Bone-like inducing grafts: in vivo and micro-CT analysis

Doctoral Dissertation

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Bone-like inducing grafts: in vivo and micro-CT analysis

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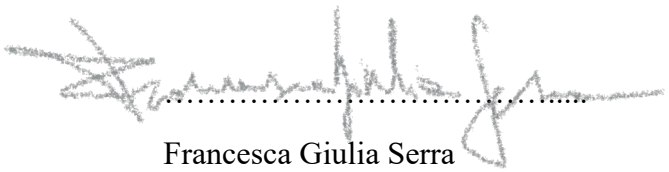

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Summary

Although the use of autografts is still considered the gold standard in bone regeneration, different biomaterials have been proposed for bone replacing. Among them, xenohybrid bone grafts showed excellent results as bone substitutes combining the already-well established features of xenograft with benefits of the blended constituents. The restoration of bone defects seems to be also supported by mesenchymal stem cells (MSCs).

The present dissertation aimed to confirm the role of MSCs, cultured on xenohybrid bone grafts, in bone regeneration promotion and to investigate their interaction/synergy with endothelial stem cells (ECs).

All the bone grafts were assessed through microcomputed tomography (micro-CT), a non-destructive and non-invasive method which allows to quantify the newly formed bone. According to the results, MSCs combined with xenohybrid bone grafts were able to osteodifferentiated and stimulate bone deposition; when MSCs are co-cultured with ECs, they showed a mutual influence on the bone formation which was confirmed in the animal trial.

Considering the potential of osteogenic cells addiction on xenohybrid bone grafts, further researches are still required to transfer the acquired knowledge in humans.

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To my beloved granny

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Introduction

Bone is a tissue with a high potential to regenerate itself after damages, although the restoration of significant defects still necessitates the use of bone grafts[1]. Indeed, the demand for bone substitutes is rising due to the several surgical bone-grafting procedures performed every year[2]. Among grafts, the gold standard is still considered the autologous bone graft (a graft moved from a donor to a recipient site in the same patient), although it involves possible complications like long surgical time, risk of vascular or nerve injuries, bleeding and pain[3].

To overcome the limitations of autografts, different substitutes have been tested. Xenohybrid bone grafts are promising substitutes, obtained from a xenograft (a graft derived from a non-human species, e.g. equine or bovine grafts) reinforced by a thin film containing collagen fragments. Compared to ordinary xenografts, they feature higher hydrophilicity, improved mechanical properties, optimal cell adhesion, and remarkable osteogenicity, [4].

Another noteworthy way to repair bone defects derives from the studies on mesenchymal stem cells (MSCs). Indeed, in regenerative medicine, the application of MSCs has been investigated to promote bone healing; easily obtainable, many clinical trials have tested the infusion of such kinds of cells to induce cartilage or bone regeneration with encouraging results[5].

Thus, an exciting field of research deals with the combination of bone grafts with osteogenic cells to confer a better osteogenicity to the implanted substitutes.

In the present dissertation, two experiments were performed in vitro to test the potential of mesenchymal stem cells, when cultured on bone graft, and their interaction with endothelial cells in co-culture. The third experiment was performed in an animal model to test the osteogenic abilities of bone graft d by MSCs, called “functionalized” bone graft.

The first chapter explains bone’s functions, components, metabolism and healing; the second chapter offers an overview of bone grafts, main features, indication and contraindications.

Before the description of experiments, the third chapter discloses the microcomputed tomography (micro- CT), the instrument used in the subsequent studies to assess the new bone formation.

The first investigation, illustrated in the fourth chapter, focuses on the ability of mesenchymal stem cells to colonise a xenohybrid bone graft and to generate bone when cultured on it. Two different kinds of MSCs were seeded and cultured: the stromal vascular fraction (SVF) and the adipose tissue-derived stem cells (ASCs), obtained from lipoaspirates.[6] According to the literature, many clinical trials tested the infusion of such kinds of cells to induce cartilage or bone regeneration with encouraging results[7]. In

the present study, SVFs and ASCs were cultured on a bone scaffold and analysed through micro-CT, scanning electron microscopy (SEM), histochemical analysis and enzyme-linked immunosorbent assay (ELISA).

In the second investigation, presented in the fifth chapter, the cross-talk mechanisms between endothelial cells and mesenchymal cells were evaluated. The proper vascularization is mandatory for bone graft survival after implantation[8], and according to scientific evidence, endothelial cells (ECs) support the osteogenic differentiation of MSCs both in vitro and in vivo[9]. Therefore, ECs and ASCs were co-cultured in a bioreactor, to recreate similar physiological conditions, and the result of their interaction was assessed through micro-CT.

In the sixth chapter, the third experiment focuses on the in-vivo regenerative potential of a "functionalized" bone graft. In a randomized clinical trial on *severe combined immunodeficient* (SCID) mice, two different xenohybrid bone grafts were implanted. In the test group, SVFs were cultured on bone grafts, whereas in the control group, ASCs were cultured on bone grafts. The osteogenic abilities of SVF and ASCs were evaluated after explant, through micro-CT 3D analysis.

In the last chapter, a discussion and conclusion on the whole results are illustrated.

Chapter 1

Bone

1.1 Introduction on bone

Histologically bone is included in the *connective tissues* because of the embryonal origin, from mesenchyme, and for the composition. Likewise, to all connective tissue, bone consists of three main components: fibres (mostly collagenous fibres), ground substance and cells. For these characteristics, it is considered a natural composite tissue and the ratio between different components is dynamic, varying with many factors, e.g., age, sex, nutritional status and health conditions. [10]

1.2 Bone functions

Bone is a multifunctional tissue with the mechanical role of protection, hematopoiesis and mineral homeostasis [11]. It has slowly become evident in recent years that bone also plays an importante endocrine role[12].

1.2.3 Mechanical functions

The mechanical functions of bone are well known and researched.
Thanks to its macroscopic structure, the bone can support various organs and offers insertion to muscles and tendons. The dense cortical bone which comprises most of the bone mass

supports the mechanical loads; although the cancellous bone supports loads, it redirects the mechanical stresses to the stronger cortical shell[11].
The microscopic composition also makes the bone well suited to prevent fractures caused by fatigue failure [13].
The bone also explicates a protective function to organs in the cranial and thoracic cavity and accommodates the hemopoietic elements of the marrow. In these locations, the bone micro-structure is organized in order to absorb maximum energy without traumatizing the bone itself [11].

1.2.4 Metabolic functions

Bone is a reservoir of ions, in particular calcium and phosphate. Calcium is essential to ensure bone stiffness and strength, but it is also involved in many metabolic processes such as clotting cascade, muscle contraction and nerve impulses transmission [14].
The levels of extracellular and intracellular phosphate control many biological processes, including energy metabolism, cell signaling, protein synthesis regulation, skeletal growth and bone integrity[15].

1.2.5 Hemopoietic and lymphopoietic functions

Bone is also a blood-forming (hematopoietic) organ[16]considered a source of red blood cells in particular in districts mainly composed of spongy bone (e.g. vertebrae, iliac crest, proximal femur).
The marrow cavity within the bone is an essential site for the creation and the maturation of blood cells and lymphocytes.

1.2.6 Endocrine functions

Bone mediates phosphate metabolism by secreting two hormones: fibroblast growth factor 23 (FGF23) and osteocalcin.
Osteocytes produce most of the human' s FGF23 that regulate bone mineralization.
Bone matrix release also undercarboxylated form of osteocalcin during resorption, that controls pancreatic β-cell proliferation and promotes insulin secretion. Bone promotes the production of

adiponectin, which can decrease insulin resistance, reduce fat in the cavities of the body and increase glucose utilization [12].

1.3 Bone Composition

Bone tissue, just like all the connective tissue, is constituted by limited cells surrounded by extracellular matrix[17].

1.3.1 Extracellular matrix (ECM)

ECM is composed of mineral component, organic component and water. The mineral component is constituted mainly by hydroxyapatite crystals, salts of calcium carbonate, phosphate and calcium fluoride and provides stiffness to the whole tissue.
The organic component is composed of connective fibres and ground substance and provides elasticity and tensile strength.
By weight, approximately 60% of the tissue is inorganic, 8–10% is water, and the rest is organic. The proportions, by volume, are roughly 40%, 25%, and 35%, respectively[17].

1.3.1.1 Mineral component

The mineral component of the bone also called the inorganic phase of the bone, is an analog of the naturally occurring mineral, nanocrystalline hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. The main substitutes are carbonate, magnesium, and acid phosphate, as well as other elements provided in amounts depending on diet and natural environment[18].
In comparison with the hydroxyapatite crystal found in nature, the bone ones have a smaller dimension (only 200 Å) resulting in more soluble than natural.
Usually, they have a flat shape (20–50nm long, 15nm wide, and 2–5 nm thick) but the measured average size of mineral crystals is highly dependent on tissue age[17].
X-ray diffraction, chemical analyses, energy dispersive electron analysis, nuclear magnetic resonance and other techniques have determined the physical and chemical properties of the bone[18]. The mineral component ensures mechanical resistance to the tissue, strengthen the

connective fibres network, and serve as a source of ions for mineral homeostasis (e.g. calcium, phosphate and magnesium).

1.3.1.2 The organic component

The organic component is constituted by connective fibres, among which by collagen fibres, and ground substance represented the largest part.[17]

1.3.1.2.1 Connective fibres

The structure of bones and cartilages are provided by collagen which controls, beyond the shape, the biomechanical properties.
In bone, collagen represents more than 90% of the organic matrix. The bone matrix consists basically of two collagen types, about 95% type I and 5% type V collagen, which are assembled into heterofibrils (Tab. 1). The framework of bone and cartilage are mainly constituited by collagen which supports minerals, glycoproteins and proteoglycans, giving to bone its peculiar features [19]. Furthermore, collagen is important for the adherence of cells and for the regulation of biological functions (e.g. migration, proliferation, differentiation and apoptosis).

Tab. 1 *Structural characteristics, functions and genetical origin of the main collagen types presented in bone (modified from Bilezikian, J. P. et al.[18])*

Proteins	Gene	Functions
Type I Collagen	17q21.23, 7q21.3-22	Scaffolds, promotion of hydroxyapatite deposition from proteins

Type X Collagen	6q21-22.3	Located in hypertrophic cartilage, no control on matrix mineralization
Type III Collagen	2q24.3-31	In trace quantity, regulation on collagen fibril diameter
Type V Collagen	9q34.2-34.3, 2q24.3-31, 9q34.2-34.3	In trace quantity, regulation on collagen fibril diameter

Elastic fibres are virtually absent in the bone tissue, except for a small quantity represented by Sharpey's "perforating" fibres.
Reticular fibres are localized in basal membranes surrounding intrabony blood vessels, but there are not present in intercellular matrix of bone.

1.3.1.2.2 Ground substance

The ground substance is distinctive and different in composition from other connective tissues. It comprises different kind of macromolecules, called *noncollagenous proteins* (NCPs) which represent the 10–15% of the total bone protein content. Only 1/4 of these components are exogenous-derivated, the remaining part synthetised by osteoblast.
NCPs are multifunctional, and although their roles are not clarified, they organize ECM, guide cell-matrix and mineral-matrix interactions, and they regulate the mineralization[18]. These proteins, although represent 2% of the bone by weight, play a fundamental role in embryogenesis and development: they control mineralization, formation and regulation of collagen fibrils and provides conduits for cellular attachment.
The NCP can be divided into several classes including *serum-derived proteins*, *proteoglycans*, *glycoproteins*, *RGD containing glycoproteins*, *siblings* and *GLA containing proteins*[11].

1.3.1.2.2.1 Serum-derived proteins

Serum-derived proteins are exogenously derived. Their affinity with hydroxyapatite expresses an effect on the mineralization of matrix and bone cell proliferation. The functions of bone proteins are not well discovered, however, according to literature, they could regulate the mineral deposition and control the metabolism of osteoblasts and osteoclasts[18]. (Tab. 2)

Tab. 2 *Structural characteristics, genetic and functions of the main serum-derived proteins in bone (modified from Bilezikian, J. P. et al.[18])*

Proteins	Gene	Functions
Albumin	2q11-13	Inhibition of hydroxyapatite crystal growth
α2hs- Glicoprotein	3q27-29	Stimulation of endocytosis, chemotaxis on monocytes, inhibition of calcification

1.3.1.2.2.2 Proteoglycans

Proteoglycans are macromolecules containing glycosaminoglycans bound to a central core protein and have multiple members of this family in the bone matrix. Their functions are not completely clear: it is believed that are involved in maintaining morphological/structural integrity of connective tissue matrixes. The primary function of proteoglycans is to regulate the

mineralization by affecting apatite nucleation and growth. Proteoglycans also influence the differentiation and proliferation of cells, modulating the activity of growth factors [18]. (Tab. 3)

Tab. 3 *Structural characteristics, genetic and functions of the proteoglycans in bone (modified from Bilezikian, J. P. et al.[18])*

Protein	Gene	Functions
Aggrecan	15q26.1	Organization of ECM, homeostasis, resilience to mechanical forces
Versican (PG-100)	5q12-14	Regulation of chondrogenesis
Decorin (Class 1 SLRP)	12q13.2	Regulation of fibril diameter, modulation activity of TGF-β, modulation on attachment to fibronectin
Biglycan (Class 1 SLRP)	Xq27	Bond with collagen, TGF-β, and other growth factors, determination of peak bone mass
Asporin (Class 1 SLRP)	9q21.3	Regulation of collagen structure
Fibromodulin (Class 2 SLRP)	1q32	Regulation of fibril formation
OsteoadhERIN (Class 2 SLRP)		Mediation of cell attachment
Lumican (Class 2 SLRP)	12q21.3-q22	Regulation of fibril formation

Perlecan	1p36.1	Regulation of cell signaling; cephalic development
Glypican-3	Xq26	Regulation of cell development
Keratocan (Class 2 SLRP)	12q22	Regulation of differentiation of osteoblasts
Osteoglycina/Mimecan (Class 3 SLRP)	9q22	Regulation of collagen fibrillogenesis
Hyaluronan—Multi-gene complex	Multi-gene comlex	Capture of space for bone deposition

1.3.1.2.2.2 Glycoproteins

Glycoproteins are proteins with diverse functions abound in the bone. (Tab.4)

Tab. 4. *Structural characteristics, genetic and functions of the glycoproteins in bone (modified from Bilezikian, J. P. et al.[18])*

Protein	Gene	Functions
Alkaline Phosphatase (bone-liver-kidney isozyme)	1p34-36.1	Carrier of calcium, inhibitors of pyrophosphates, stimulation of local phosphate concentration
Osteonectin	5q31.3-q32	Organization of collagen, deposition of hydroxyapatite, influence on cell cycle and regulation on bone deposition
Periostin	13q13.3	Organizzation on collagen, response to mechanical signals

Tetranectin	3p22-p21.3	Bond with plasminogen, regulation on matrix mineralization
Tenascin-c	9q33	Action on cell-FN interactions
Tenascin-X	6p21.3	Regulation on cell-matrix interactions
Secreted phosphoprotein 24	2q37	Association with regulators of mineralization in serum, regulation on BMP

1.3.1.2.2.3 RGD-containing glycoproteins

RGD-containing glycoproteins are phosphorylated and/or sulfated proteins which contain RGD (Arg-Gly-Asn) amino acids. The presence of RGD mediates the adhesion with many cell types, including bone cells[18]. (Tab. 5)

Tab. 5 *Structural characteristics, genetic and functions of the RGD-containing glycoproteins in bone (modified from Bilezikian, J. P. et al.[18])*

Protein	Gene	Functions
Thrombospondins (1-4, COMP)	15Q-1, 6q27, 1q21-24, 5q13, 19p13.1	Bond with heparin, platelets, types I and V collagens, thrombin, fibrinogen, laminin; activation or inhibition on plasminogen
Fibronectin	2q34	Bond with cells, fibrin, heparin, gelatin, collagen

Vitronectin	17q11	Bond with collagen, Activation or inhibition of plasminogen
Fibrillina 1 and 2	15q21.1, 5q23-q31	Regulation on formation of elastic fibers

1.3.1.2.2.4 Siblings

Siblings are small integrin-binding ligands, N-glycosylated proteins which contain RGD (Arg-Gly-Asn) but also sialic acid. These molecules allow the interaction between the intercellular environment and connective tissue cells[20].
While originally thought that the siblings were unique to mineralized tissues, it is now evident that many of them are expressed in metabolically active epithelial cells. (Tab. 6)

Tab. 6 *Structural characteristics, genetic and functions of siblings in bone (modified from Bilezikian, J. P. et al.[18])*

Protein	Gene	Functions
Osteopontin	4q21	Regualtion on mineralization, regulation on cell proliferation, inhibition on nitric oxide synthase, regulation on resistance to viral infection
Bone Sialoprotein	4q21	Regulation on bone remodeling, promotion of mineralization
DMP-1	4q21	Regulation on biomineralization, regulation osteocyte functions

Dentin sialophosphoprotein	4q21.3	Regulation of biomineralization
MEPE	4q21.1	Regulation of biomineralization regulation of phosphaturic hormone activity
Enamelin	4q21	Regulation on enamel mineralization

1.3.1.2.2.5 GLA-containing proteins

GLA-containing proteins are proteins formed in an endogenous way except for s proteins formed primarily in the liver but also by osteogenic cells [18] (Tab. 7). The residues of dicarboxylic glutamyl (gla) strengthen the calcium-binding.

Tab. 7 *Structural characteristics, genetic and functions of GLA-containing proteins in bone (modified from Bilezikian, J. P. et al.[18])*

Protein	Gene	Functions
Matrix Gla Protein	12p13.1	Action on cartilage metabolism, negative regulator of mineralization
Osteocalcin	1q25-31	Regulation of osteoclasts and precursors

Periostin	13q13.3	Regulation of response to loads
S protein	3p11-q11.2	liver product and made by osteogenic cells

1.3.1 Cellular component

Bone's cellular portion has a dual ontogenic origin that can be divided into mesenchymal stem cells and hematopoietic stem cells.

The *mesenchymal stem cells* (MSCs, also called stromal stem cells, mesenchymal stromal cells, skeletal stem cells, stromal fibroblastic stem cells) are multipotential stem cell which is considered indigenous cells of bone[21].

The *hematopoietic stem cells* (HSCs) are migrant precursor cells which derive from blood circulation.

The two major cellular lines osteoclasts and osteoblast have different origins, osteoclasts derive from hemopoietic stem cells, whereas osteoblasts derive from mesenchymal stem cells[21].

1.3.1.1. Osteoclasts

Osteoclasts, the main specialized cells that resorb bone, are derived from haematopoietics and play a key role in maintaining skeletal homeostasis. Osteoclasts are essential for the formation of the embryo's bone marrow cavity, as main cells that resorb bone. Additionally, osteoclasts 'ability to resorb bone can also control hematopoietic stem cells by releasing matrix constituents and minerals [20].

They are not indigenous cells of the bone tissue because they not belong to osteoprogenitor cells lineage. The osteoclasts precursors, so-called preosteoclasts, derive from monocyte precursors originated in bone marrow: they are related with the differentiative path of white blood cells.

Monocytes reach the bone through the blood guided by chemotactic signaling towards sites in which bone-resorbing processes will be required. Then they fuse into multinucleated cells with as many as 30 nuclei generating active osteoclasts. Active osteoclasts are syncytial elements morphologically giants (100-200μm) and polynucleate (from 4 to 20 nuclei) able to dissolve the mineral component and to digest the organic component of bone enzymatically [17].
They undergo to a continuous rearrangement of cytoskeleton according to functional moment: typical is the polarization characterized by different domini of the cytoplasmatic membrane such as ruffled border, adhesion domain, apical domain, basal-lateral domain)[20].

1.3.1.1.1. Osteoclastogenesis

Although bone formation occurs through the work of cells derived by mesenchymal stem cells, bone-resorbing occurs through the work of cells derived by hematopoietic precursors (Fig. 1).
Osteoclasts origins by hematopoietic precursor with a reduced differentiative potential exclusively to the myeloid lineage. Initially, it was though that osteoclasts originate by fusion of osteoblasts.
Precursors of osteoclast have been found in hematopoietic tissues such as the bone marrow, spleen and peripheral blood. They differentiate in monocyte able to go out and move in the bloodstream. Monocytes can enter in connective tissue and differentiate in mononuclear progenitors of osteoclasts.
The osteoclastogenesis includes different steps: differentiation in myeloid progenitors, proliferation, commitment, fusion, polarization and activation[22].

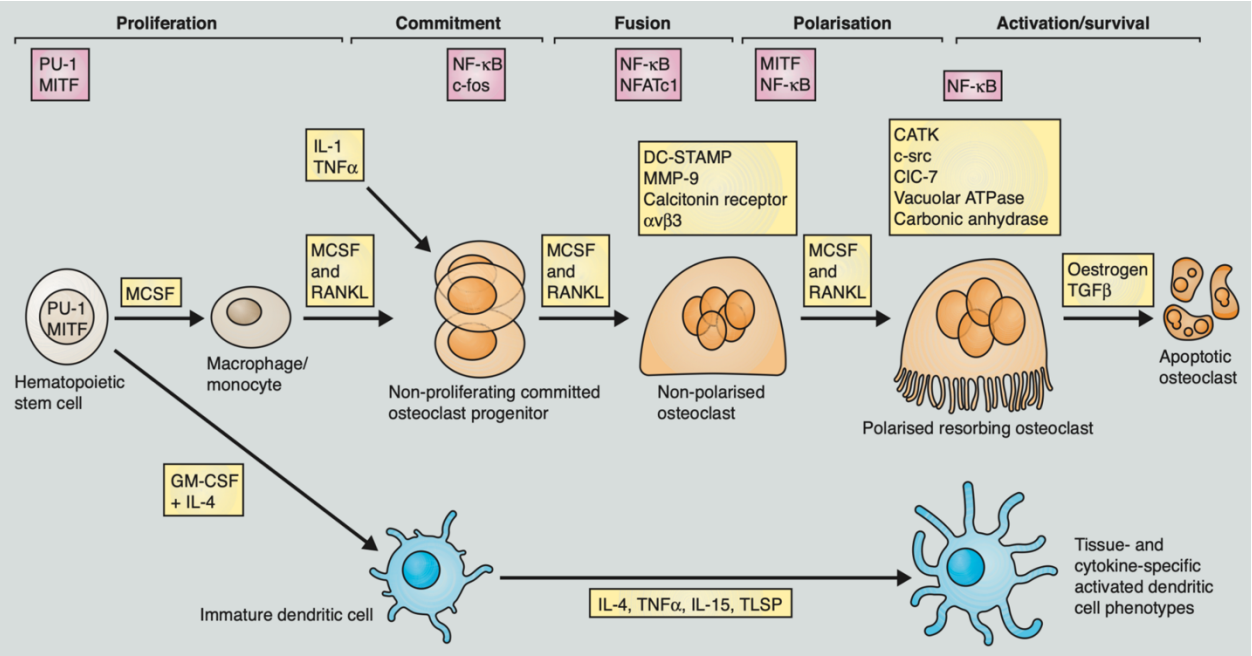


Fig. 1 Osteoclast lineage (from Crockett, J. C. et al.[23])

1.3.1.1.1.1 Differentiation in myeloid progenitors

Inside bone marrow, hematopoietic stem cells (HSCs) stimulated by IL3, IL6, SCF (stem cell factors) and more, differentiates in common myeloid progenitors (CMP). The CMP formation is supported by PU.1 that promote the expression of the colony-stimulating factor-1 receptor (c-Fms) for macrophage-colony stimulating factor (MCSF or CSF-1) and microphthalmia-associated transcription factor (MIFT). Colony-stimulating factor-1 receptor (c-Fms) for macrophage-colony stimulating factor (M-CSF or CSF-1). The M-CSF in the following steps will induce the RANK expression on osteoclast precursors. The interaction between RANK and RANKL will induce the maturation of osteoclast precursors[24].

Microphthalmia-associated transcription factor (MITF), a transcription factors that promote modification inside cells to address the differentiative pattern[25].

Recent studies suggest that plasticity exists between the different cell types originating from myeloid precursors and that mature cells can transdifferentiate into another cell type.

1.3.1.1.1.2 Proliferation

CMP stimulated by Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) differentiates in granulocyte-macrophage precursors (GMPs). M-CSF (macrophage-colony stimulating factor o CSF-1) stimulates proliferation and survival of monocyte/macrophage, osteoclast precursors[22].

1.3.1.1.1.3 Commitment/differentiation and fusion

Receptor activator of nuclear factor-kappa-B ligand (RANKL), also known as osteoclast differentiation factor (ODF), guides the differentiation from osteoclast precursors to mononuclear osteoclasts[22].
After that, the mononuclear osteoclasts fuse in not-divisible, multinuclear cells[22].

1.3.1.1.1.5 Polarization

One of the critical steps in the osteoclast maturation is the structural re-arrangement (of membrane and cytoskeleton) resulting in a polarization of cells and the formation of two domains: an apical domain, contacting the bone surface and a basal-lateral domain localized distant from bone surface[26].

1.3.1.1.1.6 Activation/survival

In the final step, the cells are activated and survive with ruffled border and sealing zone formation[22].

1.3.1.2 Osteoblasts

Osteoblasts exist in bone tissue as consecutive functional phases of the same cellular type.
They are considered indigenous cells of the bone tissue because They belong to mesenchymal progenitors which generate chondrocytes, muscle cells and adipocytes as well. The

mesenchymal cells 'commitment to the osteoblastic lineage depends on the precise activation of the transcription factors induced by morphogenetic and developmental proteins [20].

Osteoblasts are the cells responsible for the shape and formation of bone and conduct protein secretion and bone mineralisation roles of the bone matrix. Osteoblasts conduct protein secretion and bone mineralisation roles of the bone matrix. When bone matrix formation is complete, some mature osteoblasts remain trapped in the bone as osteocytes, some flatten to cover quiescent bone surfaces as bone lining cells, and the rest die from apoptosis.

In bone tissue can be found in all their evolutive phases, morphologically distinguishable: MSCs, osteoprogenitor cells (pre-osteoblasts or skeletal stem progenitor cells SSCs), osteoblasts, osteocytes and bone lining cells.

1.3.1.2.1 Osteoblastogenesis

Three osteoblast-specific genes, encoding transcription factors, control the differentiation of the osteoblast progeny: Sox9, Runx2 and OSx. *Sox9* determines the differentiation of the mesenchymal progenitor into preosteoblasts and chondroblasts, *Runx2* induces the differentiation of the mitotically-active preosteoblasts into post-mitotic osteoblasts and, together with Osterix (Osx), controls the expression of osteocalcin. Undercarboxylated osteocalcin is a specific secretory protein that enters the blood circulation to possibly stimulate insulin secretion by pancreatic β cells and testosterone production by Leydig cells. Osx, encoding a zinc-finger transcription factor, is required for the differentiation of osteoblasts into osteocytes and the function of osteocytes and chondroblasts.

The expression of the transcription factors Sox9, Runx2 and Osx, is regulated by several cell signalling pathways: hedgehog signalling, notch signalling, wnt/ β catenin signalling, bone morphogenetic protein signalling and fibroblast growth factor signalling. Hedgehog signalling, mediated by Indian hedgehog protein, is required for the differentiation of Runx2+/Osx+ osteoblasts. Notch signalling inhibits osteoblast differentiation by down-regulation of Osx activation. Wnt/ β catenin signalling stimulates osteoblast differentiation by Osx activation. Bone morphogenetic protein signalling promotes the transition of Runx2+ preosteoblasts to Runx2+/Osx+ osteoblast by enhancing the expression of Runx2 and Osx. Fibroblast growth factor signalling regulates Runx2+ preosteoblast proliferation and Runx2+/Osx+ osteoblast differentiation[27].

The osteoblastogenesis includes different steps: commitment, proliferation and matrix maturation (Fig.2).

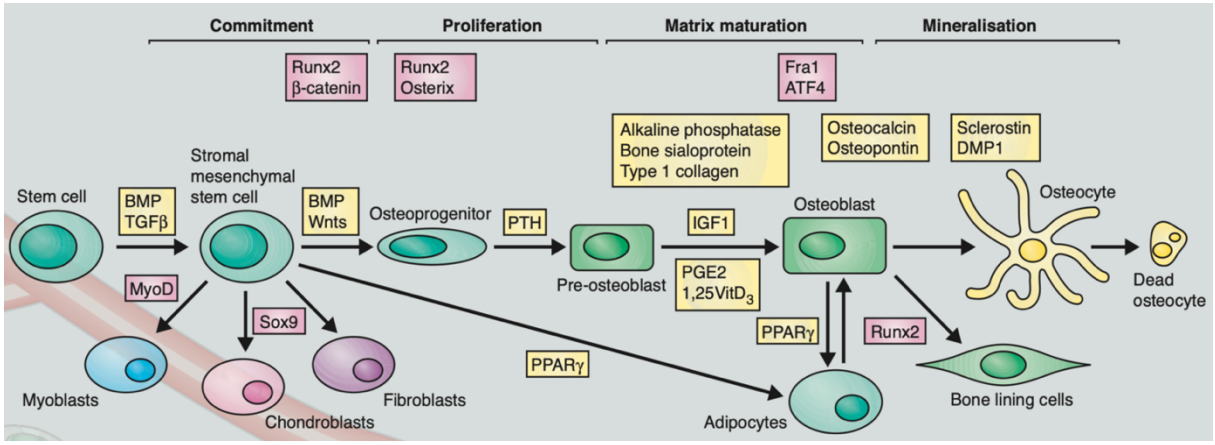


Fig. 2 Osteoblast lineage (from Crockett, J. C. et al.[23])

1.3.1.2.1.1 Commitment

Sensing to the osteoblast lineage by members of the canonical Wnt/β-catenin pathway such as Wnt10b, BMP2, and BMP4 guides the mesenchymal stem cell fate. This is achieved by suppressing the adipogenic transcription factors C/EBPα and PPARγ while activating the Runx2 and Osx osteogenic transcription. This immature osteoblast still has the ability to divide and express low levels of alkaline phosphatase activity and synthesize type I collagen, which accounts for up to 90 percent of the organic portion of bone [28].

1.3.1.2.1.2 Proliferation

Differentiation to the non-proliferating mature cuboid osteoblast actively mineralizing the bone matrix depends on the Osx transcription factor [29].

1.3.1.2.1.3 Matrix maturation

Nevertheless, it must first undergo maturation before the freshly laid matrix can be mineralised. Matrix maturation is associated with increased alkaline phosphatase production and many non-

collagen proteins, including osteocalcin, osteopontin, and sialoprotein in the bone. The incorporation of hydroxyapatite completes mineralization of bone into the newly deposited osteoid. The membranous extracellular bodies (extracellular matrix vesicles) emerging from the osteoblast promote the initial deposition of minerals by storing calcium and phosphate ions in a regulated environment [29]. These ion clusters join together to form the first stable crystals. It follows that ions are added to those crystals, resulting in their growth. Upon entombing into the bone matrix, a subset of osteoblasts may undergo further differentiation, and become osteocytes. It is assumed that the remaining osteoblasts either suffer apoptosis or be inactive bone-lining cells [22].

1.4 Bone macroscopic aspect

From a macroscopic point of view, it is possible to distinguish two variety of bone: compact bone and cancellous bone.

Compact bone, also called dense or cortical bone, is characterized by a lack of a porosity (only 3-5%, although this increases with age and with osteoporotic changes to the skeleton). Typical of the most external surface of short bones, flat bones, long bones, shafts of the long bone[30].

Cancellous bone, also known as trabecular bone or spongy bone, is characterized by a porous network of open cells. Cancellous bone has a higher surface-area-to-volume ratio than cortical bone, and it is less dense. It surrounds the medullary cavities, containing bone marrow. It ensures proper mechanical support maintaining a light-weight structure of the whole bone - typical of the short bones, flat bones and long bones[30].

1.5 Histological varieties of bone tissue

According to the size and the orientation of collagen fibres, two types of bone tissue can be distinguished: primary bone tissue and secondary bone tissue.

Primary bone tissue, also called fibrous or woven, features collagen fibres relevant in dimensions (5-10μm) with undefined spatial orientation around Haversian Canal, containing a blood vessel. Osteocytes occupy the gaps made by the unorganized collagen fibres[17].

The woven tissue is the first bone tissue deposed in the embryonal period, and it also acts in physiological fracture healing. It can be found at tendons and ligaments insertion, but the most of primary bone tissue is resorbed and replaced by the secondary one.

Secondary bone tissue, also called lamellar, represent the most widespread variety of bone, constituting almost the entire compact and significant part of woven human bones.
The collagen fibres are thinner (10-20nm) without aggregation tendency, and it is typified by bone lamellae, layered alternately and distinguished in dense or loose.
Thin or dense lamellae are about 3μm wide, rich in collagen microfibrils (oriented randomly), low in apatite crystals. They give plasticity to the whole tissue[17].
Thick, or lose lamellae are about 7μm, low in collagen microfibrils and rich in apatite crystals. They present bone lacunae containing osteocytes, and they give hardness to the whole tissue[17].
Depending on lamellae arrangement, it is possible to identify simple lamellar bone tissue and Haversian lamellar bone tissue. The first one is avascular, with a limited number of lamellae, with parallel orientation, typical of thinnest parts of woven bone trabeculae[17]. The second one consists of a variable number of lamellae (8-20) arranged concentrically around the Haversian canal, that houses a blood vessel[17] (Fig. 3).

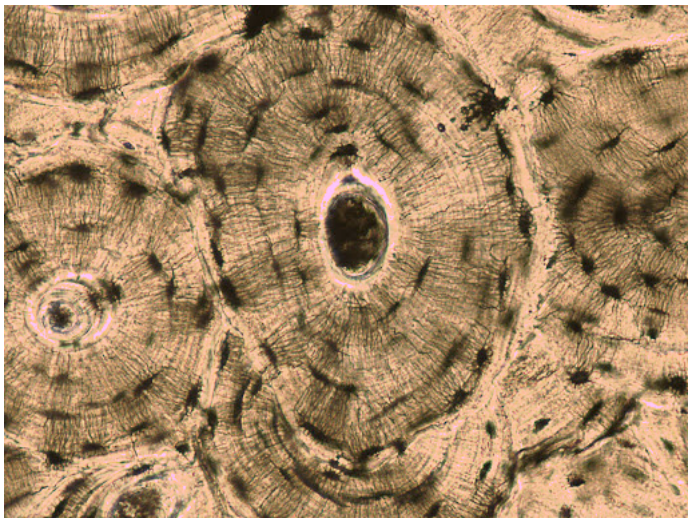


Fig. 3 Haversian system (from http://medcell.med.yale.edu/)

The elementary functional and trophic unit of Haversian bone tissue is the osteon[31] (Fig. 4). In the space, the osteons have a cylindric shape (0, 9-1,2mm long) with a longitudinal axis parallel to force vectors applied to bone segment[17]. Each osteon is independent, but there are many connections between contiguous osteons through blood vessel anastomosis (Volkmann canals)[17].
Space between contiguous osteons is occupied by interstitial lamellae consisting of previous resorbed osteons debris. Each osteon in separated from intestinal lamellae by a so-called cement line.

In the mature bones, the external surface is limited by the outer circumferential lamellar system, facing the periosteum; the marrow canal is limited by Inner circumferential lamellar system, facing the endostium[31].

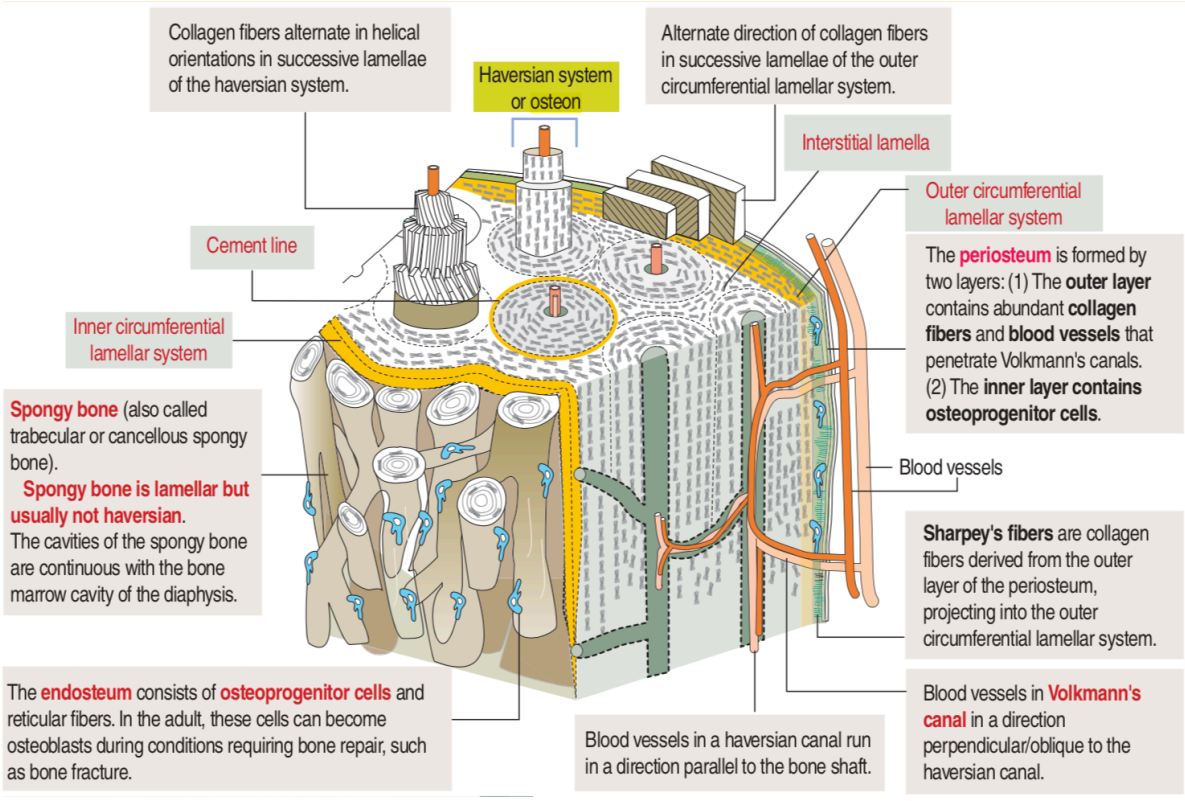


Fig. 4 Osteon structure (from Kierszenbaum, A. L. et al.[31])

1.6 Bone metabolism

The mechanisms involved in maintaining the homeostatic balance of bone tissue are *growth*, *modelling* and *remodelling*.

The growth (from the fourth month of fetal life, after the development of ossification centres, until the end of the somatic growth – about 20-22 years of age) is exclusively due to osteoblast activity.

After bone formation, two separate physiological mechanisms, modeling and remodeling, which are both used in bone fracture healing, are sustained dynamically.

1.6.1. Bone modelling

The bone modelling starts from the fourth month of fetal life, after the onset of bone growth, until the end of the somatic growth, about 20-22 years of age [32]. This continues in adulthood by improving bending resistance and adapting to functional challenges.

Modelling ensures the net increase of bone mass maintaining/modifying its shape or changing the cortical position with respect to its central axis (bone drift). It is promoted by local stresses[19], and it is typified by two independent and simultaneous processes: positive modelling (or *formation modelling*) supported by osteoblasts and negative modelling (or *resorption modelling*) supported by osteoclasts.

The process takes place in two phases: activation, recruitment of osteoblast/osteoclast precursors, and resorption/formation until the bone architecture will not adapt to local stresses.

1.6.2 Bone remodelling

The bone remodelling starts from the 6th month of fetal life and lasts for the entire life and occurs in a precisely timed sequence on the same bone surfaces. The process allows different functions, e.g. in skeleton adaptation to the mechanical and metabolic requirements and additionally, through it, bone turnover is performed [32]. Roughly 25% of trabecular bone and 3% of cortical bone are estimated to be removed and replaced each year

Remodelling ensures the replacement of bone matrix (bone turnover) and the repairing of small defects like microfractures.

It is characterized by sequential and coupled bone resorption/formation, occasional event limited in time[19].

It could be targeted, when there are local signalling promoting site-specific osteoclast activity: the two leading signals are microfractures and osteocytes apoptosis. Microfracture regions have both pro- and anti-remodeling signals which are involved in selective remodeling. Osteocytes near the microcracks express high RANKL levels and low OPG rates, thereby promoting recruitment by osteoclast. The osteocytes more distant from micro damage transmit low RANKL levels and high OPG rates [31].

When osteocytes network is damaged without micro traumatism (estrogen reduction, mechanical disuse, glucocorticoid excess) the remodelling is supported by osteocytes apoptosis.

It could also be stochastic when osteoclasts are resorbing bone without a location-specific signalling event.

Remodelling is a cyclic process, at any times there are thousands of remodelling cycles in every part of the body and each cycle is in a different stage depending on the moment in which it

starts. The whole process lasts about 4-6 months in the human, but it can be altered, for example by pathologies.
It consists of five steps[31]: activation, resorbing, inversion, formation and resting.

1.6.2.1 Activation or initiation

During the first step, the production of osteoclasts starts with the recruitment of osteoclast precursors by osteocytes, the main mechanosensing and RANKL-producing cells in the bone and the identification of skeletal micro-damage. M-CSF and RANKL promote differentiation of osteoclasts within the BRC. In addition, osteoclast precursors have to be bound to the bone matrix to differentiate into osteoclasts[31].

1.6.2.2. Resorbing

Osteocytes sense the need for bone resorption, which sends signals to lining cells. We slowly withdraw from the bone surface of a structure called the *bone remodelling compartment* (BRC). The precursors of the osteoclast penetrate the BRC by capillaries of the marrow. The bone remodelling process is promoted by the combined action of pro and anti-osteogenic cytokines (RANKL, M-CSF, OPG) that support the mature osteoclasts differentiation.
A so-called cutting cone, or resorbing cavity, is formed featuring bone matrix breakdown and release of collagen fragments (blood and urine markers of bone remodelling).
Osteoclasts can not attach themselves to the bone and begin resorption without retraction of the bone lining cells [31].
When osteoclasts have completed the bone-resorbing, the remaining collagen fragments must be removed from the exposed bone surface. It though that this is done by bone lining cells, that form a thin layer of bone matrix called cement/reversal line, rich in proteoglycan. If these fragments persist, bone deposition cannot start. Essential to the resorptive event is the osteoclast's ability to create a micro-environment between itself and the inner bone matrix called the bone remodelling compartment [31].
This compartment is acidified by an electrogenic proton pump (H+- ATPase) and a Cl- canal to a pH of 4.5. The acidified setting interferes with the bone's mineralized portion, revealing its organic matrix (consisting mainly of type I collagen). The cathepsin K lysosomal enzyme completes the breakdown of the organic matrix.

The osteoblast precursors differentiation is controlled by factors released during bone matrix resorption. Also, molecules derived from osteocytes, including sclerostin and Dkk-1, influences differentiation and bone synthesizing function of osteoblasts.

1.6.2.3 Inversion, formation and resting

During the inversion, a progressive reduction of bone resorption is registered, matched by an increase in deposition[31].
Then osteoblasts form an epithelioid layer deposing osteoid tissue, replaced by embryonic bone tissue before and secondary bone tissue layer.
Osteoblast involved in bone formation could have such different fates. The 90% of osteoblasts reach out to apoptosis and replacement by new osteoblast until new bone formation became unnecessary. A little part remains embedded inside the osteoid matrix evolving in osteocyte and the remaining stay on the bone surface as inactive lining cells, maintaining the ability to became active and start to produce bone matrix.
At the end of the remodeling process, the bone surface is replaced by bone lining cells, during which period the bone matrix within the remodeling device begins to mineralize. The most of bone surfaces are in quiescent state[31].

1.7 Bone fracture healing

On the base of the mechanism involved, it can be distinguished three main types of bone fractures: traumatic, common during the childhood and in the elderly, pathologic fractures, independent of trauma and associated with a bone alteration (such as osteoporosis or a genetic collagen defect such as osteogenesis imperfecta) and stress fractures, caused by inapparent minor trauma (microcracking) during the practice of sports.
Bone healing is regarded as a restatement of the ontological events occurring during the early stages of embryonic skeletal development. In contrast to other tissues, the damaged organ could be fully restored to its pre-damage structure and function[32].

Specific factors influencing recovery, including the degree of tissue damage, can be added to identify bone healing. The bone repair can therefore be divided into two subgroups: primary bone healing and secondary bone healing.

1.7.1 Primary bone healing

The bone gap in this cycle is assumed to be filled directly by continuous ossification and subsequent Haversian remodeling, with complete lack of models of cartilaginous or connective tissue. Callus formation is not present. It usually takes from a few months to a few years, depending on the species, before a complete fix is reached.

Direct healing usually does not tend to happen in the natural healing process of fractures because it requires the absence of any gap formation and a rigid fixation to guarantee mechanical continuity without interfragmentary strain. It represents the main goal of many orthopaedic surgeries. The primary cure of fractures will occur either by touch healing or gap healing [33] (Fig. 5).

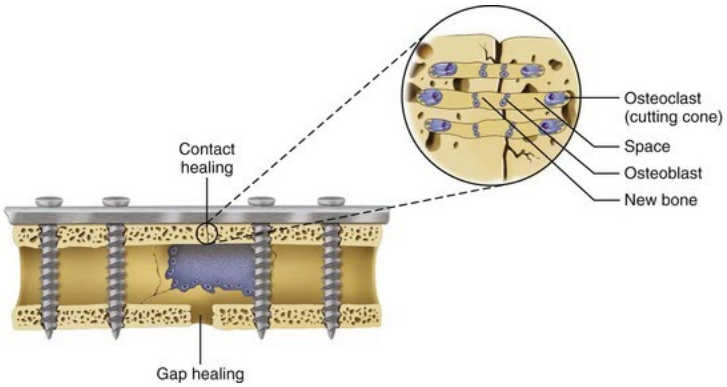


Fig. 5 Primary bone healing (from Fonseca, R. J. et al.[34])

Contact healing occurs primarily when the difference in fracture is less than 0.1 mm and the site of the lesion is stiffly rigid (interfragmentary strain is less than 2%). The osteoclasts form a cutting cone across the fracture line, creating cavities which are later filled with bone formed by osteoblasts residing at the rear of the cutting cone. The restored Haversian mechanism enables blood vessels to enter vehiculating circulating osteoblastic precursors. The transition osteons later mature by remodeling into lamellar bone resulting in a healing of fractures without development of periosteal calluses [33].

Gap healing happens if a secure anatomical reduction is accomplished, although the gap must be below 800 μm to 1 mm. Unlike the process of contact healing, the damaged site is firstly filled woven bone that is subsequently remodelled into lamellar bone.

The intermediate phase is essential to restore the anatomical and biomechanical features of the bone fully[33].

1.7.2 Secondary bone healing

Secondary healing is the most common form of fracture healing, which involves a cartilage template being replaced by bone, resembling endochondral ossification.

It occurs when the points of the fracture are less than twice the diameter of the damaged bone. It needs neither anatomical reduction nor rigidly stable conditions: occurs when there is still some small interfragmentary motion (micro-motion and weight-bearing), a condition called "relative stability" that stimulates callus formation[33].

On the opposite, micro-motion and weight-bearing strengthen this. It is stated that excessive movement and/or load causes delayed healing, or even nonunion. However, too much interfragmentary motion can lead to failure of healing nonunion.

This comprises the typical stages of injury, inflammation of haemorrhage, main soft callus formation, callus mineralization and callus remodeling (Fig. 6).

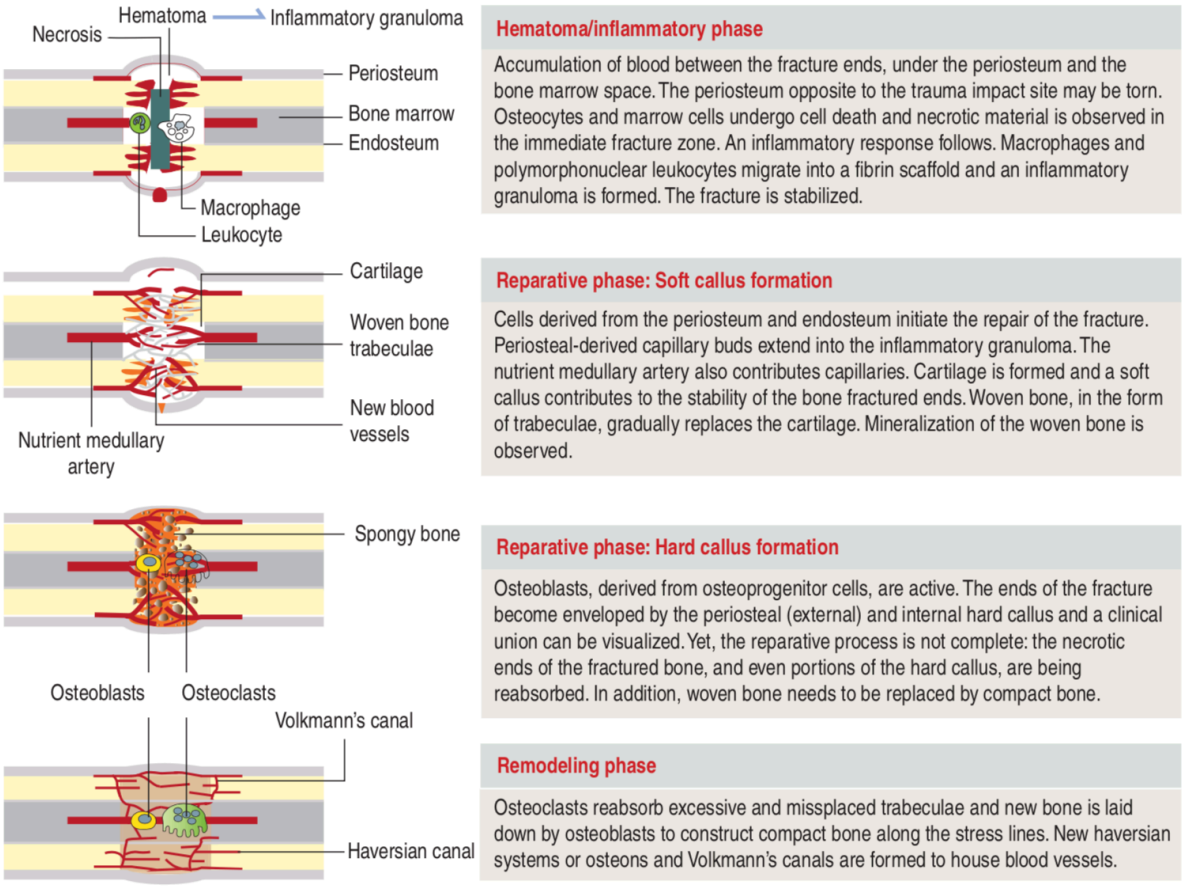


Fig. 6 Bone fracture healing (from Kierszenbaum, A. L. et al.[31])

1.7.2.1 Hematoma/inflammatory phase

The hematoma/inflammatory phase involves a significant disruption of blood vessels located in the Haversian and Volkmann's canals cause accumulation of blood and necrotic materials from osteocytes and marrow cells death at the fracture site causing an inflammatory response. Macrophages, monocytes, lymphocytes, and polymorphonuclear cells, as well as fibroblasts, are attracted to the fracture site. Inflammatory fibrin-rich granuloma bulges over the edges of the fractured bone and connects the fragments, stabilizing the fracture during the first week after injury[31].

1.7.2.2 Reparative phase

The phase consists of a soft callus formation and a hard callus formation.
During the first step, the dead cells and the damaged bone tissue are removed by phagocytic cells. Capillaries invade the granulation tissue delivering the mesenchymal stem cells (MSCs) that will give rise to osteoblasts at the periosteal and endosteal sites. The osteoblast, together with fibroblasts, initiate the healing process. A soft callus consisting of noncalcified cartilage connects the two ends of the fractured bone[31]. The cartilagineous soft callus is replaced with woven bone in the form of trabeculae, about 3 to 4 weeks after the injury, through the work of periosteal and endosteal-derived osteoblasts.
In the second phase, the soft bridging callus is expanded to hard bone callus by minerals deposition. Osteoblasts lay down woven bone on this collagen framework left by the chondrocytes. Cyclical micromotion stimulates the growth of the callus[31].

1.7.2.3 Remodeling phase

Although the hard callus is a solid structure that provides biomechanical stability, it does not completely restore normal biomechanical features of the bone. To this end, the healing fracture cascade initiates a second resorptive process to turn the hard callus into a lamellar bone structure[31].
Osteoclast activity removes the excess material of the bone callus and replaces the woven bone with compact lamellar bone between and around the injured bone fragments. The remodeling

process is performed by combining osteoclasts with rough callus resorption, and osteoblast lamellar bone deposition. This phase lasts 2-3 months after injury. Combining osteoclasts with rough callus resorption and osteoblast lamellar bone deposition, the remodeling process is performed.

Chapter 2

Bone-like inducing grafts

2.1 Introduction on Bone grafts

Bone grafts are defined, according to a definition proposed by Muschler and Lane, as any implanted material which promotes bone healing response[35].

Bone is a tissue with a high propensity for regeneration after injury, although the reconstruction of severe deficiencies caused by congenital malformations, surgery, trauma or diseases is a major health problem[1].

Bone is, indeed, the second most commonly implanted tissue in the human body, after blood. According to reports in the United States and Europe, more than half a million patients receive bone grafts annually[36] and the data is increasing, such as the ageing and growing needs of the population[37].

Therefore in the last decade, medical research focused on the restoration of damaged tissues and the development of new biomaterials and surgical techniques used for bone regeneration[38].

2.2 Historical context

Although during the Ottoman era, in 1505, Ibrahim bin Abdullah wrote a text outlining the reparation of cranial defects with xenografts of Kangal dog, the first successful bone xenograft was published in 1668 by Job Janszoon van Meekeren. The Dutch surgeon reported treatment of a sword injury to a Russian soldier, which resulted in a cranial defect, with a graft from a dog cranium[39].

After extensive investigations in the use of bone grafts in animals and humans, the French surgeon Louis Léopold Ollier, considered "the father of orthopaedic surgery", enlightened the critical function of the periosteum, writing in 1867 “Traité experimental et clinique de la régénération des os et de la production artificielle du tissue osseux”[40].

In 1879 Sir William MacEwen performed transplantation of human bone from an osteotomy on a child to a humerus of another child and reported new bone formation along with the humeral site. According to Sir Arthur Keith "The reconstruction of that boy's right humerus in Glasgow in 1880 is the first paragraph of a new chapter in the history of surgery"[41].
The first systematic description of the fundamental principles underlying the use of the bone graft in surgery is attributed to Frederik Houdlette Albee, a North American surgeon who published in 1915 a textbook named "Bone Graft Surgery"[42].
In the XX century, the bone grafts were recognized as rational and viable through the works of Georg Axhausen, Erich Lexer and Dallas Burton Phemister. Axhausen and Phemister described the process of graft incorporation, and Lexer published clinical cases of bone allografting with twenty years follow-up, showing excellent results in half of the patients[43].
Fresh bones were used for the most of grafts until in the 1912 Albee recommended the storage of tissues at 4-5°C. In 1947 Philip Wilson described freezing storage techniques for preserving bone grafts at -20°C and built a bone bank for small fragments, giving rise to the era of tissue banking[44].
The new era of bone grafting started in 1970 when Marshall R Urist discovered the "morphogenetic property" of demineralized bone matrix (DBM) which leads to the discovering of a family of proteins called Bone Morphogenetic Proteins (BMPs)[45].

2.3 Bone grafts properties

Bone grafts, to promote the healing response, must present biological and mechanical properties. The biological properties are typical of bioactive materials and could be described as osteoconduction, osteoinduction and osteogenesis. The mechanical properties are linked with the necessity to support weight-bearing and are related to the type of bone grafts.

2.3.1 Bone grafts biological properties

Bone grafts must be biocompatible, integrated into the host site with a minimum risk of biological adverse events, and progressively biodegradable, chemical reabsorbed, and substituted by new bone through osteoconduction, osteoinduction and osteogenesis[46].

Osteoconduction refers to the ability of the graft to sustain the attachment of osteoprogenitor cells to permit the migration and growth of cells. According to Wilson-Hench, the consequent new bone formation will follow the architectural structure of the graft[47].

Osteoinduction is the process which leads to the bone formation through the stimulation of osteoprogenitor cells to differentiate into osteoblasts. The process of differentiation is mediated by different growth factors which could also be applicated directly in the injured site to promote healing. The principal growth factors indagated are the following: Bone Morphogenetic Protein (BMP), Fibroblast Growth Factors (FGF), Vascular endothelial Growth Factors (VEGF), parathyroid hormone (PTH) and plated-rich plasma (PRP), described in tab.8.[48]

Osteogenesis is described as the ability of osteoblast to produce new bone.

Tab. 8 *Growth factors applied to promote bone healing.*

Growth factors	Source	Functions	Applications
Bone morphogenetic proteins (BMP)	Osteoprogentior cells, bone extracellular matrix osteoblasts	Promotes mesenchymal cells differentiation into osteoblasts	Rh BMP-2 is used in the treatment of open tibial fractures and anterior lumbar spinal fusion[49] Rh BMP-7 is used in the treatment of posterolateral lumbar spine fusion[50]
Fibroblast growth factors (FGFs)	Mesenchimal cells, osteoblasts, chondrocytes, macrophages	Stimulates mitogen in mesenchymal cells and osteoblasts. Increases angiogenesis	Promote bone fracture healing, in in-vivo animal studies[51]
Vascular endothelial growth factor (VEGF)	Platelets	Increases angiogenesis and vascular development	Promote bone fracture healing, in in-vivo animal studies[52]
Recombinant human parathyroid hormone (PTH)	Parathyroid glands	Increases osteoblasts' recruitment and survival	Accelerates bone fracture healing, in animal studies.[53]

Plated-rich-plasma (PRP)	Blood	Contains various mitogenic and chemotactic growth factors: platelet-derived growth factor (PDGF), insulinlike growth factor (IGF), fibroblast growth factors (FGFs), transforming growth factor-beta (TGF-b) and VEGF	Insufficient evidence to support its routine, utilization in orthopaedic trauma[54]

2.3.2 Bone grafts mechanical properties

The mechanical proprieties affect the capability of grafts to prevent the collapse of the surrounding tissue once introduced in the host (space-making capability) and vary in correlation to the type and shape of bone graft.

The patient's age and harvest site, in autograft, determine the mechanical resistance, in allograft, the storage (freeze-drying) reduces the mechanical strength by 20%, in bovine xenografts the elastic modulus is similar to cortical bone[55,56].

Mechanical resistance to compression is affected by the bone graft's shape: in particulate bone substitutes the granule size >1mm present higher mechanical resistance, contributing to maintaining the space-making capability.

The pore size and the porosity also influence the space-making capability.

In the early stage of graft's integration, the maintenance of the augmented bone volume is a critical factor of success. The presence of interconnected pores and the porosity, indeed, allows the bioabsorbability of bone, the chemical degradation of bone graft which precede the bone remodelling by osteoclasts and new bone formation.

The pore size (diameter) acts on the diffusion of nutrients and the migration of cells; ideally, a diameter of 200–350 μm is considered optimal for the growth of new bone[57].

Porosity, pore size and interconnectivity of grafts promotes the migration of vessels and ingrowth into the material as well as subsequent cells infiltration. However, increased pore size, over 400 μm, and high porosity decrease the mechanical resistance and must, therefore, be balanced against the space-making capability[58].

2.4 Bone graft incorporation

The formation of new bone in the host site is correlated to the healing response resulting in the incorporation of the bone graft. The rate of incorporation depends on different factors like the graft material and the host inflammatory reaction[59]. The graft incorporation, indeed, involves the release of cytokines and growth factors, cells migration, vascularization, differentiation and proliferation.

The process of incorporation could be modified not either by alterations of the host inflammatory response, but also by mechanical stability, quality of tissues, vascularization, infections and drugs.

The mechanical stability is necessary to guarantee the formation of granulation tissue and fibrosis[60]. The good vascularization is essential to provide the migration of mesenchymal cells and progenitors of endothelial cells, leading to adequate osteogenesis[61]. The assumption of immunosuppressants, bisphosphonates or radiant therapy could compromise the new bone formation[62].

2.5 Type of bone grafts

Bone grafts could be classified according to the source: same individual (autograft), different individuals (allograft, isograft) or different species (xenograft). Synthetic bone substitutes and composite materials represent other categories of bone like-inducing grafts.

2.5.1 Autograft

Autograft is the term to define autologous bone graft, move from one site, donor, to another site, recipient, in the same individual.

Autologous bone graft has become widely used and is still considered the gold standard in treatment for bone defects. The reason is ascribable to some factors: the easiness to obtain, the osteoconductive, osteoinductive and osteogenic favourable proprieties, the absence of adverse immune response or infectious diseases. Furthermore, autologous bone is an ideal option when structural stability is required; indeed, the graft can be withdrawn in a variety of forms and sizes from different donor sites. The main donor site remains the iliac crest which supplies necessary

quantities of cortical and cancellous bone for most clinical settings. Alternative donor sites encompass the greater trochanter, the proximal and distal part of the tibia and the distal end of the radius.

Despite the broad applications of autologous bone grafts, long surgical time, pain, bleeding, haematoma, risk of vascular or nerve injuries and cosmetic deformities, represent possible complications[3].

2.5.1.1 Type of autografts

Autologous bone grafts could be classified like cortical, cancellous and corticocancellous bone grafts.

The type of autografts selected is related to the defect size, according to some authors the use of non-vascularized graft is not recommended for bone defects > 5cm and the use of free vascularized corticoncellous grafts is recommended for bone defects >12cm, in order to improve the graft healing[63].

2.5.1.1.1. Cortical Bone Graft

Cortical autografts present good osteoconductivity and provide immediate mechanical stability. The osteoinductive and osteogenic proprieties are lower expressed, because the cortical matrix is dense and present relatively slow revascularization and perfusion. The principal indication is in structural defects[64].

2.5.1.1.2. Cancellous Bone Graft

Cancellous bone grafts are the most common grafts applied for their rapid incorporation and remodelling. The primary indication is in small defects which require rapid healing, like arthrodesis and treatment of nonunions[65]. The large surface of porous trabeculae is linked with high osteoinductive and osteogenic abilities but low osteoconductive property. After implantation, the high porosity of graft and the presence of survival donor osteocytes, in combination with local cytokines, promote the mesenchymal stem cells recruitment. The

consequence is the differentiation of stem cells into osteoblasts and the deposition of new bone after a few weeks[66].

2.5.1.1.3. Corticocancellous Bone Graft

Corticocancellous bone grafts offer the advantages of both cortical and cancellous bone: the cortical bone provides a good osteoconductive ability and the cancellous bone excellent osteoinductive and osteogenic properties. Corticocancellous bone graft could be harvested with a vascular pedicle[67].

2.5.2 Allograft

The allograft is the term to define allogeneic bone graft, move from a donor to a recipient of the same species but genetically different[68]. Bone allografts are considered an alternative to autografts, indicated in the treatment of significant bone defects.

Allogenic bone grafts obtained from living donors or cadavers present the advantages of the availability and avoidance of morbidity related to harvesting. The excellent source is considered the multiorgan donor, thanks to the seroconversion which keep the transplanted bone safer with regard to disease transmission. Otherwise, bone from living donor records a percentage of rejections over 50% and bone from post-mortem donors records a higher risk of transmitting infections because of the lack of control.

Allografts obtained from cadavers have osteoinductive and osteoconductive proprieties but lack osteogenic properties because the preparation process leads to the absence of viable cells[69].

As a consequence of immunogenic and infection risks, the fresh grafts are not commonly used, but the process of frozen or freeze-dried has influences on the graft properties. Frozen bone is stored at -80°C, but the cryopreservation solves immunogenic problems maintaining adequate mechanical characteristics whereas freeze-dried bone is stored at room temperature, the irradiation process is not virucidal for the human immunodeficiency virus (HIV) and present minor mechanical resistance[70].

The possibility to machine and to customize the grafts makes them available in a variety of forms like DBM, cancellous chips, corticocancellous and cortical grafts and osteochondral segments[48]. Despite the advantages, allogenic bone grafts demonstrated a higher failure rate both for immunogenic and infective problems. The major histocompatibility complex (MHC)

activates itself during the initial phase of osteoinduction, causing the necrosis of osteo-progenitor cells[71]. The risk of viral transmission has been reported, even though the development of tissue banks and processing technology have improved the potential risk of contamination[72]. Cancellous allografts, because of their poor healing promoting ability, have been mainly applied as filler materials in cavitary skeletal defects[68]. Cortical allografts, thanks to their excellent mechanical properties, are applied where immediate loading-bearing resistance is required, for filling large bone defects[48]. DBM presents 40% of the mineral content of bone matrix and remaining collagens, non-collagenous proteins and growth factors. The presence of the growth factors stimulates osteoinductive properties and new bone formation, permitting an incorporation mechanism similar to autogenous grafts[73]. The lower mechanical resistance imparts the application of DBM for filling bone defects[74].

2.5.3 Isograft

Isograft is a term used to identify a graft procedure between individual developed from one zygote (monozygotic twin). The bone graft is moved from the first monozygotic twin, donor, to the second monozygotic twin, recipient.

2.5.4 Xenograft

Xenograft is a term used to identify a graft moved from a donor of a species to a recipient of a different species.

The antigenicity of xenograft is significantly higher, compared to isografts or allografts, because derived from a non-human species. Therefore, the processes of sterilization required have a consequence in mechanical resistance and could result in a minor osteoinductive ability and bioabsorbability. Nonetheless, xenografts bone are suitable in orthopaedic surgery, in particular, bovine based bone, because they are available and not expensive.

The bovine-based xenografts are made of calcium hydroxyapatite (HA, $Ca_5(PO_4)_3(OH)$) and present a chemical and morphological porous structure similar to human bone, but the high temperature (>1000°C) destructs the structure augmented the frailty. A limited number of studies evaluate the incorporation of the bovine grafts, in animal models, they show no sign of biodegradation and low new bone formation, in human studies, the xenografts show proper clinical and radiological incorporation[75].

2.5.5 Synthetic bone graft substitutes

The synthetic bone substitute is a term to define different biomaterials which can provide provisional mechanical support and new bone formation once implanted in the host.
Among bone grafts, the autologous bone graft is still considered the gold standard despite the disadvantages illustrated before. To overcome these limitations, during the past decades, bioengineering and regenerative medicine developed synthetic substitutes[76].
Indeed, an ideal bone substitute should have specific characteristics, both biological and mechanical, which can influence bone regeneration. From a biological point of view, substitutes should be biocompatible and resorbable (before replacement by new bone), cost-effective and easy to use[77]. From a mechanical point of view, porosity and pore size should ensure adequate vascularization, nutrients' supply and cells migration, mechanical flexibility and compressive strength should guarantee the distribution of loading forces by surrounding tissues[78].
Currently, synthetic biomaterials could be classified according to their composition in metal-based, ceramic-based and polymeric-based[79].

2.5.5.1 Metal-based bone graft substitutes

Although metals were considered non-bioactive material with no osteoinductive or osteoconductive properties, in the last decades several papers demonstrated the bioactivity of certain metals like titanium. The surface of metals, indeed, could interact with the environment and stimulate protein adhesion and cell interaction.
Nevertheless, the metal-based substitutes should be limited to small bone defects due to the high modulus of elasticity, compare to bone's modulus of elasticity, which could lead to fatigue fracture under cyclic loading[80].
Beside the surface's treatment of metal, a further concept is the impregnation of scaffolds with metal ions, which have a role in osteogenic, osteoblastic and osteoclastic differentiation naturally[81].

2.5.5.2 Ceramic-based bone substitutes

Bioactive glasses were introduced in the 1970s by Larry Hench as a group of synthetic silicate-based ceramics, constituted by silicon dioxide (SiO_2), sodium oxide (Na_2O), calcium oxide

(CaO) and phosphorus coxide (P_2O_5)[82].They are wildly applied as bone substitutes because they show excellent osteoinductive and osteogenetic properties. When the bioactive glass is implanted in the host site, a layer of bone-like apatite is formed on its surface promoting the cells adhesion, differentiation, proliferation and new bone formation[83]. Despite the bioactivity the bioactive glasses present low mechanical strength and fracture toughness which restrict their applications. To overcome this disadvantage, novel methods of fabrication, e.g. thermal treatment, and combination with polymer, were introduced.

Bioinert ceramics, like aluminium oxide (Al_2O_3) and zirconium dioxide (ZrO_2), present excellent mechanical properties (high strength, elastic moduli similar to human cortical bone, wear resistance) which make them useful for manufacturing of prosthetic bearings. Although the bioinert ceramics present good biocompatibility they are not applicated as bone substitutes because they do not present bioactivity. Therefore, calcium phosphate bioceramics, with a structure similar to the mineral phase of bone, were introduced[84].

Calcium phosphate ceramics are constituted by hydroxyapatites (HA), which possesses similar composition to the mineralized part of the bone, and can be applied in different ratios: hydroxyapatite alone (HA), tricalcium phosphate (TCP) and biphasic calcium phosphate (BCP).

In regenerative medicine, hydroxyapatite was firstly used for grafting in the 1950s[85]. Hidroxyapatite Ca10(PO4)6(OH)2 is wildly applicated as a bone substitute because of its similar mechanical and chemical structure with natural bone.

In the host site, HA shows a behaviour similar to cancellous bone, weak under tension but resistant to compressive loads. After several months the resistance to compressive loads decreases by 30-40%[86]. HA is a stable and low soluble calcium phosphate ceramics with variable mechanical properties. The bending, compressive and tensile strength usually are in the respective ranges of 38-250 MPa, 120-150 MPa and 38-300MPa. The modulus of elasticity varies from 35 to 120 GPa, and the hardness is 3-7 GPa. The normal ranges are depending on porosity, density, crystal size and other characteristics which can be influenced by the microstructure and sintering ability. Densely sintered scaffolds present fine grains and show higher toughness compared to sintered with larger grains[87]. A shows good biocompatibility, osteoconductivity and osteoinductivity thanks to the presence of calcium and phosphate ions and the interaction between apatite and tissues. When HA is implanted, an organic layer, constituted by carbonated calcium apatite, interacts with bone-forming chemical bonds. The phenomena lead to the initial stabilization of the substitute, cells adhesion, proliferation and differentiation. The progressive reabsorption of HA allows the deposition of bone.

The osteoinduction and biodegradation of HA depend on the surface area, microporosity, topography and surrounding biofluids. The dissolution is a process mediated by osteoclast cells and macrophages, and it is inversely proportional to Ca/P ratio and crystallinity. Usually, HA is more stable than tricalcium phosphate, in a study on HA cylinders implanted in rabbits' bone, only 5,4% volume reduction was observed after six months. In contrast, tricalcium phosphate had a reduction of 85,4%. The presence of the remaining HA reduces the formation of new bone, and the consequence was a lower strength of bone at the implant site due to the decrease of mechanical properties[86]. Therefore, HA alone is more applied as implants coating than the bone scaffold.

Recently, to overcome this issue, nanocrystalline HA has been developed. Nanocrystalline HA shows improved sinterability and improved mechanical properties due to a greater surface area. The main advantages of a higher surface are the possibility to be sintered at low temperature, reducing issues associated with high-temperature sintering, and the possibility to control characteristics features, enhancing mechanical and biological performances. Nevertheless, the higher surface increases the resorption rate, but in clinical observation, it is not noticeable[88].

Tricalcium phosphate Ca3(PO4)2 was first studied by Fred H. Albee in 1920s as a stimulus to bone growth[89]. The rhombohedral form, β-TCP, is wildly applied for scaffold materials because of its availability and ease of sterilization. The material is biocompatible and demonstrates a right balance among absorption and degradation due to the Ca/P ratio lower than hydroxyapatite[90]. TCP scaffolds, compared to HA, present more interconnected porous structures which can promote endothelial invasion and bone replacement at the expense of mechanical properties[91] TCP is usefull for filling bone defects but present an unpredictable biodegradation profile because TCP is partially degraded. After the implantation, indeed, a portion of TCP is converted in HA and, despite TCP is resorbed by phagocytosis, the remaining part of HA is not degraded for years[92].

Biphasic calcium phosphate (BCP) is a macroporous ceramic obtained from the association between HA e TCP in different concentrations. The formulation can be adjusted to enhance dissolution rate and mechanical properties; in this way, BCP combines the advantages of HA and TCP and can be used as a scaffold or a coating. [32]

Although the bioactive ceramics have shown excellent biological and mechanical properties, their widespread is still limited by their low fracture toughness and inherent brittleness.

2.5.5.3 Polymer-based bone substitutes

Synthetic polymers have been investigated and wildly applied for design of biodegradable scaffolds for bone regeneration. The polymeric materials, compared to other synthetic materials, present some advantages because of their biocompatibility and the possibility to control the degradation rate allowing the substitution of the scaffold with new bone. Furthermore, their composition can be precisely controlled through chemical or mechanical modification during the fabrication of scaffolds[93]. Most synthetic polymers are produced via condensation (step-growth polymerization) or addition (chain-growth polymerization) and are hydrolytically degraded.

Polyglycolide (PGA) was one of the first polymer used in tissue regeneration thanks to the excellent biocompatibility and mechanical properties and surface characteristics which favour the cell viability.

Polylactide exists in four distinct polymers derive from two stereoisomeric forms of lactic acid (L-lactide and D-lactide). Poly-L lactide (PLLA) has been applicated in orthopaedic and dentistry to fix devices, e.g. in ligament reconstruction or alignment of bone fragments. On the one hand present excellent mechanical strength, reasonable degradation rates and non-toxic degradation products, on the other the surface do not aid cell adhesion and proliferation.

Food and Drug Administration (FDA) approved poly lactide-co-glycolide (PLGA) in human and, thanks to the easiness of fabrication, it can be commercialised in different shapes. PLGA possesses excellent osteoinductive properties even if the acid accumulation derived from degradation can cause an inflammatory response in the host[94].

Polycaprolactone (PCL) and polyvinyl phosphonic co-acrylic acid (PVPA) copolymer present excellent osteoconductive properties, presenting a significant increase in bone fill percentage compare to sole PCL. The increase is linked with the presence of calcium and phosphorus, which lead to the formation of the extracellular matrix around the defect, favouring bone healing[80].

2.5.5.4 Composite materials

Composite materials consist of two or more constituents which are combined in order to produce a different material with different properties. Among composite materials, the research has been focused on collagenated synthetic biomaterials and bioactive composite scaffold.

Despite different synthetic bone substitutes have been developed and clinically applied, regenerative properties are restricted because the synthetic biomaterials do not have osteoinductive properties[32]. To overcome this issue, bioactive agents such as collagen, hyaluronic acid or osteoinductive molecules were added to synthetic materials.

Collagen, extracted from an animal source, or recombinant collagen could be added to ceramic, synthetic bone substitutes and can be fabricated as three-dimensional scaffolds or hydrogel.

The three-dimensional scaffolds can be prepared by precipitating calcium phosphate powder into a collagen solution, followed by a cross-linking and lyophilization process, or by precipitating calcium and phosphate precursors on a cross-linking and then lyophilizing collagen material.

Several clinical trials were performed on patients showing a healing rate comparable with autogenous bone. In the dentistry field, the application of composite materials in the regeneration of periodontal bone defects shows clinical and radiological bone augmentation[95].

Bioactive composites scaffold are bioactive glasses with a polymer matrix which combine the mechanical properties of bioglass with the flexibility of polymers. The scaffolds are obtained by a dipping method which is the immersion of the bioactive glass in a polymer solution. In this way, the polymer displays a coating on the ceramic scaffold, creating a structure similar to bone with higher toughness and compressive strength compared to ceramics alone[96]. Despite the results, the composite materials still present some critical issues such as the mechanical, which should be improved[97].

Chapter 3

Microcomputed tomography

3.1 Introduction on microcomputed tomography (micro CT)

Micro-computed tomography (micro CT), through the use of x-rays, provides high resolution three-dimensional (3D) images preserving the original specimen. Micro CT scanners capture and process a series of two-dimensional (2D) trans-axial projections, denominated "slices", into a 3D representation.

The non-destructive imaging acquisition, compared to other techniques, e.g. ultrasound or magnetic resonance imaging, provided a superior resolution of a small-scale sample microstructure's and compared to routine histology do not require any preparation, staining or slicing of specimens[98,99].

3.2 Historical context

The first micro CT system, inspired by clinical computer-assisted tomography, was developed in the 1980s by Lee Feldkamp. Feldkamp cooperating with Michael Kleerekoper worked on the scientific use of micro CT to scan bone tissues[100]. Steven Goldstein named the technique "microcomputed tomography" and with the collaboration of Feldkamp and Kleerekoper published the first article on the micro CT analysis of bone architecture. Through the years, the use of micro CT became fundamental in the fields of bone biology and biomechanics[101].

3.3 Physical principles of micro CT

Micro CT scanner consists of four main components: micro-focus X-ray tube (synchrotron emitter), collimator, specimen stand, and charge-coupled device (CCD) camera (Figure 7). The

synchrotron emitter produces a monochromatic beam which is collimated to either a fan or cone-beam projection. The radiation emitted passes through the specimen and hits the CCD camera covered by a phosphor coating which converts x-rays to visible light.
Through the rotation of the sample or the emitter and detector, the scanner acquires images at different angles of rotation and reconstructs a 3D rendering[102].

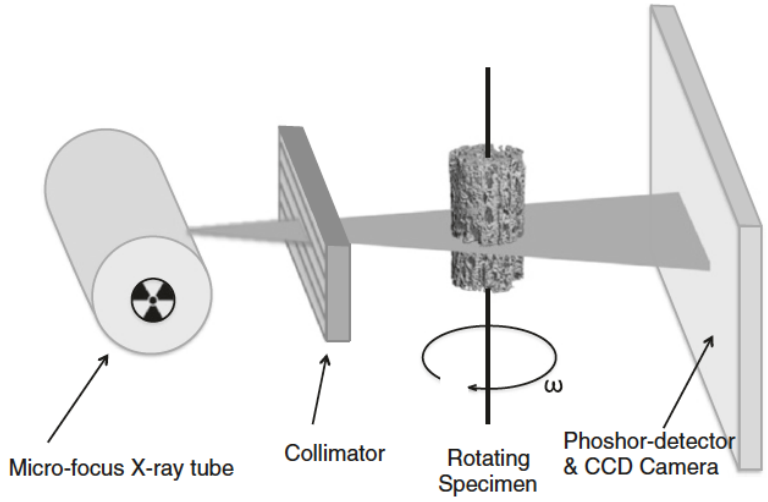


Fig. 7 Schematic representation of micro CT scanner (from Boerckel et al.[102])

The images produced by scanner principally derive from the physical x-ray interaction with matter: attenuation, photoelectric absorption and Compton effect[103].

3.3.1 Attenuation

The x-ray attenuation is based on the probability of x-rays to interact with atoms, according to the equation $Ix = I0\ e^{-\mu x}$: where Ix represents the intensity of the beam at a certain distance, x, from the source, I0 is considered the intensity of the incident beam and μ is the linear attenuation coefficient. Conforming to the theory, the sample characteristics (thickness and material) and the source of energy could exponentially decrease the intensity of the beam; therefore the attenuation could be used to quantify the density of the analysed tissue.

3.3.2 Photoelectric effect

The photoelectric effect describes the interaction of a low energy photon with an electron. The electron is removed from the atomic shell, and the incident photon is completely absorbed. To stabilize the atom, another electron is emitted as an x-ray photon (figure 8).

The photoelectric absorption is related to the atomic number (Z) of the absorbing element and to the energy (E) of the incident photon therefore if Z increase the photoelectric effect increase if E increases the photoelectric effect decrease.

The theory described is favourably applied in micro CT where the use of samples with higher Z (e.g. bone, containing Ca++) and the application of a lower x-ray energy guaranty a superior imaging result.

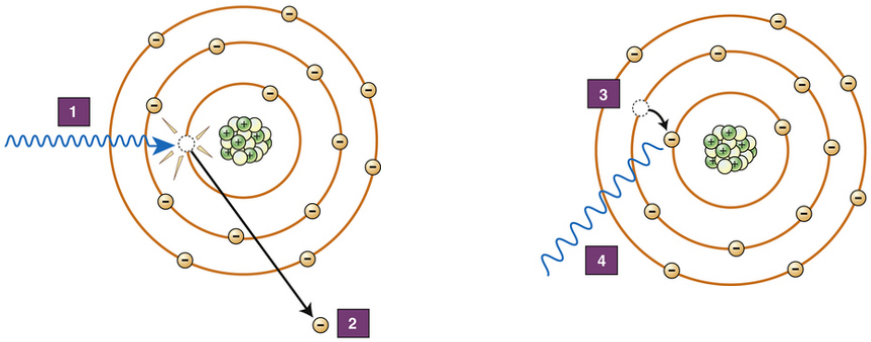


Fig. 8 Photoelectric effect 1-2. Incident photon interacts whit an electron in an inner orbital: the photon is absorbed and gives all its energy to the electron which is ejected (photoelectron). 3-4. An electron fills the void in orbit, producing a secondary photon. (Modified from Cittadini et al.[104])

3.3.3 Compton effect

The Compton effect is the consequence of the interaction with photons which are not absorbed but lose energy during the interaction. The scattering photons change direction, and an electron (secondary electron) is ejected, causing scattering artefacts (Figure 9).

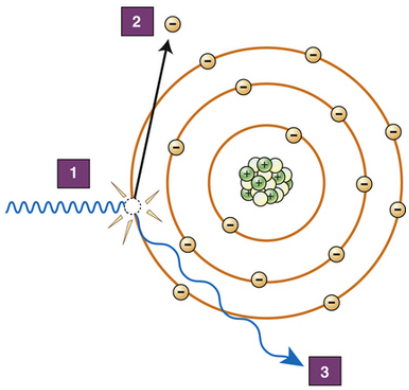


Fig.9 Compton effect 1-2. Incident photon interacts with an electron in an outer orbital, the electron receives part of its energy and is ejected (Compton electron). 3. Incident

photon continues on an altered path, scattered, with less energy. (Modified from Cittadini et al.[104])

3.4 Micro CT image formation

The formation of a CT image could be distinguished in three phases: scanning, reconstruction and conversion from digital to analogue.
In the first phase the beam throughs the specimen, as prior described, and is measured by the detector.
In the second phase, for any slices acquired and processed a digital image (a matrix of pixels) is produced. The reconstruction method is denominated "filtered back production", referring to digital image processing algorithms used to improve image quality. During the reconstruction, the slices of a specimen are divided into a matrix of voxel (volume elements) and the CT number is calculated for each pixel. The CT number derives from x-ray linear attenuation coefficient value, which is depending on density, on Z of materials and on E of photons. Therefore, CT numbers are strictly related and determined by the density of tissues.
In the final phase, the digital image is converted into a visible analogue image, represented by different shades of grey or brightness level[105].

3.5 Artefacts in micro-CT

Artefacts in micro-CT derive from dissimilarities between the mathematical modelling and the physical imaging process. An artefact is defined as a detected structure in the acquired data which is not present in the object investigated. The artefacts derive from the discrepancy between the physical conditions of the measuring set-up and the simplified mathematical models used for 3D reconstruction. The principal artefacts are: noise, ring artefacts and beam hardening[106].

3.5.1 Noise

The noise could be described as an unwanted change in pixel values in a homogenous image. In micro CT noise depends on the small size of the x-ray source and limits the x-ray flux and the maximum source power. Associated with noise an artefact which could affect the

acquisition is the "quantum mottle", a consequence of the stochastic distribution of photons striking the elements[107].
In general, the noise is measured via the signal to noise ratio (SNR) and could be reduced by increasing the signal, increasing the dose of the scan or decreasing the thickness of slices.

3.5.2 Ring artefacts

Ring artefacts derive from the continuous rotation of the object-camera and from a not uniform x-ray detection. Usually, samples with a high density are affected by ring artefacts, which could be removed through the correction of the "flat-field" to obtain a uniform image background[108].

3.5.3 Beam hardening

Beam hardening is the result of a mixture of low and high energy x-ray. The polychromatic beam passes through the sample, the low energy photons are removed, and the remaining photons present higher energy creating a “hard” beam. The effects can be reduced, but not entirely removed, by placing a filter (e.g. a thin aluminium foil) or correcting the “flat field”[109,110].

3.6 Applications of micro CT to bone scaffolds analysis

Micro CT could be considered a standard tool in the field of bone tissue engineering. Thanks to its non-destructively modality, rapidly 3D images processing and a high degree of accuracy, it has been employed to quantify the bone scaffold geometry, neovascularization and newly formed bone.
Several works describe the application of micro CT to evaluate morphology and topology of scaffolds, in particular the ability to quantify bone thickness and to obtain a pore size distribution 3D thickness map[111,112].
Among studies on bone scaffolds, the angiogenesis has been evaluated; after injecting blood vessels with barium sulfate, micro CT performed a 3D imaging of microvasculature[113,114].
In longitudinal studies of bone ingrowth micro CT, through a spatial resolution between 5-40 μm to evaluate scaffolds with pore size between 50-200 μm, unbiased quantifies the morphometry of newly formed bone on scaffolds, 3D imaging investigates the connectivity between bone ingrowth and the scaffold[115,116].

3.6.1 Bone morphometry

Quantitative morphometry uses morphometric indices to describe bone architecture.[117] The leading morphometric indices are: bone volume, total volume, bone volume fraction, cone surface, bone surface density, trabecular thickness, trabecular separation and trabecular number, as shown in table 9.

Tab 9 *Morphometric indices*

Parameter	Abbreviation	Description
Bone volume	BV	Volume of the region segmented as bone
Total volume	TV	The surface of the region segmented as bone
Bone volume fraction	BV/TV	The ratio of the segmented bone volume to the total volume of the region of interest
Bone surface	BS	The surface of the region segmented as bone
Bone surface density	BS/TV	The ratio of the segmented bone surface to the total volume of the region of interest
Trabecular thickness	Tb.Th	Mean thickness of trabeculae, assessed using direct 3D methods
Trabecular separation	Tb.Sp	Mean distance between trabeculae, assessed using direct 3D methods
Trabecular number	Tb.N	The measure of the average number of trabeculae per unit length

According to several studies, mechanical proprieties of a 3D structure could be described estimating the anisotropy of trabecular bone, expressed by the mean intercept length (MIL), the measurement of structural anisotropy[118]

A fundamental topologic measurement is used to characterize the redundancy of trabecular connections, expressed by the connectivity density (Conn.D), the measure of the degree of connectivity of trabeculae normalized by TV.[119]

Chapter 4

Bone regenerative potential of MSCs culture on a xenohybrid scaffold

4.1 Introduction

In regenerative medicine, the use of mesenchymal stem cells (MSCs) to promote bone healing has been widely investigated. Their popularity has grown up in particular for their easy acquisition (e.g. from adipose tissue): it is not surprising the increasing number of clinical trials that have tested infusion of such kind of cells to induce cartilage or bone regeneration with encouraging results over the years[5].

Furthermore, to restore significant bone defects, different grafts have been proposed with the purpose to generate new bone mimicking a physiological response: among bone grafts, a promising family of substitutes, denominated xenohybrid bone grafts (xenografts reinforced by polymers and containing collagen), seem to fulfil this request. Compared to ordinary xenografts, which feature a structure similar to human bone but which are partially destructed by the process of sterilization used to contrast the antigenicity, xenohybrid bone grafts present some advantages. Indeed, the addition of polymer confers a significant mechanical resistance and the presence of collagen augments the cell adhesion and osteoinductivity[32].

In the present experiment the non-cultured fraction of MSCs, obtained after collagenase treatment of adipose tissue, called *stromal vascular fraction* (SVF)[120] and *adipose tissue-derived stem cells* (ASCs)[121] , obtained from SVF, were cultured on xenohybrid bone grafts

The aim was to compare the osteogenic potential of ASC and SVF once cultured on grafts.

4.2 Materials and method

4.2.1 Bone scaffold

In the experiment discs of xenohybrid collagenated bone (Smartbone®, IBI S.A., Mezzonico-Vira, Switzerland) were used. Smartbone® (SB) is a new composite scaffold, made of a bovine-derived matrix reinforced with bioresorbable aliphatic polymers poly (l-lactic-co-ε-caprolactone) and RGD-containing collagen fragments (extract from purified bovine gelatin). The substitute combines the excellent biocompatibility and osteointegration of the xenografts with the mechanical properties of synthetic materials close to the human bone ones. The bovine-derived matrix is made of calcium hydroxyapatite (HA, $Ca_5(PO_4)_3(OH)$, similar to human bone but with a more fragile structure; furthermore its porous structure is partially destroyed by the sterilization process, reducing cells adhesion. The addition of a polymeric coating reduces the fragility, and the addiction of RGD-containing collagen fragments increases the hydrophilicity of the scaffolds improving cell adhesion[122].

SB, before commercial release, was studied in vitro with SAOS-2 and MG-63 cells, in an animal model, passing biocompatibility tests performed according to ISO 10993 specifications, and lastly in human[122].

SB discs (7x3mm) were washed with saline buffer solution and kept in a minimum essential medium (Alpha MEM, Voden Medical Instruments SPA, Meda, Italy) to improve hydrophilicity and cell adhesion.

4.2.2 Cell cultures

SVF derived from the enzymatic treatment of the fresh adipose tissue obtained from lipoaspirates. The lipoaspirates derived from 7 patients, who consent the treatment according to the approval of the Ethical Committee[123]. The lipoaspirates were treated with Collagenase NB4 (SERVA Electrophoresis, Heidelberg, Germany), then after washes with saline solution, a cell lysis solution (Promega, Milan, Italy) was applied to obtain SVF.

ASCs were obtained from the seed in T25 flasks and culture of SVF in a basal medium (Dulbecco's Modified Eagle Medium, DMEM, Sigma-Aldrich, St. Louis, Missouri, USA), with 1% antibiotics, 2mM glutamine and 10% fetal bovine serum, FBS, (Gibco, Thermo Fisher

Scientific, Waltham, USA); the medium was then replaced after 24 hours to eliminate the nonadherent cells.
SVF and ASCs were cultured on plastic discs and SB discs for 60 days in Alpha MEM or osteogenic medium (OM) containing Alpha MEM supplemented with FBS, ascorbic acid dexamethasone and beta-glycerophosphate (Sigma-Aldrich, St. Louis, Missouri, USA).
To monitor the osteoblasts differentiation, cellular cultures were stained for alkaline phosphatase (ALP), and the mineralization activity was evaluated through von Kossa staining.

4.2.3 Micro-CT analysis

Scaffold discs were analysed through micro-CT (SkyScan 1172, Bruker, Billerica, USA) before and after SVF and ASC colonization. The Intensity of 80kV was used to keep acquisitions with a 0,4° rotation step, 360° scan at 4x frame averaging. An aluminum filter of 0,5 mm was applied at a resolution of 6 μm. NRecon software (Bruker) reconstructs datasets, and DataViewer software (Bruker) measures the amount of mineralized tissue. To allow the identification of mineralized tissue was used a colour contrast mask.

4.2.4 Scanning Electron Microscopy (SEM) analysis

The *scanning electron microscope* (SEM) is an electron microscope which use a beam of electrons to scan the surface of samples. The interaction between electrons and surface produce different signals which contain information on the topography of specimens. SEM (EVO, 50EP Instrumentation, Zeiss, Oberkochen, Germany) was used to scan the surface and obtain data on the composition of SB seeded discs. Samples were fixed in glutaraldehyde solution and evaluated at 15, 30 and 60 days, both the external surface and the inner surface.

4.2.5 Histochemical analysis

SB samples were fixed in a neutral buffer with 4% formaldehyde, washed and decalcified. Discs were then dehydrated, embedded in paraffin and treated with a paraffin removal (EZ Prep, Ventana Medical System, Inc., Hoffmann-La Roche, Basilea, Switzerland) before immunohistochemical analysis was performed. The sections were incubated with mouse monoclonal antibody: COLL-1 (ab34710, at 1:400 dilution), OCN (ab93876, at dilution 1:250) and TGFβ (ab92486, at dilution 1:150). The addition of a biotin-free system (ultraView

Universal DAB Detection kit, Ventana Medical System, Inc., Hoffmann-La Roche, Basilea, Switzerland) inhibited the endogenous peroxidase activity. Samples were counter-stained with Mayer's hematoxylin solution, set with Kaiser's glycerol gelatin and processed. Slides were analysed in a double-blind and photographed with a microscope (Olympus BX51) equipped with a camera (Nikon DCS E995).

4.2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

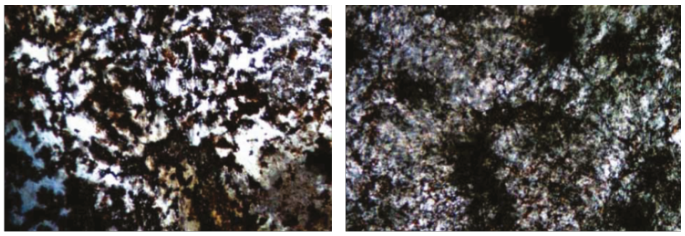
The ELISA is a technique used to detect, through antibodies, and quantify specific proteins in samples where a mixture of proteins is present.
To quantify the osteogenic properties of ASCs and SVF growth on SB, the expression of endothelin-1 (ET-1) and Vascular Endothelial growth factor (VEGF) in cell culture supernatants was determined through a Quantikine ELISA kit (R&D Systems, Minneapolis, USA). Supernatants samples were evaluated at 4, 15, 30 and 60 days of culture and data were expressed as mean values.

4.2.7 Statistical analysis

GraphPad Prism 4 (Graphpad Software Inc., La Jolla, USA) was used to perform statistical analysis. Data were collected like means and standard error and were analysed by one-way ANOVA through Bonferroni's multiple comparison tests. To assess significant differences in the formation of new bone, 150 samples were evaluated, considering significant a p-value minor than 0.05.

4.3 Results

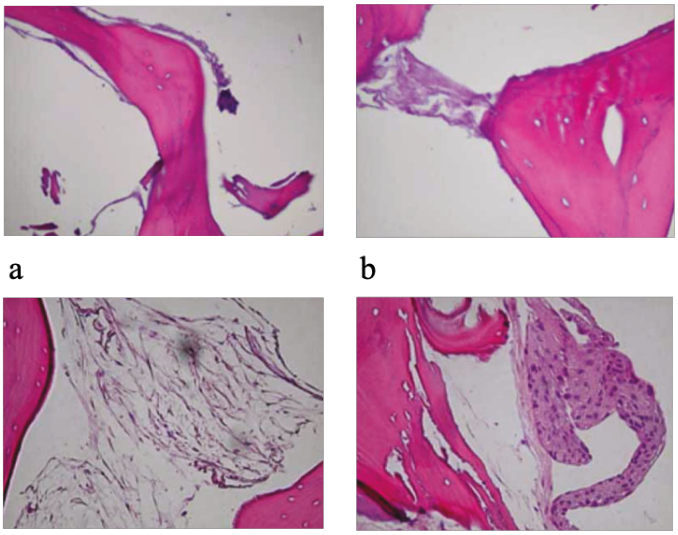
In vitro, ASCs and SVF display the ability to differentiate in osteoblasts towards the expression of ALP. Both cellular lines were cultured on plastic discs in absence or presence of OM for 60 days showing different results. In the control groups, in Alpha MEM without OM, ASCs did not produce ALP whereas SVF were ALP-positive. In OM both ASCs and SVF were ALP-positive.
Von Kossa staining was applied to monitor the mineralization activity: in the absence of OM, both ASCs and SVF did not mineralize while they did in the presence of OM (Fig.10).



a b

Fig.10 Mineralization activity evaluated through von Kossa staining. Both in ASCs (A) and SVF (B), at a magnification of 5x, the images show the formation of nodules in osteogenic medium (OM).

ASCs and SVF were also cultured on SB discs showing the ability to colonise the material and generate new bone. The new tissue formation was evaluated at 15, 30 and 60 days in cells cultured with or without OM (fig.11).



a b

c d

Fig.11 H&E staining to detect colonization of SB by SVF. At 15 days of culture, the presence of new bone is evident both in osteogenic medium, OM (b) and in Alpha MEM (a). At 60 days of culture, the tissue is increased both in SVF culture in OM (d) and Alpha MEM (c).

To quantify the new bone formation, the specimens were evaluated through micro CT, which show a progressive increment of tissue from 15 to 60 days (fig 12, 13). The presence of OM did not influence osteogenesis; indeed, the new bone formation was also detected in the absence of OM, suggesting that SB owns the osteoinductive property. SVF cultured on SB with OM were significantly more osteogenic then ASCs cultured in the same conditions. Whereas in the

absence of OM, ASCs were significantly more efficient in bone formation compared to SVF cultured in the same conditions.

The osteogenesis was also evaluated through SEM analysis which corroborates the findings. (fig 14)

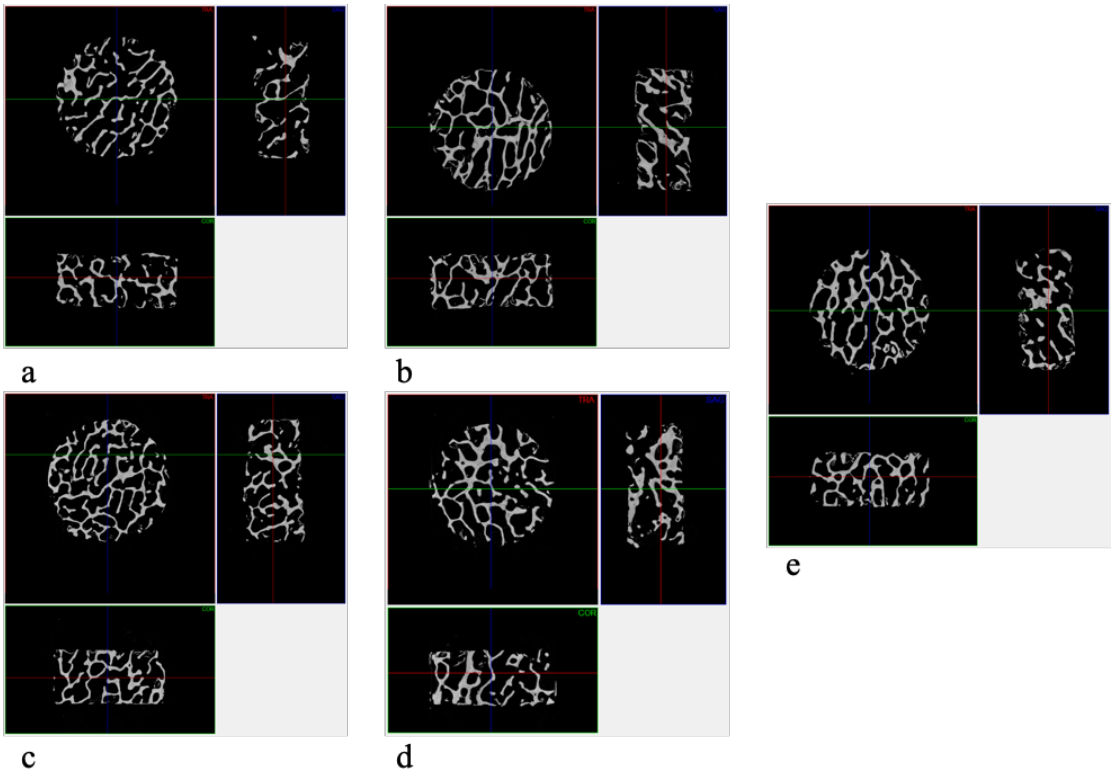


Fig.12 MicroCT images of new tissue at two different time points. A) ASCs formation at 15 days. B) ASCs at 60 days. C) SVF at 15 days. D) SVF at 60 days. E) Control at 60 days.

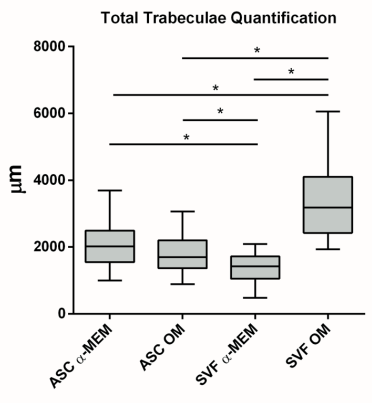


Fig. 13 Micro-CT quantification of newly formed bone. Expression of total trabeculae in ASC and SVF culture di AlphaMEM and osteogenic medium (OM). *p<0.05

The expression of osteoblasts was evaluated through markers and protein staining in different groups: SB, ASCs cultured on SB and SVF cultured on SB with or without OM.

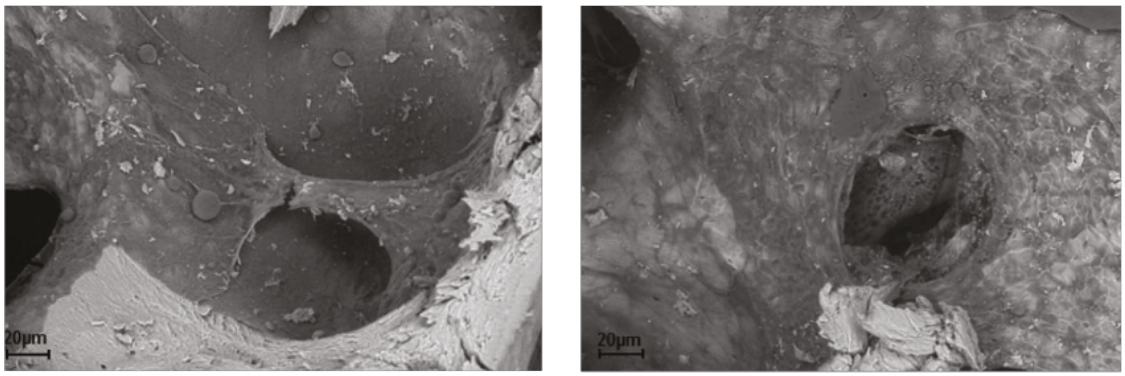


Fig.14 SEM analysis of newly formed bone. Image of SB disc after 30 days of culture respectively with ASCs (A) and SVF (B).

The expression of mature osteoblast markers (ALP, OCN and COLL-1) and osteogenic marker (Runx2) increase until a maximum expression at the 60th days both in ASCs and in SVF cultures.

COLL-1 was highly expressed in SVF cultures and weakly represented in ASCs cultures, while in the presence of OM, COLL-1 stained the newly formed bone.

The protein staining with ALP, osteocalcin (OCN) and TGFβ show a progressive increase from the 15th to the 60th days, while it was negative on SB alone. OCN was weakly express in the absence of OM, whereas TGFβ confirms the presence of new bone both in the presence and in the absence of OM.

To assess the osteogenic potential of cell culture supernatants, the secretion of Endothelin-1 and VEGF by ASCs and SVF growth on SB was evaluated through the ELISA technique. The level of ET-1 decreases over time, while VEGF is constant. Both ET-1 and VEGF were expressed with or without OM.

4.4 Discussion and conclusion

The study investigated the ability of MSCs, derived from adipose tissue, to generate new bone when cultured on a xenohybrid bone scaffold (SB)[124]. To elucidate any potential differences, two different types of cells were cultured: SVF, the non-cultured fraction of MSCs, and ASCs, derived from SVF. SVF contains either mesenchymal stem cells (ASCs) and other cells, e.g.

leukocytes, red blood cells and endothelial cells. According to the findings, the presence of ASCs confers the ability to SVF to osteodifferentiated, but the presence of other cells confers a better osteoinductive ability compare to ASCs itself.

Indeed, regarding the ability to differentiate in osteoblasts, on plastic discs, both ASCs and SVF were able to express ALP in OM, showing the ability to osteodifferentiate. Nonetheless, SVF expressed ALP also in the absence of OM, probably because of the presence of different cells. Although they were not positive at Von Kossa staining in the absence of OM, showing that the presence of a specific environment can influence the ability to mineralize.

Monitoring tissue growth on SB, it was reported an increase of new bone in cultures both with OM and without OM. The findings suggest that xenohybrid bone seems to have osteointegration ability by itself[125]. Moreover, the colonization and the growth of SB by ASCs and SVF confirmed its osteointegrative capability.

The osteoinductive property of SB and the new bone formation was also noticed by micro-CT and SEM analysis. With micro-CT, it was possible to quantify and compare the new tissue in ASCs and SVF cultures. According to the results, SVF shows a better osteogenic ability, in the presence of OM, compared to ASCs.

To evaluate, molecularly, the new bone formation osteogenic markers were detected. The expression of early osteogenic markers (Runx2) and mature osteoblast markers (ALP, OCN and COLL-1), as expected, increased over time, confirming the osteogenic ability of cells. The protein expression (COLL-1, OCN and TGFβ) confirmed that SVF, also in the absence of OM, were able to generate new tissue[126].

All the results suggest that SVF could be applied for regenerative medicine; indeed, they stimulate mesenchymal activity and the presence of other cells create a microenvironment fundamental to bone formation[127]. The interplay between different cells type was indagated by other studies that showed an interaction between mesenchymal cells and endothelial cells[128].

In a study on animal models, VEGF showed a role in osteoblast differentiation: scaffold seeded with mesenchymal cells, derived from bone marrow, released VEGF which stimulate new bone regeneration[129].

Therefore, in the experiment, VEGF and ET-1 were dosed, confirming an increased expression of VEGF and the osteogenic role of mesenchymal cells.

In conclusion, xenohybrid bone grafts are suitable for bone defects repairing; they are able to induce cellular colonization and growth. It has been noted that SVF has better osteoinductive abilities compare to ASCs.

Moreover, the absence of manipulation of SVF and the easy way to obtain them could represent a benefit for widespread applications.

Chapter five

Co-culture of MSCs and ECs on a xenohybrid bone graft

5.1 Introduction

The angiogenesis, the formation of new blood vessels from preexisting ones, is a fundamental stage for bone formation, modelling-remodelling process and fracture healing.

In bone remodelling, the necessity of resorption is sensed by osteocytes and mediated by osteoclasts. The adequate blood supply is essential to guarantee a gradual increase of mesenchymal cells, in particular of osteoclast precursors which reach the bone remodelling compartment throw the marrow capillaries[31].

In fracture healing, capillaries invade the granulation tissue delivering the mesenchymal cells (MSCs), which differentiate in osteoblasts. Osteoblast together with fibroblast initiates the healing process. Inhibition of angiogenesis can lead to a decrease in bone formation, resulting in a fracture non-union[130].

The angiogenesis also plays an essential role in the survival of bone grafts after implantation[8]. Indeed, the differentiation of osteoblasts from osteoprogenitor cells, during the osteoinduction process, is mediated by different growth factors. Through them, the vascular endothelial growth factors (VEGF) stimulates vascular development and can be applicated directly in injured tissue to promote bone healing.

Nonetheless, the mechanism of angiogenesis in bone formation, remodelling and bone graft implantation are still not elucidated[131].

According to literature, endothelial cells enhance the osteodifferentation of MSCs[132]; instead, MSCs promote survival of endothelial cells, in vitro and in vivo[133].

To understand any relations between endothelial cells and mesenchymal cells in bone formation, the co-culture of adipose tissue-derived stem cell (ASCs) and human microvascular endothelial cells (HMEC) was assessed, in the present experiment,

5.2 Material and methods

5.2.1 Cell culture

Human Microvascular Dermal Endothelial Cells (HMECs) together with *Adipose-Derived Stem Cells* (ASCs) were employed.
A culture media kit (EndoGRO-MV, Sigma-Aldrich) was used to cultivate HMECs combined with a broad-spectrum aminoglycoside antibiotic.
ASCs were obtained, after Local Independent Ethics Committee approval, from adipose tissue aspirated from patients treated for knee osteoarthritis[134].
The Lipoaspirates were firstly digested with NB4 collagenase and subsequently washed with saline solution and processed with a cell lysis solution to discard blood cells. The resulting ASCs were gathered, isolated and counted, and their purity was assessed through flow cytometry.

5.2.2 Osteogenic cell differentiation

ASCs were cultured in osteogenic medium combined with 10mM β-glycerophosphate, 50 μg/ml ascorbic acid and 0.02mg/mL dexamethasone to promote osteogenic differentiation.
Dexamethasone was subsequently removed to avoid the inhibitor behaviour on endothelial cells as previously reported in literature[135].

5.2.3 Co-cultures of HMECs with ASCs

For migration, tubulogenesis and proliferation experiments, inserts of 0,4μm pore polycarbonate membrane filter were used for co-culture two cellular type: ASCs ($2x10^4$ cells/ml) were seeded into 24-well culture inserts in the upper chamber, HMECs were seeded into 24-well plates in the bottom chamber.
For Real-time PCR analysis, HMECs were seeded into 6-well 0,4μm pore inserts and ASCs were seeded into 6-well plates.

5.2.4 Proliferation analysis

The cell proliferation was investigated counting cells, disposed in 24-well culture dishes with a density of 2500 cells/well, using Celltilter-glo® (Promega, Milan, Italy) at first, third and seventh day according to manufacturer's instructions. The number of viable cells in the culture is determined by quantifying the amount of the ATP present, that is directly proportional to the number of cells.

5.2.5 Migration assay and chemotaxis analysis

Cell motility into a wound was assessed using a motorized inverted microscope (Nikon Eclipse Ti-E) with 4x magnification and acquisition obtained by software (Metamorph, molecular devices, Sunnyvale, California, USA) and expressed as cell migration ratio[136]. Throughout the experiment, cells were kept at a constant temperature of 37°C with 5% of CO_2. Three independent analysis for each field was performed.
Total migrated cells were counted after using a cotton swab to remove the non-migrated ones. HMECs were seeded into 8μm pore polycarbonate membrane filter and maintained into culture media kit (EndoGRO-MV, Sigma-Aldrich) and subsequently inserted into 24-well plate with ASCs maintained in growth medium or osteogenic medium. Paraformaldehyde was used to fix, and 4′,6-diamidino-2-phenylindole (DAPI) was used to stain inserts after 4hours.

5.2.6 Angiogenesis assay

Angiogenesis Analyzer for ImageJ on gelatinous protein mixture (Matrigel, Corning, Tewksbury, USA) assessed formation of capillary-like structures. It was analysed through an inverted microscope (Nikon Eclipse Ti-E) with 10x magnification. Three independent analysis for each field was performed.

5.2.7 RNA extraction and Real-time PCR analysis

Total RNA was isolated using a column-based kit (Purelink RNA mini kit, Ambion, Life Technologies, Italy). The *quantitative real-time polymerase chain reaction* (qRT-PCR) was performed through high-capacity cDNA reverse transcription kit (Multiscribe® reverse transcriptase, Thermo Fisher Scientific, Waltham, USA) transcribing 1μg of total RNA in complementary DNA. Subsequently, real-time PCR analysis was performed using hydrolysis

probes on a sequence detection system (7900ht fast Real-time PCR system, Applied Biosystem, Life Technologies, Italy)[137].

Universal Probe Library - Assay Design Center (Roche life science software) was used to design specific primers and probes. The mRNA expression of the following genes was tested:

ANGPT1 (Angiopoietin 1)

ANGPT2 (Angiopoietin 2)

VEGF-A (Vascular Endothelial Growth Factor A)

PDGF-B (Platelet-Derived Growth Factor Subunit B)

TGFB1 (Transforming Growth Factor β 1)

FGF2 (Fibroblast Growth Factor 2)

BMP2 (Bone Morphogenetic Protein 2)

SPP1 (Osteopontin)

5.2.8 Bioreactor and scaffolds

Xenohybrid collagenated bone graft (Smartbone®, IBI S.A., Mezzonico-Vira, Switzerland) was used as a scaffold, and ASCs and HMECs were simultaneously placed in co-culture condition. A bioreactor (Livebox2 bioreactor, IVTech, Italy) was used to support proper cell growth on bone scaffold[138]. A controlled environment was maintained for bioreactor performance: a humidified atmosphere of 5% CO_2, temperature of 37°C, using 0,5 ml/min flux.

5.2.9 Micro-CT analysis

The new bone deposition was assessed using high-resolution X-ray microtomography (Skyscan 1172, Bruker, Billerica, USA). The qualitative analysis was performed using a colour contrast mask that permits identification of newly formed mineralized tissue. The quantitative analysis was performed calculating the mineralized tissue length through measuring software (DataViewer, Bruker, Billerica, USA).

5.2.10 Statistical analysis

All statistical analysis was performed with GraphPad Prism 7 (Graphpad software Inc., La Jolla, USA) and Microsoft Excel (Microsoft, Redmond, USA) with the exception of angiogenesis images analysed by Angiogenesis analyzer plugin of ImageJ (Wayne, Rasband, NIH, USA).

Mann-Whitney non-parametric test or two-way Anova with Tukey's multiple comparison test was performed. A p-value of <0.05 was considered significant.
Ordinary two-way ANOVA with Sidak's multiple comparison test for grouped analyses or Mann-Whitney test for column analyses were performed to assess qRT-PCR data.

5.3 Results

The osteodifferentiation ability of ASCs was evaluated through the cell culture in OM and GM. After that period, real-time PCR analysis shows the expression of Collagen type 1 and Runx2 in ASCs culture in OM compared to ASCs culture in GM.
After seven days of culture in OM, osteodifferentiating ASCs express a higher level of ALP compared to undifferentiated ASCs.
The effect of osteodifferentiating ASCs on HMECs was assessed after 24 hours of co-culturing. The proliferation of HMECs, evaluated through the quantitation of ATP and cell count, was higher in the presence of ASCs compared to the control group.
The migration and chemotaxis assays show that, after 8 hours from an introduced wound, the migration rate of HMECs was higher in the presence of osteodifferentiating ASCs and the chemotaxis assay confirm the ability of ASCs to enrolled endothelial cells compared to undifferentiated ASCs.
Significantly, HMECs show better migratory abilities in the presence of osteodifferentiating ASCs.
Angiogenesis was evaluated in vitro on Matrigel coating after 8 hours in different conditions: in co-culture with osteodifferentiating ASCs, in co-cultured with undifferentiated ASCs and in HMECs without ASCs (Fig. 15). The new vascular endothelium was estimated in terms of the number of segments, master junctions and nodes, measuring the capillarity of the network. HMECs co-cultured in osteodifferentiating ASCs present a more complex network of capillaries compared to other samples.

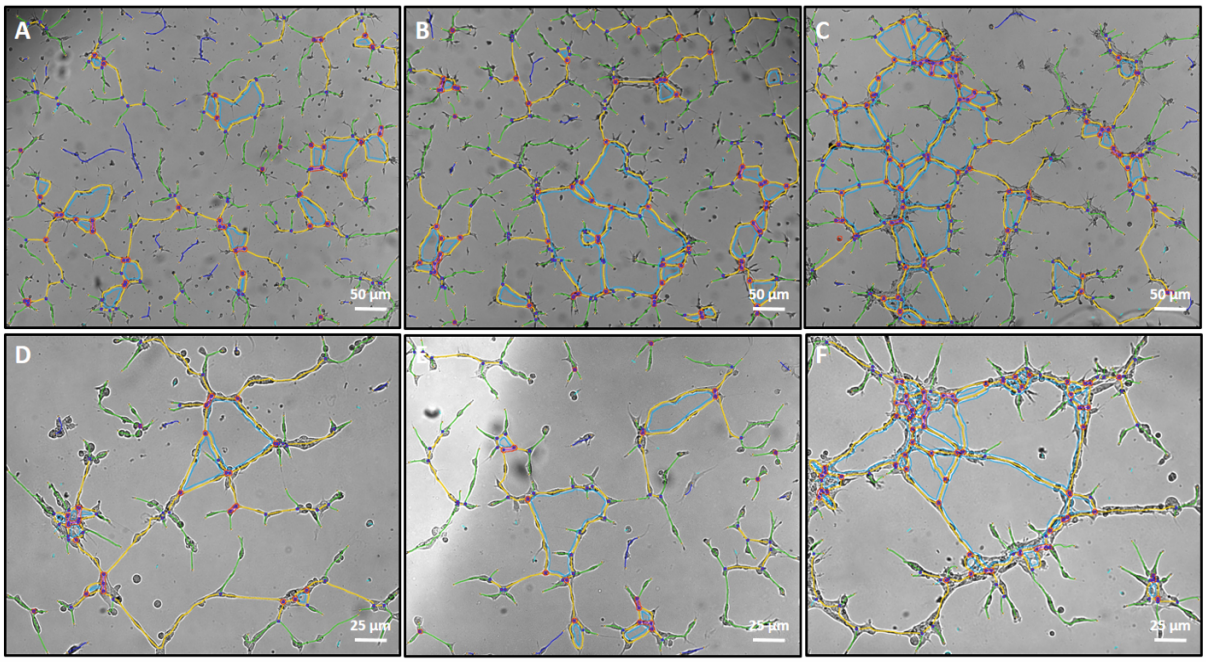


Fig 15 In vitro angiogenesis. The formation of capillary-like structures on Matrigel coating was evaluated after 8 hours through Angiogensis Analyzer for ImageJ. (A,D) Assays performed on control group (HMEC alone), (B,E) assays performed on HMEC co-cultured with undifferentiated ASCs, (C,F) assays performed on HMEC co-cultured with osteodifferentiating ASCs.

The Real-time PCR was used to study the expression of pro-angiogenic factors released by osteodifferentiating and undifferentiated ASCs. The results indicate that the expression of ANG-1, ANG-2, VEGF, PDGFβ, TGFβ and FGF-2 is higher in osteodifferentiating ASCs. The gene expression of the same factors was also higher in HMECs co-cultured with osteodifferentiating ASCs compared to the basal condition without HMECs.

To evaluate the interaction of HMECs and ASCs in the process of osteodifferentiation, the gene-expression of BMP2 and OPN were analysed. The expression of osteogenic factors increased in HMECs co-cultured with osteodifferentiating ASCs.

The bone production was studied through the co-cultured of HMECs and ASCs on bone scaffolds. The scaffold was kept in a perfused bioreactor applied to obtain a state with specific characteristics, similar to the physiological environment. After one month of culture, bone scaffolds were analysed through micro-CT to quantify new bone formation (Fig. 16). The findings demonstrated that HMECs co-cultured with ASCs on scaffold increased the new bone production (Fig. 17).

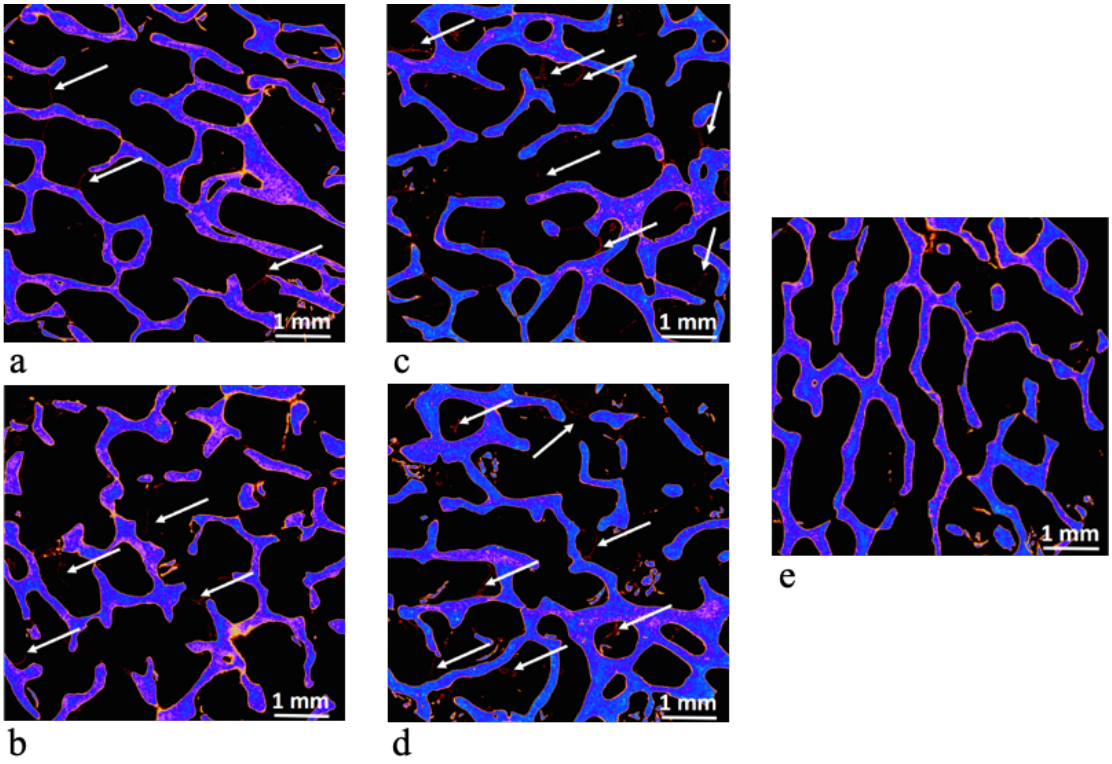


Fig 16 Micro-CT analysis. Quantification of the newly formed mineralized tissue (in red) on bone scaffolds (in blue). A) ASCs cultured in growth medium (GM). B) ASCs cultured in osteogenic medium (OM). C. HMEC co-cultured with ASCs in growth medium. D) HMEC co-cultured with ASCs in osteogenic medium. E) Control group, HMEC cultured alone.

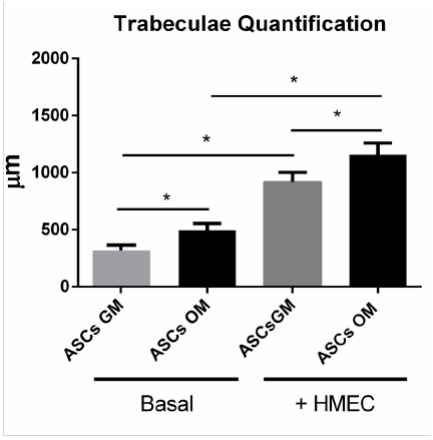


Fig 17 Micro-CT quantitative analysis. The histogram represents the newly formed trabeculae after one month of culture. The bars show an increase in new bone formation in group of HMEC coculture with ASCs both in growth medium (ASCsGM+HMEC) and in osteogenic medium (ASCsOM+HMEC). *p< 0,05

5.4 Discussion and conclusion

In the previous experiment, it was demonstrated the crucial role of mesenchymal stem cells in bone regeneration. In the present experiment, the coculture of endothelial cells and adipose tissue-derived stem cells was indagated to understand the complex cellular cross-talk and to analyse any possible influences on new bone formation.

The advantage of the use of ASCs in regenerative medicine was reported in the literature: a study in mice proved a more efficient treatment of bone defect with ceramic scaffolds, containing ASCs and bone marrow stromal cells, compared to scaffolds with ASCs alone[139].

According to the results, after seven days of culture in OM, ASCs express a higher level of ALP, showing a better ability to osteodifferentiate, compared to ASCs cultured in GM.

HMEC were co-cultured with osteodifferentiating ASCs to evaluate any potential influence of ASCs on HMEC proliferation and function. To avoid potential anti-angiogenic effects, during the coculture, the GM was deprived of dexamethasone, and the data show that pre-osteodifferentiation of ASCs have a consequence on HMEC recruitment[140]. In coculture, the ATP level was higher in the presence of ASCs, and the migration of HMEC was elevated in the presence of osteodifferentiating ASCs, suggesting the influence of ASCs on the proliferation of HMEC. A protocol was followed to quantify the ability of HMEC, co-cultured with ASCs, to form a network of capillaries[141].

The high number of nodes suggests the ability of cells to form a functional vascularization, essential for bone healing and remodelling.

On the one hand, all the data supports a crucial role of mesenchymal cells on enrolment and proliferation of endothelial cells, on the other, the endothelial cells can develop a vessels network essential for new bone formation.

The Real-time PCR express impressive results on the expression of pro-angiogenic and osteogenic factors. Osteodifferentiating ASCs express specific key factors involved in angiogenesis (ANG-1, ANG-2, VEGF, PDGFβ, TGFβ and FGF-2), showing a positive influence on vascularization. Moreover, endothelial cells increase the expression of osteogenic factors (BMP2, OPN). The mutual influence of ASCs and HMEC was following other studies on 3D culture systems which show increased production of angiogenic factors in coculture of ASCs with Human Umbilical Vein Endothelial Cells (HUVECs)[142].

The cross-talk between angiogenic and osteogenic factors have been investigated in the literature. In a study, the release of VEGF has been linked with osteogenic differentiation of ASCs[143]. In another study, BMP2 stimulated the production of VEGF[144]. According to the results of the present experiment, the influence of endothelial growth factors on ASCs, and the influences of osteogenic factors on HMECs were enlightened.

The 3D culture of ASCs and HMECs on the bone scaffold was performed in a bioreactor to mimic the physiological conditions, thus to eliminate any potential confounding factors. After 30 days of culture, the formation of new trabecula on the bone scaffold was analysed through micro-CT. The application of micro-CT allows a quantitative analysis of new tissue without damaging the samples and to overcome the traditional limitations of histological analysis.

The coculture on scaffolds showed an increased area of new tissue, confirming the role of both cells on osteogenesis.

The experiment confirms the cross-talk between endothelial and mesenchymal cells and the potential application of them to stimulate new bone formation; therefore, the application of functionalized scaffolds can be tested in the final in-vivo experiment.

Chapter six

Bone regenerative potential of "functionalized" bone graft in animal

6.1 Introduction

Although mesenchymal stem cells (MSCs) have been used to repair injured tissues, they present some limitations in the clinical application due to the restrictive regulatory authorisation in transforming them into a pharmaceutic product[145]. Therefore, several studies have proposed the use of stromal vascular fraction (SVF), the noncultured fraction of MSCs, derived from the enzymatic treatment of adipose tissue. SVF could be useful in the regeneration of bone defects because they contain different types of cells: endothelial cells, mesenchymal cells, red blood cells, pericytes and leukocytes[6].

To fill bone defects, different scaffolds have been generated through the application of several biomaterials. Xenohybrid bone grafts are considered promising substitutes, constituted of a bovine bone matrix reinforced by bioresorbable aliphatic polymers and RGD-containing collagen fragments[122].

Considering the exciting results of SVF cultures on bone grafts in vitro, the present experiment aimed to investigate, in vivo, the integration, osteoinduction and osteogenesis of scaffolds d by SVF. Several animal models are available to study bone tissue engineering, but the implantation of human cells in immunodeficient mice is essential to understand the complex biological scaffold-host interactions without immunological interferences.

The study was conducted on *severe combined immunodeficient* (SCID) mice, homozygous mice for an autosomal recessive mutation on chromosome 16. The mutation causes a deficient function in lymphocytes B and T, which permits the use of SCID mice in studies on lymphocytes development and function[146].

Moreover, SCID mice could be recruited to study implantation of xenograft materials[147].

In this *randomized clinical trial* (RCT) ten mice underwent to the surgical implant procedure. Bone grafts implanted were cultured with SVF or ASCs and randomly SCID mice were

assigned to the test (SVF cultured on bone grafts) or control group (ASCs cultured on bone grafts).

6.2 Material and methods

6.2.1 Preparation of bone scaffolds

In the experiment xenohybrid bone graft, Smartbone® (SB) was applied. SB is a bone substitute constituted of a bovine bone matrix reinforced by bioresorbable aliphatic polymers and RGD-containing collagen fragments[122]. SB chips for the study were washed with saline buffer solution and kept in a minimum essential medium (Alpha MEM, Voden Medical Instruments SPA, Meda, Italy) to improve hydrophilicity and cell adhesion.

6.2.2 Cell cultures

SVF derives from the enzymatic treatment of the fresh adipose tissue obtained from lipoaspirates, obtained from patients, who consented the treatment according to the approval of the Ethical Committee[123]. The lipoaspirates were treated with Collagenase NB4 (SERVA Electrophoresis, Heidelberg, Germany), then after washes with saline solution, a cell lysis solution (Promega, Milan, Italy) was applied to obtain SVF.
ASCs were obtained from the seed and culture of SVF in a basal medium (Dulbecco's Modified Eagle Medium, DMEM, Sigma-Aldrich, St. Louis, Missouri, USA), with 1% antibiotics, 2mM glutamine and 10% fetal bovine serum, FBS, (Gibco, Thermo Fisher Scientific, Waltham, USA); the medium was then replaced after 24 hours to eliminate the nonadherent cells.

6.2.3 Cell seeding on SB

SVF and ASCs were cultured on SB discs for 15 days in Alpha MEM, or osteogenic medium (OM) containing Alpha MEM supplemented with FBS, ascorbic acid, dexamethasone and beta-glycerophosphate (Sigma-Aldrich, St. Louis, Missouri, USA). To monitor the osteoblasts differentiation, cellular cultures were stained for alkaline phosphatase (ALP).

6.2.4 Animal model

Ten SCID mice, sexually mature and weighing 20-25g were used for the experiment. They were maintained under specific pathogen-free conditions and fed on sterile food and water[147]. Randomly they were assigned in two groups: a control group and the experimental group. In the experimental group (A), bone grafts functionalised with SVF were implanted, whereas in the control group bone grafts with ASCs (B) were tested.
Under general anaesthesia with pentobarbital sodium, an incision was performed at the middle of the dorsum. SB chips were subcutaneously transplanted into the pocket, and the incisions were sutured. SCID mice were monitored for signs of illness, infections or distress and weighed weekly.
After two months they were euthanised with CO_2, and subcutaneous scaffolds were explanted, immediately fixed in 10% neutral buffered formalin and store at 4°C. After three days the scaffolds were transferred to 70% ethanol for storage at 4°C.

6.2.5 Micro-CT analysis

Scaffolds were analysed, after explant, through micro-CT (SkyScan 1172, Bruker, Billerica, USA). The intensity of 80kV was used to keep acquisitions with a 0,4° rotation step, 360° scan at 4x frame averaging. An aluminium filter of 0,5 mm was applied at a resolution of 6 μm. NRecon software (Bruker) reconstructs datasets, and DataViewer software (Bruker) measures the mineralised tissue. A 3D visualisation method, volume rendering, was used to rendered directly without decomposing the samples into geometric primitives.

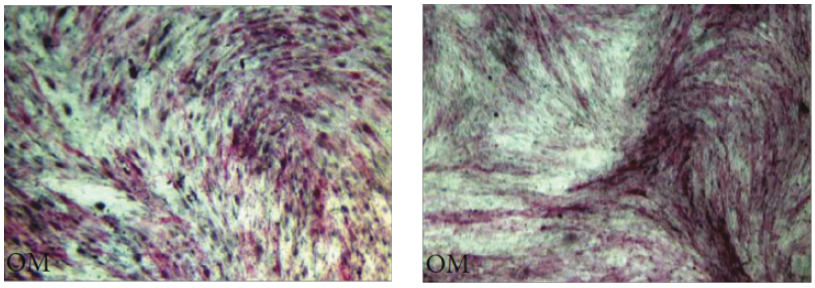
6.2.6 Statistical analysis

Graph Pad Prism 6 software (Graphpad Software Inc., La Jolla, USA) was used for statistical analysis. Data were analysed using one-way ANOVA test with Bonferroni's multiple comparison tests. Results were considered significant with $p < 0.05$.

6.3 Results

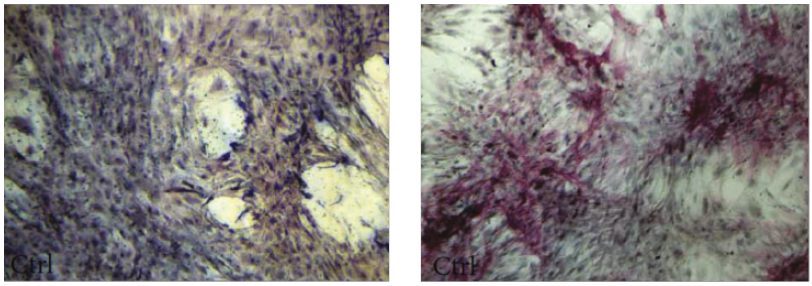
In vitro, ASCs and SVF were cultured in the presence and absence of osteogenic medium (OM), for 15 days, to evaluate the osteodifferentiation. The ability to differentiate in osteoblasts was detected through the expression of alkaline phosphatase (ALP). Both cellular lines, cultured on SB in the presence of OM, resulted in ALP-positive, confirming the ability to

osteodifferentiated (Fig.18). In the absence of OM (control groups), ASCs were ALP-negative, whereas SVF were ALP-positive (Fig.19).



a b

Fig.18 In vitro osteoblastic differentiation of ASCs and SVF cultured in osteogenic medium (OM). After 15 days of culture in OM, both ASCs (a) and SVF (b) expressed alkaline phosphatase (ALP).



a b

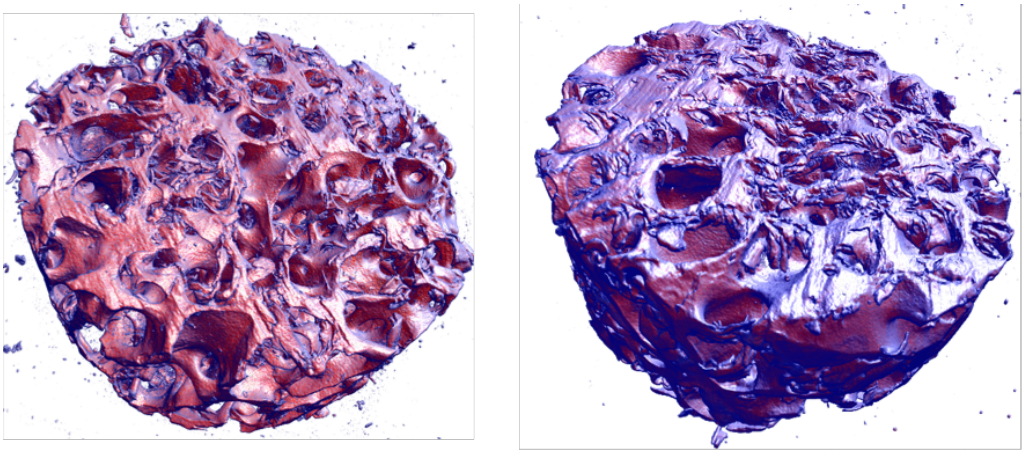
Fig.19 In vitro osteoblastic differentiation of ASCs and SVF cultured in Alpha MEM (Control groups). After 15 days of culture in the absence of osteogenic medium, ASCs (a) were ALP-negative, whereas SVF (b) were ALP-positive.

To evaluate the ability to generate new tissue, the scaffolds were tested in vivo. Ten SCID mice were randomly assigned in two groups: a test group (A) and a control group (B). In the test group, five mice received a bone scaffold functionalized with SVF, whereas in the control group bone scaffolds functionalized with ASCs were transplanted.

After two months, the scaffolds were explanted and evaluated through micro-CT.

Micro-CT allows to evaluate the connectivity between host bone and scaffold and to compare, through a 3D investigation, the new bone formation in test and control group.

The bone ingrowth was statistically significant in the test group, whereas in the control group, it was less evident (Fig.20).



a b

Fig. 20 Images of 3D reconstructions of samples. A) Sample explanted from the control group. B) Sample explanted from the test group. In the test group, the new bone (in blue) is more represented than in the control group.

In the 3 D analysis the scaffold porosity, the interconnection of pores and the extracellular matrix production were considered. In the test group, the extracellular matrix production, the porosity and the interconnection of pores were higher than in the control group.

6.4 Discussion and conclusion

According to the literature, scaffolds designed to regenerate bone should provide specific minimum requirements. The structure should be porous enough to support cell attachment, proliferation and extracellular matrix production and the pore should be interconnected to promote nutrient exchange. The chemical characteristics of the surface should enhance cell attachment, proliferation and differentiation; the mechanical properties should support the implantation. Besides, scaffolds should be biocompatible and resorbable[148].

To repair large bone defects, the culture of cells on osteoconductive scaffolds has already studied in animal models and humans[149].

In the present experiment, two types of MSCs were cultured on xenohybrid bone grafts and implanted in vivo to compare the scaffold integration and new bone formation.

The bovine bone blocks have been used as xenografts to repair bone defects because of its properties similar to human bone, although they present some limitations. The reinforcement by bioresorbable aliphatic polymers and RGD-containing collagen fragments has enhanced the

osteoinductive properties[150]. Thus, the culture of mesenchymal stem cells on SB were evaluated in term of osteodifferentiation before the experiments in mice.

Therefore, ASCs and SVF were cultured in the presence or absence of osteogenic medium (OM), to highlight their osteodifferentiative abilities. SVF was able, also in the absence of OM, to express ALP showing an osteodifferentiating behaviour. According to the findings, SVF were ALP-positive because the presence of other cells confers a better osteoinductive ability compare to ASCs.

In accordance with RCT, "functionalized" bone grafts were implanted in SCID mice; the scaffolds were subsequently explanted and assessed through micro-CT.

Micro-CT, compared with traditional methods, e.g. histological and radiological techniques, provides an efficient, non-destructively, three-dimensional images of bone microstructure[151]. Furthermore, using a 3D dedicated reconstruction software, it is possible to visualise samples in all directions[115].

The 3D reconstructions of the explanted samples showed an excellent integration and osteogenic abilities of both grafts. Although, in terms of new bone quantity and quality, the test group showed better results. Indeed, in the test group, the rate of new bone was higher and also the porosity, the interconnection and the extracellular matrix production were better represented.

The findings suggest that SB is an appropriate scaffold and SVF has better osteogenic abilities compare to ASCs. The variety of cells presented in SVF (e.g. adipocytes, mesenchymal cells, fibroblasts, blood vessel cells) seems to confer better osteoinductive properties. Indeed, the presence of different cells could be essential to reproduce a physiologic-like microenvironment for bone formation[152].

Chapter seven

Discussion and conclusion

In the last decades, the demand for bone grafts to improve healing response, to repair and to regenerate lost bone, as a result of disease or trauma, is increasing. It is reported that over 2.2 millions of bone grafts are applied annually worldwide[153].

To promote healing response, bone grafts must present biological and mechanical properties: the biological properties are measured in terms of osteoinduction, osteoconduction, and osteogenesis, the mechanical properties are linked with the necessity to support weight-bearing and related to the type of bone grafts.

Among bone grafts, three types are the most suited: autografts, allografts and xenografts.

Autologous bone grafts, which are transplanted from one site to another in the same patient, have become widely used and are still considered the gold standard in treatment for bone defects. The reasons are correlated to the easiness to obtain, the osteoconductive, osteoinductive and osteogenic favourable proprieties and the absence of adverse immune response or infectious diseases. However, its use is limited by long surgical time, pain, bleeding, haematoma, risk of vascular or nerve injuries and cosmetic deformities[3].

Allografts, which are transplanted from cadavers or living donors to recipients, present the benefits to eliminate the donor site morbidity and issues related to their limited supply. However, their use is limited by the lack of osteogenic capacity and the risk of infectious agents or immune rejection once implanted[154].

Although both types of bone grafts have been widely applied, their limitations have prompted the search for other grafts, like xenografts[155].

The bovine-based xenografts present a chemical and morphological porous structure similar to human bone, but the high temperature (>1000°C), applied to contrast the antigenicity, destructs the structure augmenting the frailty and resulting in a minor osteoinductive ability. To overcome those shortcomings, promising substitutes have been introduced. Xenohybrid bone grafts, obtained from the reinforcement of xenografts with polymers and collagen, show a

significant mechanical resistance, increased hydrophilicity, augmented cell adhesion and osteoinductivity[32].

Another way to improve bone defects repairing derives from the infusion of mesenchymal stem cells (MSCs). In regenerative medicine and tissue engineering, the application of MSCs has been studied to promote bone healing. Easy to isolate, they can be induced to differentiate into bone, with encouraging results[5].

In the present dissertation, the first and the second experiments were conducted in vitro to analyse the osteogenic potential of mesenchymal cells, when cultured on bone grafts, and the interaction of mesenchymal cells with endothelial cells in co-culture. The third experiment tested, in vivo, in an animal model, the osteogenic abilities of "functionalized" bone grafts.

In the first experiment the *stromal vascular fraction* (SVF)[145] of MSCs and the adipose tissue-derived stem cells (ASCs)[6] were cultured on xenohibryd bone scaffolds to indagate the ability to osteodifferentiat and generate new bone.

The osteodifferentiation of SVF and ASCs was indagated through microcomputed tomography, scanning electron microscopy, histochemical analysis and enzyme-linked immunosorbent assay. The results showed that SVF presented better osteogenic properties compare to ASCs and that the xenohybrid bone scaffolds could induce cellular colonization and growth.

The ability of SVF to differentiate in osteoblast was detected when cells were cultured in the absence of osteogenic medium, and it was corroborated through the expression of early and mature osteogenic marker (COLL-1, OCN and TGFβ) in the absence of osteogenic medium[126].

Microcomputed tomography and scanning electron microscopy analysis corroborated the findings showing a significant production of new bone in samples d by SVF.

A reason to explain the phenomena is that SVF contains mesenchymal stem cells and other different cells (e.g. leukocytes, red blood cells and endothelial cells), which conferred a better osteoinductive ability, compared to ASCs alone, because they create a microenvironment for bone deposition[127].

In the second experiment, the co-culture of ASCs and endothelial cells (ECs) was evaluated to understand the complex cellular cross-talk and to analyse any possible influences on new bone formation. According to literature, the vascularization is mandatory for bone graft survival after implantation[8], and endothelial cells can support the osteogenic differentiation of MSCs in vitro and in vivo[9]. Therefore, ASCs were firstly cultured in osteogenic medium to osteodifferentiate and secondly co-cultured with ECs on scaffolds, maintained in a bioreactor, to recreate similar physiological conditions. The scaffolds were subsequently evaluated with micro-CT.

The results showed that after seven days of culture in osteogenic medium, ASCs presented a better ability to osteodifferentiate, compared to ASCs cultured in GM. ECs were co-cultured with osteodifferentiating ASCs to evaluate any potential influence of ASCs on ECs proliferation and function. In co-culture, the migration of ECs was elevated in the presence of osteodifferentiating ASCs, suggesting the influence of ASCs on the proliferation of ECs.

All the data demonstrated both the role of mesenchymal cells on enrolment and the role of endothelial cells to develop a vascular network essential for new bone formation.

The Real-time PCR confirmed the mutual influence of ASCs and ECs. ASCs displayed a role on vascularization, expressing markers involved in angiogenesis (ANG-1, ANG-2, VEGF, PDGFβ, TGFβ and FGF-2) and ECs increased the expression of osteogenic factors (BMP2, OPN).

The co-culture of stem cells and osteoblasts or mesenchymal cells is a common approach in literature and the mutual influence of ASCs and ECs was in accordance with other studies on 3D cultured systems[156].

In recent years, several studies have employed co-culture systems in a bioreactor with the purpose of understanding processes that occur in vivo during the bone healing[157]. Therefore, 3D cultures of ASCs and HMECs on the bone scaffold was performed in a bioreactor to eliminate any potential confounding factors. Micro-CT analysis detected, after 30 days of culture in a bioreactor, the formation of new trabeculae on the bone scaffolds.

The coculture of cells on bone scaffolds seems to stimulate new bone formation; therefore, the third experiment was conducted in vivo to test the effect of a "functionalized" bone graft.

A clinical randomized trial was conducted on SCID mice. Two groups have been generated: the test group underwent to the implantation of xenohybrid bone graft obtained in cultured with SVF whereas the control group received a xenohybrid bone graft cultured with ASCs.

In different studies, on animals or humans, the implantation of cells cultured or seeded on bone scaffold has been investigated[134,149].

To better understand the osteogenic abilities of SVF and ASCs cultured on xenohybrid bone grafts, they were evaluated after the explant, through micro-CT analysis.

ASCs and SVF were cultured in the presence or absence of an osteogenic medium, to highlight their osteodifferentiative abilities. The results, in accordance with the first experiment, showed that SVF were able, also in the absence of OM, to differentiate in osteoblasts.

In vivo, the micro-CT 3D reconstructions showed an excellent integration of both grafts but emphatised a better osteogenic ability of SVF. The test group presented a higher rate of new bone, porosity and interconnection compared with the control group. The results also showed

a better production of extracellular matrix in the test group underlining the potential to incorporate mesenchymal cells into a scaffold which mimic the physiologic bone microenvironment.

Taken together, the results of the three experiments suggest the crucial role of mesenchymal stem cells cultured with bone grafts and the peculiarity to mimicking the natural bone healing process, i.e. promoting the new bone formation stimulating the vascularization process.

The in vivo interplay between osteoblasts like cells and endothelial cells can be simulated in co-culture systems.

Also in vivo, the results confirmed the excellent osteoinductive and osteoconductive abilities of xenohybrid bone grafts and the osteogenic properties of SVF.

The need to display and measure the architectural features of scaffolds has been satisfied through the micro-CT. This 3D non-destructive and non-invasive method which does not require any particular treatment of samples allows the identification of the newly formed bone and the comparison of different bone scaffolds[158].

In conclusion, the dynamics of bone tissue microenvironment is still unknown and require interdisciplinary collaboration to find an ideal bone substitute able to mimic natural bone. At the moment the present dissertation confirms the utility of MSCs in the regeneration of bone and the possible application of 'functionalised' bone grafts to better mimicking the physiological microenvironment.

The application of micro-CT analysis could be an ideal method to indagate the new bone formation on samples without damage the tissues.

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