607), 1,5 mg/ml BSA (Sigma, A7906) and 1% pen/step (Gibco, 15070-063). The cells (2.5 $x10^5$) were placed in a 1.5 ml eppendorf and centrifuged at 2000 rpm for 5 min in order to form a pellet at the bottom of the tube. The pellet was cultured in 500 µl of expansion medium. The following day we changed media and we treated negative control with CDM alone and experimental group with CDM +, that is CDM supplemented with ITS 100X (Gibco, 41400045), 10 ng/ml TGFβ (PeproTech, 100-36E), 20 µg/ml Dexamethasone (Sigma, D8893), 50 mg/ml Ascorbic acid (A8969, Sigma) and 4.7 mg/ml linoleic acid (Sigma, L5900). Cell pellets were fed twice per week. After 21 days the pellet was fixed in 4% PFA for 10 minutes and embedded in 2% agarose. After 24 h the samples were dehydrated in graded series of ethanol solutions and embedded in paraffin-wax using an automatic tissue processor (Leica ASP300). The slices cut with a microtome were stained with Alcian Blue and counterstained with Nuclear Fast Red Solution (Sigma-Aldrich) for the detection of hyaluronic acid and the assessment of chondrogenic differentiation.

3.2 3D cell culture.

For 3D studies and scaffold analysis we used BM-MSCs which were isolated from healthy donors and stored in liquid nitrogen. Frozen BM-MSCs were thawed in a 37° water bath, then expansion medium was added and the cell suspension was centrifuged in order to discard the freezing medium. After the centrifugation the pellet was resuspended and cultured in T-175 flasks in BM-MSC

expansion medium containing Dulbecco's Modified Eagle's Medium (DMEM, Gibco, 61965) supplemented with 10% FBS (Gibco, 10270-106), 1% penicillin/streptomycin (Gibco, 15070-063) and bFGF2 (5ng/mL, Prospect Biosciences, CYT0218) added to promote cellular expansion. The cells were maintained at 37° C in normoxic conditions. Cells were expanded until passage 3 and then used for the experiments.

To detach BM-MSCs from the culture plastic, they were washed with 1X PBS and incubated with 5 ml trypsin solution for 5 minutes at 37° C. Afterwards, expansion media was added to inhibit the trypsin and cell suspension was centrifuged. The pellet was resuspended in 5 ml of expansion medium (without bFGF) and the cells were counted using a haemocytometer. A total of 370000 cells were seeded on the top of each scaffold. To promote cell adhesion, after 3h from the seeding, 1ml medium was added to each well (24-well plates, Thermo-Fisher). Finally, the following day one group was maintained in expansion medium, whereas in the other study group the expansion medium was replaced with osteogenic one consisting of basic medium (DMEM, Gibco, 61965) supplemented with 100 nM dexamethasone (Sigma, D8893), 50 μM indomethacin (Sigma, I7378) and 0.5 mM 3-isobutyl-1 methyl xanthine (IBMX) (Sigma, I7018).

BM-MSCs seeded scaffolds were cultured for 24h, 21, 28 and 35 days from the plating. For each time point biochemical, histologic and immunofluorescence analyses were performed in order to study cellular viability, proliferation and differentiation on scaffold.

For the experiment, SBN plates of 10x10x4 mm were employed; to improve cell adhesion the scaffolds were washed and incubated in expansion media O.N.

3.2.1 Cell viability assay.

After 24 h and 35 days from cell seeding on the scaffold, live/dead assay was performed to assess the viability of cells. The method consists of calcein-AM and ethidium homodimer-1 stainings (EthD) which label living and dead cells respectively. The former indicates intracellular esterase activity of live cells; the enzyme cleaves AM from calcein which turns nonfluorescent into an intensely green fluorescent probe. On the other side EthD penetrates cells with a compromised membrane, labels exposed DNA, producing red fluorescence.

Briefly, the scaffolds were washed with 1X PBS and incubated in 1X PBS containing 2 μM calcein AM and 4 μM EthD for 30 minutes at 37 °C. Then the samples were washed again in 1X PBS and

analysed using a scanning confocal microscope (Leica SP8) at excitation/emission wavelengths of 495/515 nm for calcein and 495/635 nm for EthD.

3.2.2 Histological analysis.

After each time point (24h, D21, D28 and D35) the media was removed and the scaffolds were rinsed twice with 1X PBS and prepared for the histology. All samples were fixed with 4% PFA O.N.. They were then incubated in Decalcifying Solution-Lite (Sigma-Aldrich) for 1 week in order to decalcify the sample before tissue processing. The scaffolds were dehydrated in graded series of ethanol solutions, cleared in xylene and embedded in paraffin wax. Finally the samples were sectioned at a thickness of 8 µm by a rotary microtome (Leica, Microtome RM2235). Sections were stained with hematoxylin and eosin (H&E), picrosirius red and goldner's trichrome stainings or they were used for immunofluorescence (IF). All histological stainings were carried out using an automatic staining machine (Leica 5010 autostainer). Finally the sections were imaged using an Aperio ScanScope slide scanner.

3.2.3 Immunofluorescence analysis.

Sections were deparaffinized and rehydrated through varying ethanol grades using an automatic staining machine (Leica 5010 autostainer). Then, sections were treated with 35 U/ml pronase for 25 minutes at 37° C and with 4000 U/ml hyaluronidase for 45 minutes at 37° C. Then, samples were blocked for 60 minutes at room temperature with a blocking buffer consisting of 10% of donkey serum, 1% w/v BSA (w/v) and 1% Triton 100X. Finally, sections were incubated ON at 4° with rabbit monoclonal anti-Collagen I antibody (ab138492, 1:100) in the blocking buffer. The samples were rinsed with 1X PBS and incubated with Alexa Fluor® 647 donkey anti-rabbit secondary antibody (ab150075; Abcam, 1 : 500) and 4’,6-diamidino- 2-phenylindole (DAPI) for 1 h at room temperature in the dark. After three washing steps with 1X PBS, slides were mounted with ProLongTM Gold Antifade (Invitrogen) and fluorescence was detected using a confocal laser scanning microscope (Olympus Fluoview FV1000) at excitation 652 nm and emission 668 nm.

3.2.4 Biochemical analysis.

Cell proliferation on the scaffold was analysed by DNA quantification using picogreen assay at 24 h, D21, D28 and D35. At each time point the scaffolds were washed with 1X PBS and treated with 1 ml of lysis buffer composed of 10mM Tris, 1 mM EDTA (EDS, Sigma Aldrich) and 0.1% Triton X-100 (T8787, Sigma Aldrich). The scaffolds were immersed in the solution and frozen (-70°C). To lyse cell and release DNA, the samples were thawed and frozen three times. The assay was performed according to the manufacturer‘s instructions. Briefly, a standard curve was created using a serial dilution of a known concentration of a lambda DNA solution, which ranged from 2 µg/ml to 40 µg/ml. To prepare the working solution of the reagent, picogreen solution was diluted in 1X TE buffer. 100 µl/well of standard and samples were pipetted in triplicate on black flat bottomed 96 well plate. Lastly, 100 µl/well of PicoGreen working dye solution was added. After 5 min of incubation in darkness, the samples were spectroscopically measured (excitation=480 nm, emission=520nm), and the fluorescence values were converted into the DNA concentration using the standard curve. Statistical analysis between the two groups (XPAN and OSM) were performed using GraphPad v6.01 (GraphPad Software, Inc.) and one-way ANOVA.

3.2.5 Calcium assay.

To analyse calcium content in the samples treated with MSC expansion or osteogenic media, calcium assay was performed, using a reagent (17667H, Sentinel Diagnostics) which forms a red colored complex when it reacts with calcium ions, producing a colour intensity that is absorbed at 570 nm and is proportional to the concentration of calcium contained in the samples.
After 1X PBS washings, the samples were treated with 1M Hydrochloric acid (HCl) and placed on a rotator at 60 °C overnight. Following sample preparation, the scaffolds were removed and the supernatant was collected for the analysis. We prepared the standards and 10 µL of samples and standards were added to 140 µL of working solution made of 2-aminoethanol buffer (MEA) 1.0 mol/L and HCl 0.12 mol/L. After 10 minutes of incubation, the plate was read using a microplate reader at 570 nm absorbance. Calcium was quantified and calculated via a standard curve. Statistical analysis were performed using GraphPad v6.01 (GraphPad Software, Inc.) and one-way ANOVA.

3. 3 BMP-2 adsorption onto scaffold.

The incorporation of BMP-2 onto the scaffold was performed by embedding technique. According to previous studies (Eichholz at al., 2020), BMP-2 used amount was determined, the growth factor

was dissolved in 1X PBS at concentration of 50 µg/ml and 20 µl of solution containing 1µg BMP-2 was pipetted on each scaffold and incubated for 4 h at 37°C, then 200 µl of expansion medium was added to each support. At different time points (24 h, 3, 7, 10 days) the media was collected and stored at -80 until *in vitro* BMP-2 release experiments, and fresh media was added.

3.3.1 Enzyme linked immunosorbent assay (ELISA).

To detect BMP-2 levels in cell culture supernatant and growth factor release from scaffold, ELISA was performed using Human BMP-2 DuoSet ELISA (DY355, R&D System). According to the manufacturer's protocol, a 96 well microplate was coated with human BMP-2 Capture Antibody diluted in 1X PBS overnight at room temperature. 100 µL of each sample and standards in reagent diluent were added in the 96 well microplate and incubated for 2 hours. After 3 washings, human BMP-2 detection antibody which is conjugated with horseradish peroxidase (HRP) was added to the plate. In order to remove unbound components other 3 washing steps were performed and streptavidin-HRP was added to each well, then the samples were incubated with substrate solution and finally, stop solution was added to inactivate enzyme activity. Using a microplate analyzer the absorbance was measured at 450nm.

4. Results

4.1 MSC culture and characterization

hMSCs were harvested from bone marrow of patients using commercial tubes and plated at three cellular densities (2 x 10^6 , 5 x 10^6 , 1 x 10^7 cells/cm^2). Moreover, culture medium supplemented with 10% of either PRP or FBS was employed. After 24h of culturing, most cells showed a round shape and floated in culture. They began to adhere to culture flasks in the subsequent days. In all conditions, the initial cultures consisted of a heterogeneous population with variable shape, including rounded cells and spindle-shaped MSCs. Over time, we observed a gradual reduction in the heterogeneity of the population and an increase in the number of fibroblast-like cells that formed colonies and proliferated **(figure 3)**.

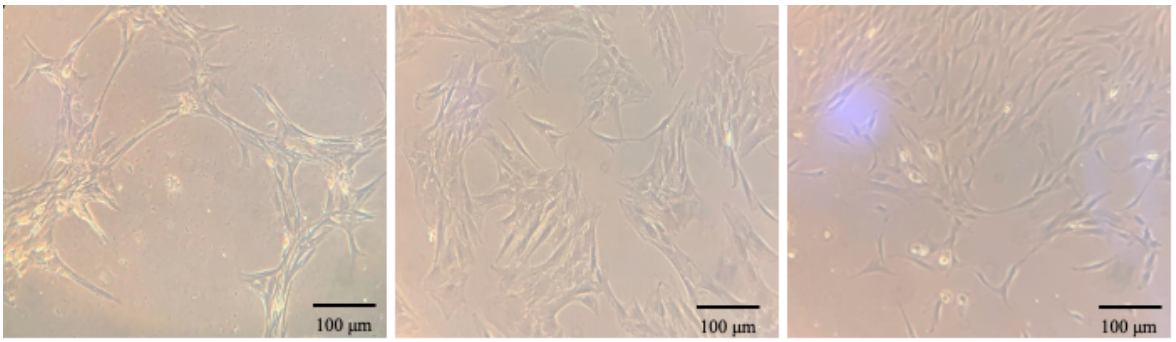

Figure 3*: Morphological appearance of hMSCs at passage 1. The cells treated with DMEM (Dulbecco's modified Eagle's medium) supplemented with PRP, presented here, showed a fibroblast-like morphology typical of MSCs expanded in FBS. Scale bar of ~100 µm.*

According to the guidelines of the International Society of Cell Therapy, we characterized isolated cells by analyzing the expression of MSC surface markers (CD105, CD90 and CD73) and assessing their tripotentiality. The labeling pattern of CD105, CD90 and CD73 was identical in BM-MSCs expanded in DMEM supplemented with FBS or PRP, and the expression of all three markers was observed in all analysed cells **(figure 4)**.

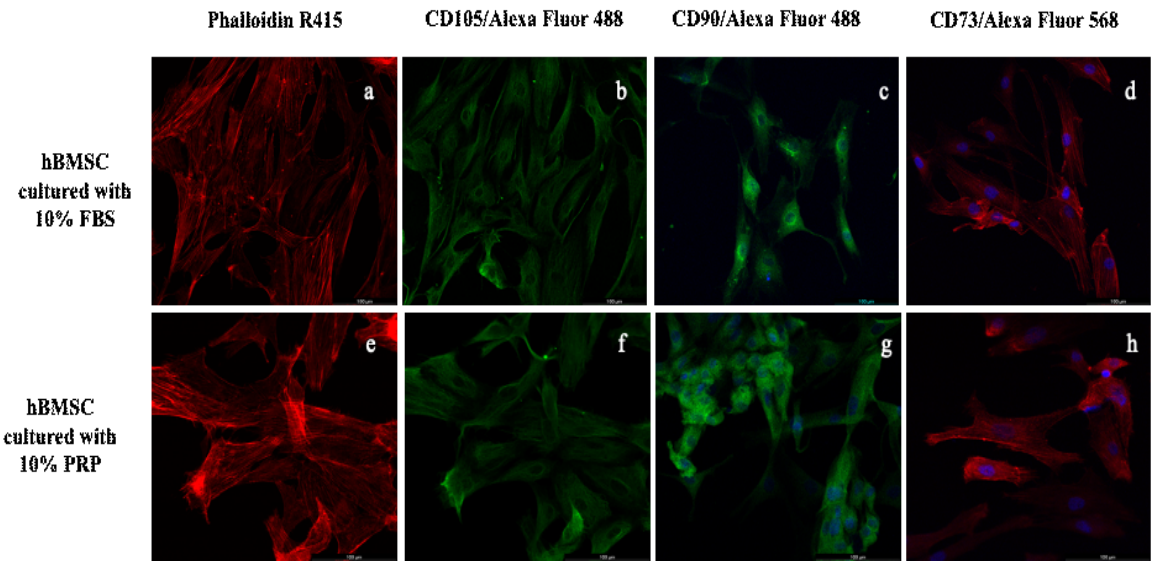

Figure 4: *Immunofluorescence stainings of hMSCs. (a-d) Cells were expanded in DMEM supplemented with 10% FBS. (e-h) Cells were treated with DMEM and 10% PRP. (a and e) Phalloidin R415 staining to outline cell morphology; (b and f) CD105/Alexa Fluor488, (c and g) CD90/Alexa Fluor 488; (d and h) CD73/Alexa Fluor568. Scale bar 100 µm.*

The cells were then differentiated into three distinct cell lineages: osteogenic, adipogenic and chondrogenic. We used $6x10^4$ cells for osteogenic and adipogenic differentiation and $2x10^6$ cells for chondrogenic induction to assess MSCs tripotentiality. During the process, the cell morphology changed and after 14 days of osteogenic and adipogenic differentiation, alizarin red and oil red o stainings were performed to evaluate matrix deposition. Light microscopy images confirmed the osteogenic differentiation, since MSCs produced calcium deposits which were detected by alizarin red **(figure 5)**. Similarly, cells in adipogenic media produced abundant lipids that were stained with oil red o, confirming also the adipogenic potential **(figure 6)**.

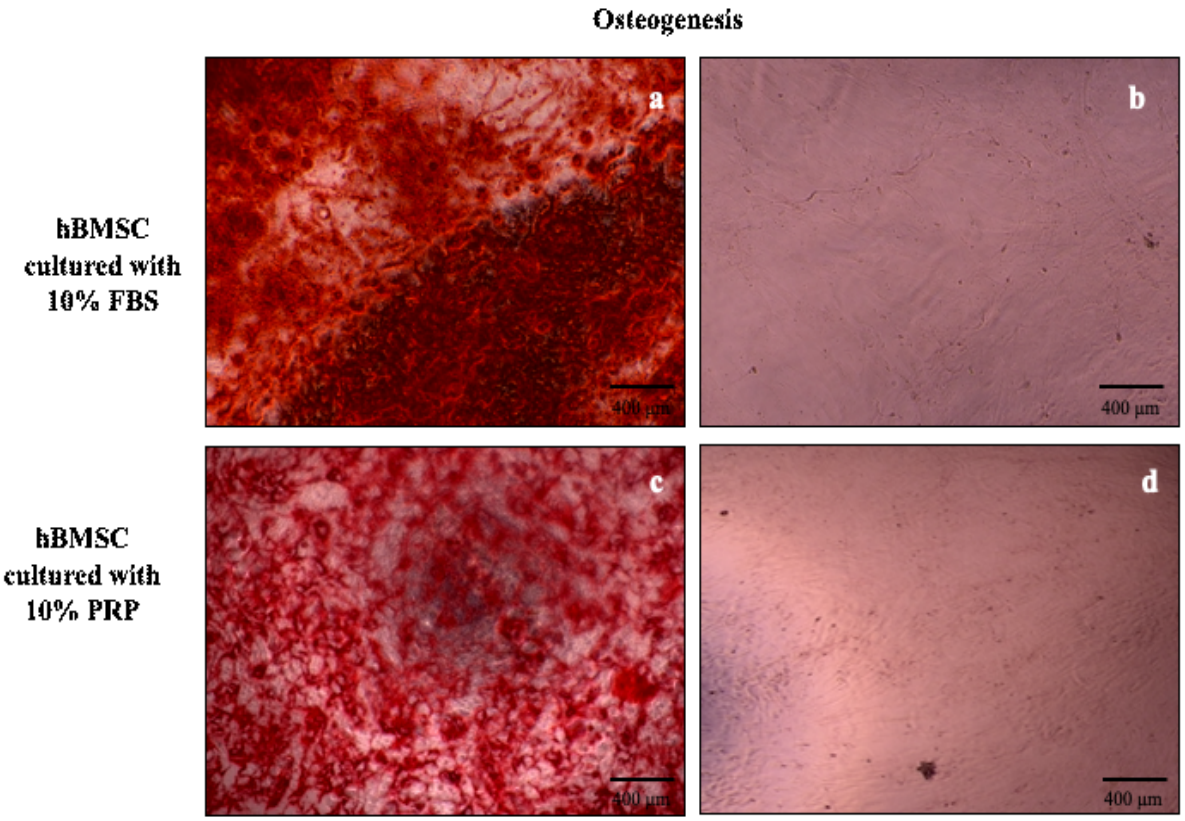

Figure 5: *Osteogenic differentiation analysis of hBMSCs cultured in two different media, performed by alizarin red staining. After 14 days of differentiation induction, the cells show a consistent osteogenic capacity (a and c) in comparison with negative control (b and d). Scale bars 400 µm.*

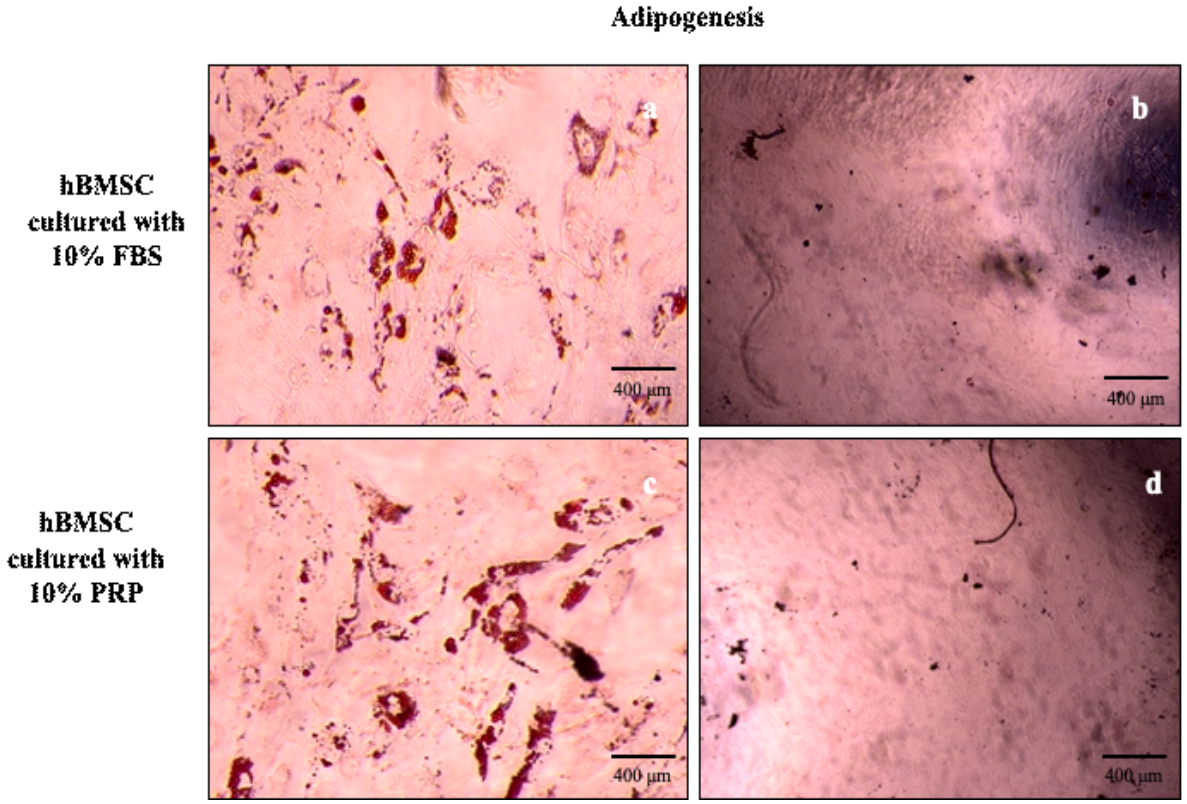

Figure 6: *Adipogenic differentiation potential of hBMSCs cultured in FBS (a,b) and PRP (c, d) supplemented media detected by Oil Red O staining. hBMSCs showed adipogenic differentiation ability (a,c), while no signal was detected in negative controls (b,d). Scale bar of 400 µm.*

Finally, the cells treated with chondrogenic differentiation media for 21 days showed stronger expression of glycosaminoglycan, demonstrated by positive Alcian Blue staining, that is indicative of chondrogenic differentiation **(figure 7)**.

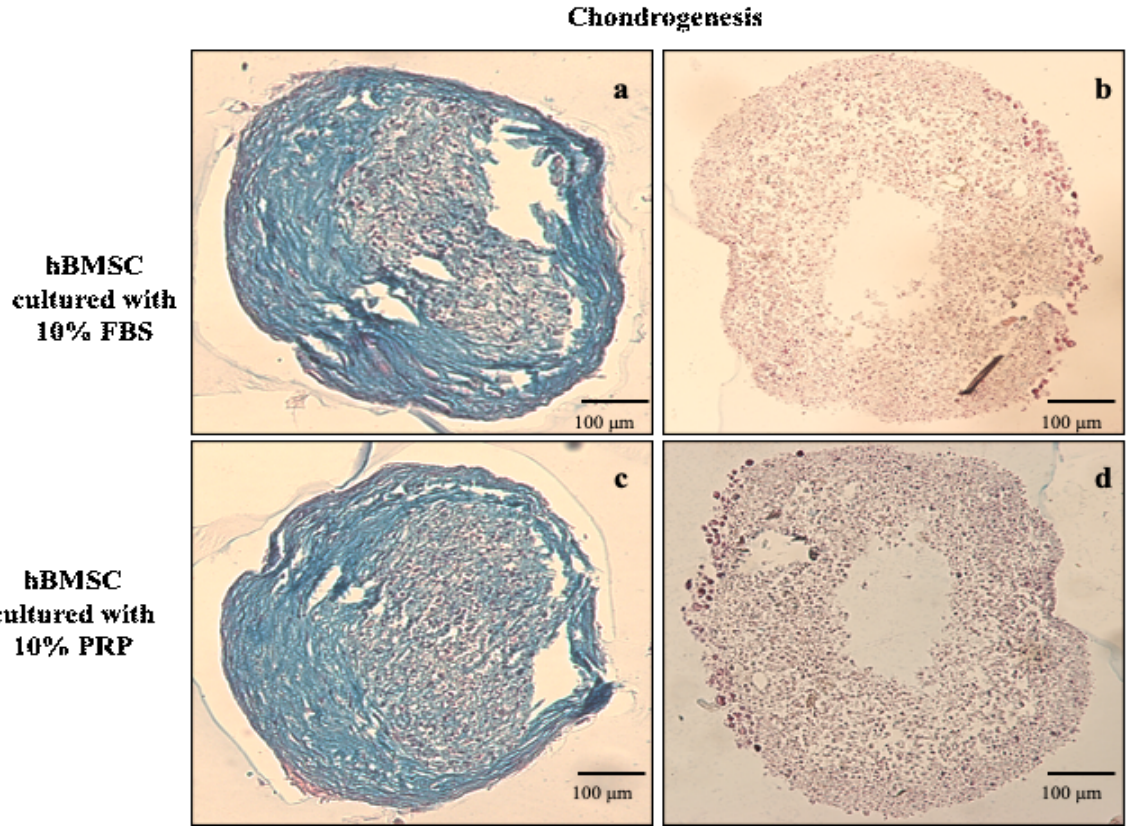

Figure 7: *Chondrogenic differentiation of hBM-MSC detected by Alcian blue staining. hBMSCs cultured in FBS (a,b) and PRP (c, d) supplemented media, showed a positive signal after chondrogenic induction (a,c), while no signal was detected in negative controls (b,d). scale bar of ~100 µm.*

These results confirmed successful cell isolation and demonstrated that both culture conditions did not affect MSCs properties and preserved MSC markers expression and their capacity for multilineage differentiation. Indeed, when cellular populations were expanded under DMEM conditions supplemented with FBS or PRP, they differentiated in all three cell lineages.

4.2 Analysis of hBM-MSCs seeded on scaffold

The project aims to investigate the ability of a xeno-hybrid graft called SmartBone® (SBN), to support hBM-MSCs attachment, proliferation and differentiation. For this purpose, the cells seeded on the scaffold were cultured in both MSC expansion and osteogenic media. Live/Dead assay, performed after 24 h from cell seeding, assessed the efficacy of the process; many living cells in green color and few dead cells in red were detected. Confocal images confirmed cell attachment and showed their distribution on the scaffold; the cells adhered on the scaffold but they did not cover its

entire surface. The cellular aggregation in specific regions could be attributed to the seeding methods, because the cell suspension was pipetted onto different localized areas of the scaffold, and after 24 h from seeding the cells did not start the proliferation and elongation processes **(figure 8)**.

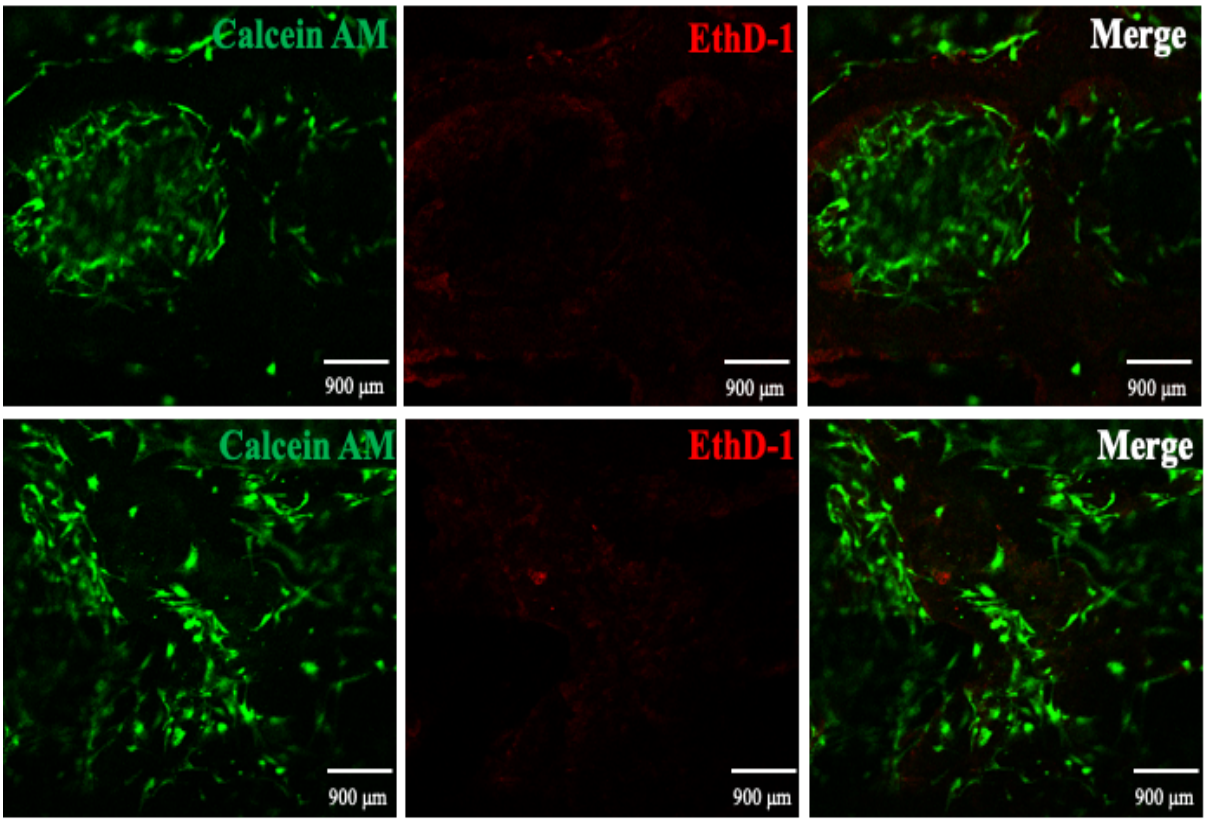

Figure 8: *Live/dead staining of hBM-MSCs 24h post seeding on the bovine-derived scaffold. The cells were attached and localized in several areas including scaffold pores. Scale bar 900 µm.*

The same assay was performed at the end of the experiments (D35). The results did not differ from the analysis at 24 h regarding cellular viability: most cells were still viable, as visualized by green fluorescence, while most red signal was aspecific from scaffold and it just stained a few dead cells. Cells appeared more abundant compared to 24 h analysis and they homogeneously covered all scaffold surface (**figure 9)**.

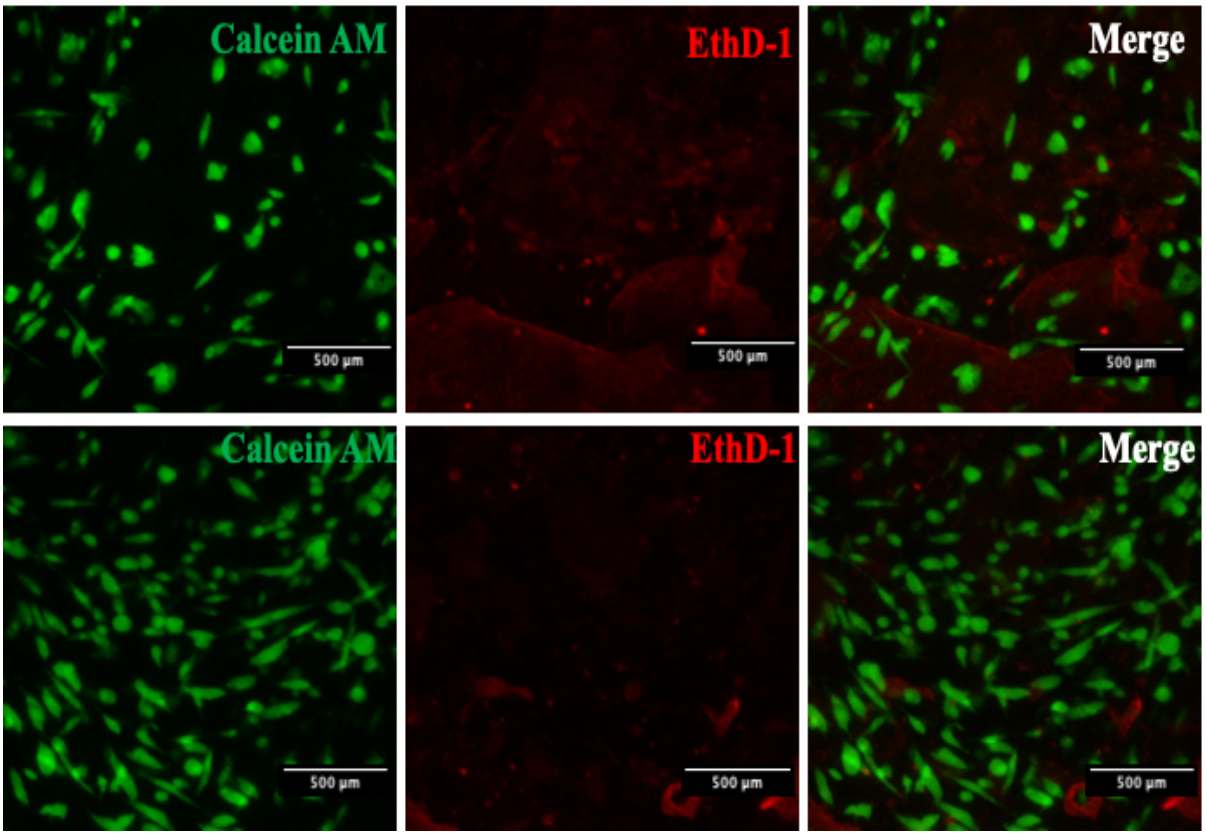

Figure 9: *Live (green)/dead (red) staining of hBM-MSCs at 35D after seeding. The analysis of scaffold surface showed mostly living cells. Scale bar 500 µm.*

After 24 h from the seeding, the expansion medium was replaced with the osteogenic one to induce cellular differentiation in one group, while the other experimental group was maintained in the expansion medium. In both cases the cells were treated for up to 35 days. Cellular proliferation on the scaffold was measured by the analysis of DNA content using PicoGreen assay at different time points. At D21, cellular proliferation was significantly higher in the expansion medium than the osteogenic one, suggesting the differentiation process was ongoing in the latter group. Later, DNA content increased in both groups, cell proliferation appeared to be stable and reached a plateau phase at D28. From D28 to D35 no significant difference was observed between the two groups **(figure 10)**.

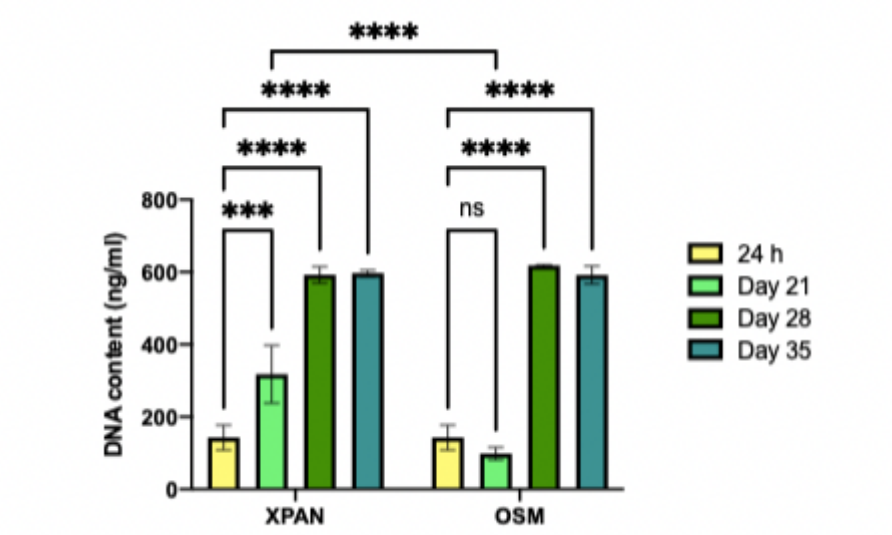

Figure 10: *Graphic of DNA content (ng/ml) of hBM-MSCs in expansion (XPAN) and osteogenic (OSM) media. Cell proliferation increases in both groups at D28 and remains stable up to D35. *** p<.0,0005; **** p< 0,0001*

Data thus confirmed cell adhesion and demonstrated the excellent biocompatibility of SBN scaffold, which supported cell viability for all the duration of the study (D35).

To assess MSCs osteogenic differentiation, calcium assay was performed in order to quantify calcium levels produced by osteoblast derived by BM-MSCs on the scaffold. In addition to the samples, calcium content of the scaffold alone (without cells) was analyzed. No significant differences were detected among samples, suggesting that calcium levels detected in the scaffold were not associated with differentiation or mineralization processes, but they were attributed to scaffold composition which exhibited high calcium content. Thus, in this contest, the assay was not useful to assess cellular osteogenic differentiation. Consequently, H&E, picrosirius red and Masson's trichrome stainings were performed to examine tissue formation. For this purpose, the samples were decalcified for histological analysis and we could not investigate the mineralization process by alizarin red staining which detects mineral calcium. H&E stained sections revealed the basic structure of the scaffold, characterized by trabeculae and empty lacunae. They pointed out cellular colonization and distribution on the scaffold; most cells were localized on the border of the support where they produced bone matrix, since a large amount of matrix was evident in the pores of the scaffold in all analyzed samples **(figure 11).**

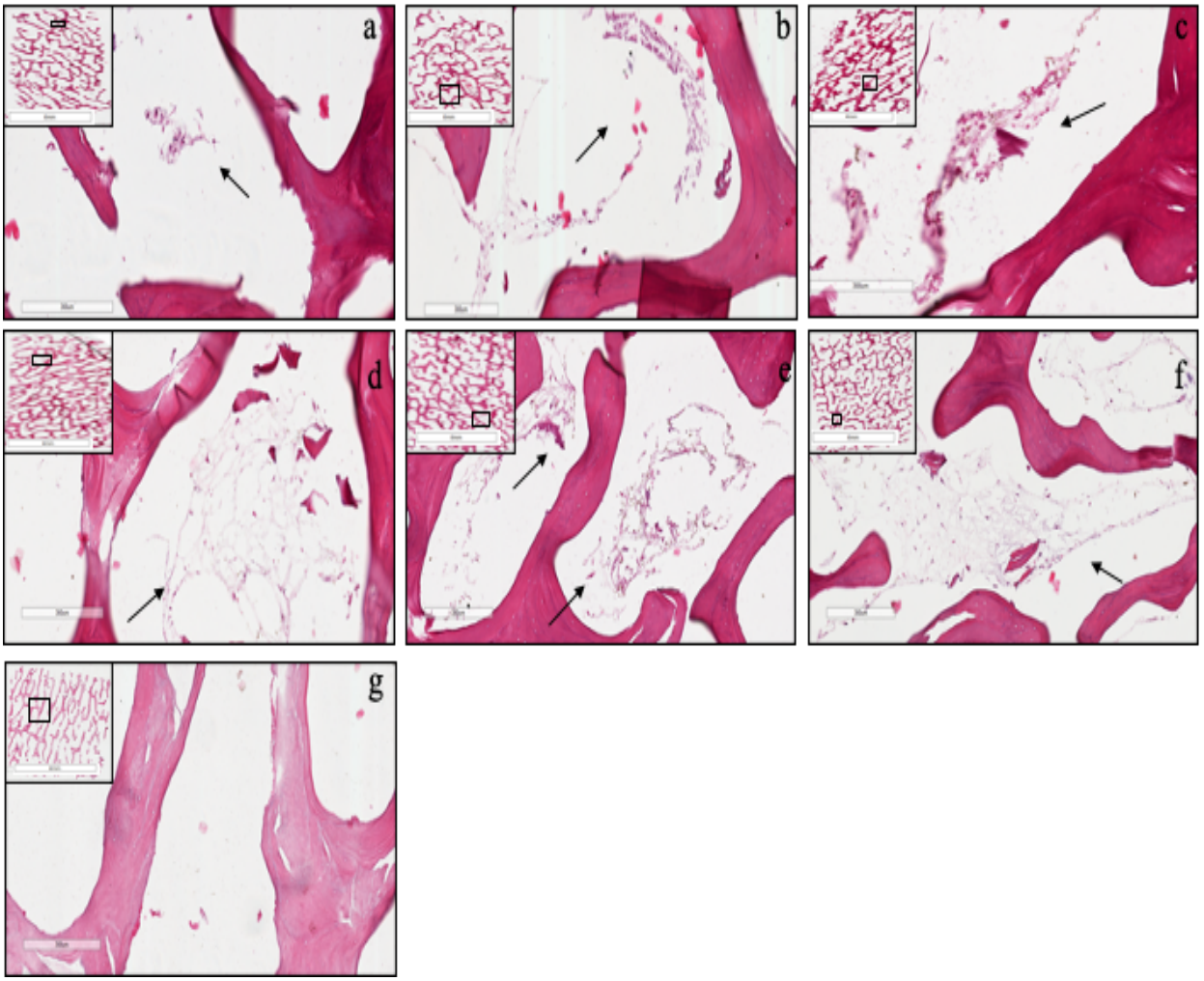

Figure 11: *Hematoxylin and eosin staining indicating the histology of hBM-MSCs seeded on the scaffold in expansion (a-c) or osteogenic media (d-f) and of the scaffolds without cells (g), at D21 (a,d), D28 (b,e) and D35 (c,f). The cells were distributed in the scaffold and new matrix bone was deposited in the pores (arrows). Images at low magnification (10X) are shown in the inlet. High magnification (20X) of the area delimited by the rectangle is presented. Scale bar 300 µm*

Picrosirius red binds collagen fibers and, unfortunately, it stained the scaffold itself, so it did not allow detecting any native bone matrix and no difference among the samples was found. Finally, the collagen component of the scaffold was stained in green by Masson's trichrome which combines three stainings, namely haematoxylin for cell nuclei (black), fuchsine for cytoplasm and erythrocytes (red) and light green for collagen (green). As illustrated in **figure 12** and pointed out by arrows, black cell nuclei were detected among the pores, which were surrounded by green matrix

deposition; moreover, starting from D21, a colour change in the stainings was evident, and scaffold seeded with cells appeared red, suggesting woven new bone.

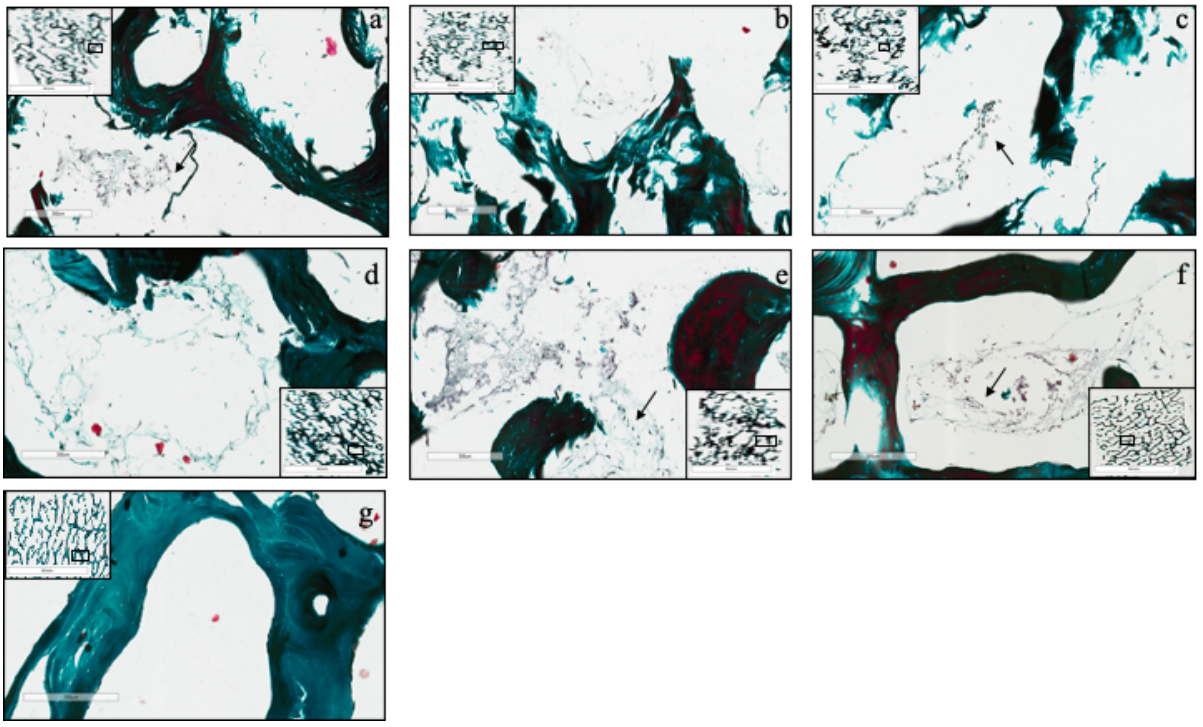

***Figure 12**: Masson's trichrome stainings of hBM-MSC seeded on the scaffold, treated with MSC expansion media (a-c) and osteogenic induction media (d-f). The stainings (scale bar 300 µm) indicate new matrix deposition among scaffold pores at D21 (a,d), D28 (b,e) and D35 (c,f). Arrows point to cell nuclei stained by haematoxylin. Each image includes a scaffold staining of 4 mm scale bar. The stainings were compared with a control group consisting of scaffolds without cells (g).*

To support histological findings we characterized new matrix deposition by Col1A1 immunofluorescence analysis. Collagen is a main component of extracellular matrix, which is synthesized by osteoblasts and is considered an osteogenic marker. Confocal images exhibited strong staining for Col1A1 from both the scaffold itself and the matrix formed in the pores **(figure 13)**. Interestingly, collagen expression was detected in both expansion and osteogenic media.

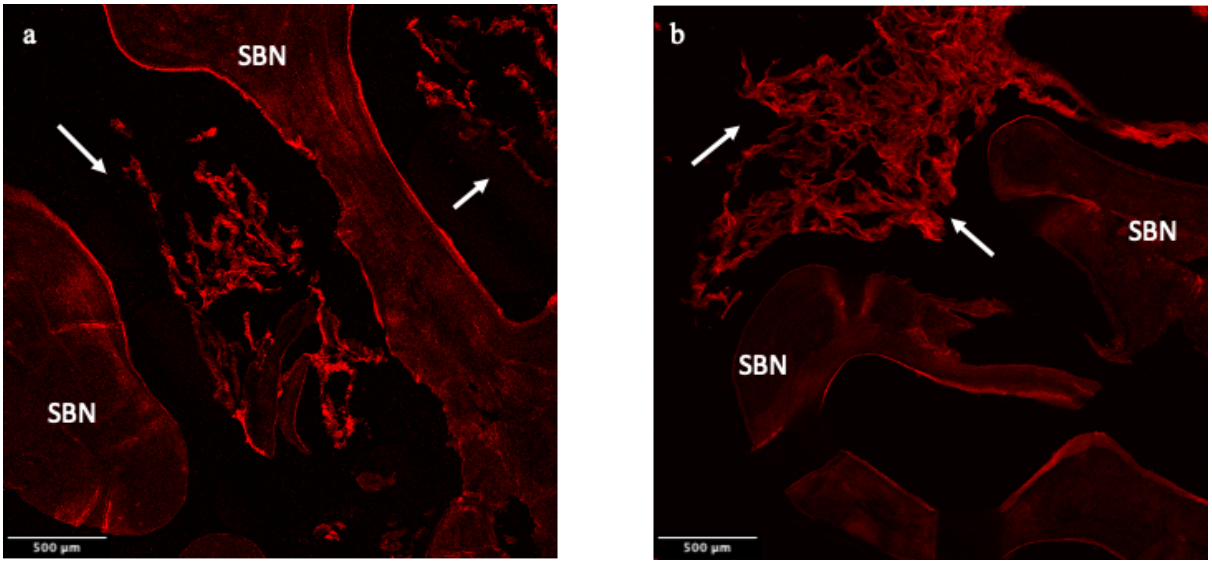

***Figure 13**: Detection of collagen (Col1A1) by immunofluorescence staining in hBMSCs cultured in expansion media (a) and osteogenic one (b) at day 28. Arrows indicate new collagen deposition Scale bar 500 µm.*

4.3 BMP-2 implementation on the scaffold.

In order to improve osteogenic properties, BMP-2 was loaded on the support and preliminary experiments were performed to investigate its release kinetics from the scaffold by ELISA. We defined BMP-2 loaded concentration and seeding process according to a publication previously reported in the literature (Eichholz at al., 2020). *In vitro* release analysis was performed at different time points. No significant differences were detected in BMP-2 release over time and the results showed low and negligible amount of BMP-2 (< 100 pg/mL) in the supernatant collected from each scaffold loaded with BMP-2 **(figure 14)**. Data indicated a high level of adsorption, suggesting that the scaffold retained the majority of growth factors. However, additional experiments are required to assess BMP-2 release from the scaffold and the effect of growth factor implementation on cellular proliferation and differentiation.

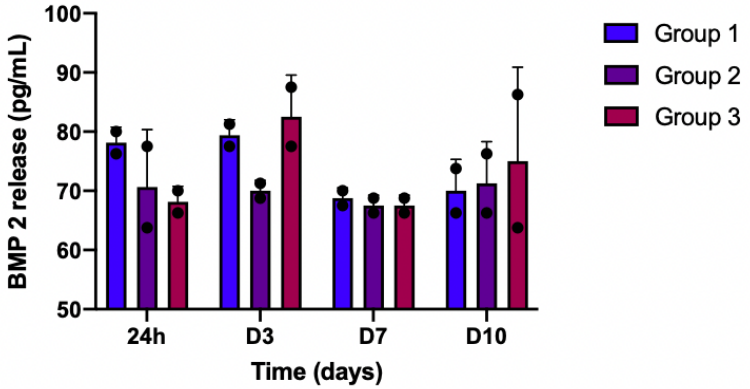

Figure 14: *Analysis of BMP-2 release up to D10 from scaffold (n=3). Low BMP-2 concentrations in the media were detected. Two-way ANOVA test was performed but no statistical significance was found among the samples.*

5. Discussion and conclusions

Many research efforts are ongoing to develop new therapeutic approaches in order to overcome the limitations of current therapies and stimulate bone healing process in critically sized defects. At present, autografts characterized by cells, growth factors and proteins that stimulate bone regeneration remain the gold standard. Autografts show excellent osteogenic and osteoinductive abilities but, due to the lack of supply and patient morbidity, alternative strategies are required. In this context, the majority of the studies have focused on the combination of stem/progenitor cell populations with biomaterials. Cell behaviour and activity depend on biophysical and biochemical cues from the microenvironment and extracellular matrix. Tissue bone engineering aims to recapitulate natural bone architecture and create a tissue-engineered bone graft which accelerates cell differentiation and new bone formation. The aim of my thesis was to improve bone healing process by optimizing the mesenchymal stem cells isolation protocol. Then, in order to provide mechanical and biological support and enhance osteogenic differentiation, we combined mesenchymal stem cells (MSCs) with an appropriate scaffold.

MSCs therapeutic applications have raised a lot of attention in the regenerative medicine field. The cells exert a critical role in bone healing, they migrate to the injured site and differentiate into several cell types. Moreover, the most interesting aspect is their trophic activity, as they secrete

various bioactive molecules that modulate the immune response and promote the recovery of injured sites. In the field of bone regeneration, bone marrow-derived MSCs (BM-MSCs) have been widely used, because of their great differentiation ability toward osteoblast lineage cells, but they represent ~ 0.001–0.01% of bone marrow mononuclear cells (Chu et al., 2020; Hernigou et al., 2005). The low abundance of BM-MSCs is a significant limitation for the clinical application, because expansion processes are required to obtain an adequate number of cells. Culture methods are not precisely defined (Drela et al., 2020) and they provoke cellular genetic instability, senescence and alteration of differentiation potential and proliferation rate, therefore an appropriate method for BM-MSCs expansion needs to be established.

In terms of culture conditions, traditional media are supplemented with fetal bovine serum (FBS), which application is limited for clinical use, since it represents a potential source of xenogenic antigens and zoonotic infections. Therefore, xeno-free culture protocols are required for efficient cellular production. The first part of the project aimed to assess the use of platelet-rich plasma (PRP) as an alternative to FBS. PRP is a plasma fraction containing a high platelet population. It is harvested directly from a patient blood sample, and it is a storage of multiple growth factors, such as Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Transforming Growth Factor beta (TGF-β), Platelet-Derived Growth Factor (PDGF), that support cellular proliferation. In clinical practice PRP application has greatly increased over the past decade (Nazaroff et al., 2021) for the treatment of orthopaedic injuries, wound healing and nerve regeneration (Wang et al., 2017; Albanese et al., 2013; Anjayani et al., 2014). Many studies have confirmed positive outcomes (Wang et al., 2015): PRP injection results in the release of multiple growth factors which are involved in the healing process and activate cellular signaling cascades that provoke tissue repair and regeneration (Everts et al., 2006; Everts et al., 2012). For example, PDGF plays a chemotactic role and induces the migration of neutrophils, monocytes and stem cells to the injured site, whereas VEGF promotes new blood vessel formation. However, some critical aspects are associated with PRP application including non-standardized PRP processing protocols, component characterization, platelets concentration and the number of doses required for clinical improvement. All of these factors contribute to the uncertainty and skepticism on PRP efficacy (Nazaroff et al., 2021). Moreover, other authors have proposed PRP as a safer alternative to FBS. In particular, its application was studied for adipose-derived mesenchymal stem cells (AT-MSC) expansion, demonstrating that PRP promotes AT-MSC proliferation without modifying cellular phenotype and multipotency (Atashi et al., 2015). For our experiments, hMSCs were isolated from the bone marrow of patients, after receiving their written informed consent, and expanded in

parallel in two different media, one supplemented with FBS and the other with PRP. Based on literature data (Anitua et al., 2022), we decided to analyse the effect of 10% PRP, which is considered to be the optimal PRP dose. We obtained PRP from a venous blood sample from the same patient from whom we got MSCs, using a commercial device which allows the isolation of plasma containing PRP after one centrifugation. In both culture conditions, the cells showed a fibroblast-like morphology. After expansion we characterised the cells by analysing the expression of MSC surface markers and assessing *in vitro* tri-differentiation ability. According to the criteria approved by the International Society for Cellular Therapy (ISCT) we analysed the expression of CD105, CD90 and CD73. IF stainings demonstrated the cells preserve the co-expression of MSC markers in both PRP and FBS supplemented media. In addition to cell surface markers analysis, classical differentiation assays were performed. The cells were differentiated into three distinct cell lineages: osteogenic, adipogenic and chondrogenic. During the differentiation process the cells change morphology and produce a matrix which was analysed to assess MSC differentiation ability. In particular, following osteogenic or adipogenic inductions we detected calcium deposition by Alizarin Red staining and lipid droplets accumulation confirmed by Oil Red O staining, confirming osteogenic and adipogenic differentiation, respectively, finally, we examined GAG accumulation for chondrogenic potential assessment. These experiments confirmed the tri-potentiality of cells isolated from human bone marrow and expanded in a basal medium supplemented with FBS or PRP. Indeed, all stainings revealed a positive signal in the cells differentiated toward the specific cellular lineage and no signal in the negative controls consisting of cells cultured in MSC expansion medium. Several studies have investigated the possibility to replace xenogeneic culture with PRP for BM-MSC expansion *in vitro*. As previously reported in the literature, our results revealed no significant difference between cells cultured in FBS or PRP supplemented media (Amable et al., 2014) in terms of cellular morphology and surface marker expression. After expansion, the cells maintained a spindle-shaped fibroblast-like shape and no difference in the expression of CD105, CD90 and CD73 was found between the cells treated with two media. As regards differentiation potential, data reported in literature showed that PRP promotes the chondrogenic differentiation capacity of MSCs (Mishra et al., 2009; Gottipamula et al., 2012; Prins et al., 2009). In contrast, our results did not confirm this trend, and MSCs expanded in PRP or FBS had a similar differentiation ability toward the three cellular lineages. Taken together, our results confirmed that PRP-supplemented medium could replace FBS, because it preserves BM-MSC characteristics and does not interfere with any lineage differentiation. However, we did not directly study the

expression of chondrogenic, osteogenic or adipogenic markers but we analysed the matrix produced by differentiated cells. Future studies are thus needed to further address this issue.

Cellular fate is influenced by the microenvironment. For this reason, in the second part of the project, we investigated the role of the scaffold to induce cell osteogenic differentiation. We performed an *in vitro* analysis and we seeded hMSCs on a bovine-derived scaffold. For this study a large number of cells was required so we used commercial hMSCs isolated from the bone marrow of healthy donors. In recent years, different tissue-engineered bone grafts have been introduced, but each graft presents its own advantages and limitations. Our study aimed to characterize the properties of a xenograft-derived scaffold which originates from a bovine bone matrix. The biohybrid scaffold used for the experiments is made of calcium hydroxyapatite (HA, $Ca_5(PO_4)_3(OH)$) and reinforced by a mixture of polysaccharides and polymers plus RGD-containing collagen fragments (Pertici et al., 2015; Pertici et al., 2014). To analyze the effect of the bovine-bone scaffold on cells, we tested hBM-MSCs seeded on the scaffold and cultured under two conditions: expansion and osteogenic media. The *in vitro* studies included the assessment of proliferation and differentiation to evaluate the possibility that xenograft-derived scaffold supports long-term BM-MSC adhesion and viability. To assess the efficacy of the seeding process and monitor cellular health, a live/dead assay was performed at two timepoints (24h and D35 after seeding). Cells had an excellent adhesion and they spread over the scaffold confirming the efficacy of the seeding process. Indeed, by looking at the confocal images of the analyzed samples at D35, most attached cells were alive and only a few dead cells were detected, indicating that the scaffold provided an appropriate microenvironment and supported cellular adhesion and viability. To confirm these results we quantified the DNA content and monitored the proliferation of cells seeded on the scaffold at different timepoints (24h, D21, D28 and D35). An evident increase in DNA content was detected from day 21 to day 28 when the cells reached a plateau phase. The results indicated that the cells were viable and proliferated for the entire duration of the experiments, showing that the scaffold possessed a good cellular compatibility.

The assessment of BM-MSC osteogenic differentiation was more difficult and challenging, and we explore several solutions. We attempted to quantify calcium concentration using a calcium assay (Calcium Liquid). Calcium content was analysed at different timepoints after cell seeding on the scaffold (24h, D21, D28 and D35) and compared with scaffold alone as a negative control. The study revealed the same calcium levels in all samples, suggesting that the amount of calcium detected was not attributable to MSC-derived osteoblast activity and production but rather to the

scaffold itself. These results indicated that calcium measurement is not the right method to assess osteogenic differentiation. Thus, we shifted to histological analyses and we could demonstrate the osteogenic properties of the scaffolds. The cells were seeded on a bovine-bone scaffold, which is made of calcium hydroxyapatite, and requires a decalcification protocol for histologic processing of samples. The decalcification process destroys calcium deposits, therefore we excluded Alizarin red staining as a tool to assess osteogenic differentiation, however we employed other stainings including H&E and masson's trichrome. The scaffold shared a typical structure of bone tissue with trabeculae and empty lacunae as already indicated by other studies (Roato et al., 2018; Bari et al., 2021). All performed stainings highlighted cellular colonisation of the scaffold. Osteoblastic cells were detected on the border of the scaffold, where they synthesized new matrix filling the pores of the scaffold. These results were in accordance with data reported in literature (D'Alessandro et al., 2017) and confirmed scaffold capacity to promote the formation of bone matrix. New bone formation among the pores was also characterized by immunofluorescence, revealing a strong Collagen I expression which supported the deposition of the new matrix. We monitored matrix production until D35 post seeding. Stainings suggested increased matrix deposition over time. Taken together, our findings demonstrated that scaffolds influenced cellular characteristics including their viability, proliferation and differentiation and supported bone formation. Specifically, we hypothesize that osteogenic differentiation of cells may be related to the scaffold potential, which showed good osteoconductivity. Since new bone matrix deposition was detected in both expansion and osteogenic conditions, we hypothesize that scaffold stimulate new matrix formation rather than osteogenic growth factors. As in previously published studies (Nie et al., 2020; Amini et al., 2021), our work showed that decellularized bovine-derived scaffold can be considered as an effective alternative to autologous bone grafts. The combination of natural and synthetic components results in a highly biocompatible scaffold which favours the adhesion and growth of hMSC.

Finally, in the last part of this study, we investigated whether the addition of Bone Morphogenetic Protein-2 (BMP-2) to xenograft-derived scaffolds may improve hBM-MSC osteogenic differentiation. Many studies have identified BMP-2 as a potent osteogenic growth factor, which promotes more efficient bone formation and is widely used to enhance MSC differentiation. The protein binding and release capacity depends on scaffold composition. For these reasons, we decided first to characterize BMP-2 interaction with our scaffold. Our preliminary results were very promising and highlighted the strong capacity of the scaffold to retain BMP-2, indeed the amount of

growth factor detected in the supernatant collected from scaffold was negligible. We hypothesize that BMP-2 embedding may provide new bioactive signals and increase osteoconductivity and osteoindutivity of the scaffold, promoting cell differentiation and stimulating new bone deposition. Future experiments should assess the effectiveness of BMP-2 binding and its effect on hBM-MSCs. Several studies have reported that BMP-2 immobilization onto scaffold facilitate bone regeneration and promotes bone healing (Li X et al 2019). Thus, comparative analysis will be performed; BM-MSCs will be seeded on both scaffolds (alone and BMP-2 modified one), then we will evaluate cellular attachment and proliferation, which are crucial for facilitating osseointegration and differentiation, by the analysis of early and late osteogenic markers.

In summary, we investigated MSCs properties alone or in combination with scaffold and growth factors and analyzed their proliferation and differentiation *in vitro*. Our study revealed interesting results in culture media definition, suggesting PRP as a promising FBS substitute, since it stimulates cellular growth without interfering with their differentiation potential and the expression of surface markers. However additional studies on a larger cohort of samples are needed to confirm our results. The study on scaffold suggested that it had an excellent biocompatibility and a positive effect on cell proliferation. It provided a good microenvironment that promoted hBM-MSCs growth. Moreover, our findings demonstrated that SBN acted as a stimulator and supported BM-MSCs osteogenic differentiation and new bone matrix formation even in proliferation media, suggesting thas the scaffold itself has osteoconductive properties.

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