MODULATION OF THE BONE REGENERATION PROCESS IN SYSTEMIC IMPAIRED CONDITIONS
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“Live as if you were to die tomorrow.
Learn as if you were to live forever.”

Mahatma Gandhi
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ABBREVIATION LIST

AP40 – Bioactive Silica-Phosphate Glasses
ACTH – Adrenocorticotrophic Hormone
ALB - Albumin
ALP - Alkaline Phosphatase
AU – Arbitrary Unit
BMC - Bone Mineral Content
BMD – Bone Mineral Density
BMP - Bone Morphogenetic Proteins
BMU – Basic Multicellular Unit
BPs – Bisphosphonates
BSAP - Bone-specific Alkaline Phosphatase
BSV/TV - Bone Substitute Volume per Tissue Volume
β-TCP - Beta-tricalcium phosphate
BV/TV - Bone Volume per Total Volume
C – Control
Ca - Calcium
CD - Connective Density
Col I – Collagen type I
CK - Creatine kinase
CSD – Critical Size Defect
CT – Quantitative Computed Tomography
DXA – Dual-Energy X-ray Absorptiometry
ECM - Extracellular Matrix
EMP – Enamel Matrix Proteins
e-PTFE – Polytetrafluoroethylene
ER-a - Estrogen Receptor-alpha
ER-b - Estrogen Receptor-beta
FDA - Food and Drug Administration
FMDUP – Faculdade de Medicina Dentária da Universidade do Porto
FRAX - Fracture Risk
FSH - Follicle-stimulating hormone
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GBR - Guided Bone Regeneration
GH - Growth Hormone
GnRH - Gonadotropin-releasing Hormone
GTR - Guided Tissue Regeneration
HA – Hydroxyapatite
HPG – Hypothalamic Pituitary Gonadal
HRT – Hormone Replacement Therapy
ICSI – Institute for Clinical Systems Improvement
IGF I - Insulin-like Growth Factor-I
IOF - International Osteoporosis Foundation
IL-1 – Interleukin-1
IL-6 – Interleukin-6
IP – Intraperitonial
LF - Luteinizing Hormone
M- Membrane group
MB – Membrane and Biomaterial group
M-CSF - Macrophage Colony-stimulating Factor
Mpa – Megapascals
MSCs - Mesenchymal Stem Cells
NF-κB - Transcription Factor Nuclear kappa B
OC - Osteocalcin

OPG – Osteoprotegerin

Ovx – Ovariectomy

P - Phosphorous

PDGF – Platelet-Derived Growth Factor

PGAs - Polyglycolides

PLAs - Polylactides

pQCT - Peripheral Quantitative Computed Tomography

PCR – Polymerase chain reaction

PPAR-g - Peroxisome Proliferator-activated Receptor Gamma

PTH – Parathyroid Hormone

QCT - Quantitative Computed Tomography

QU S – Quantitative Ultrasound

RA - Radiographic absorptiometry

RANKL – RANK Ligand

ROI – Region Of Interest

RUNX2 - Runt-related transcription factor 2

RUTH -Raloxifene Use for The Heart

SD – Standard deviations

SERMs - Selective Estrogen Receptor Modulators

Sham – Control

SPA – Single-Photon Absorptiometry

SPSS – Statistical Package for Social Sciences

Tb.N - Trabecular Number

Tb.Sp - Trabecular Separation

Tb.Th – Trabecular Thickness

TG - Triglyceride
TGF – Transforming Growth Factor
Ti – Titanium
TNF – Tumor Necrosis Factor
TP – Total protein
WHO – World Health Organization
VOI - Volume Of Interest
ZrO₂ – Zirconia
µCT - Microcomputed Tomography
ABSTRACT

Osteoporosis is characterized by a reduction in bone mass and disruption of bone architecture, resulting in increased bone fragility and fracture risk. These fractures are widely recognized as a major health problem in the elderly population. Osteoporosis is of additional relevance in women entering the menopause, due to the lack of estrogen production. Hormonal disequilibrium is known to favour bone resorption, which in turn leads to skeletal fragility and increased risk of fracture. Additionally, preliminary data reports that the bone regeneration process, in osteoporotic conditions, may be somewhat limited mainly due to a decrease of new bone formation, nevertheless specific mechanisms have not been stated.

In this way, this experimental work aims to address the bone regeneration process in an animal model of osteoporosis mediated by a biomaterial in two experimental designs: in the absence of biomechanical load (implementation in the calvarial bone), and in the presence of biomechanical load (implementation in the mandibular bone).

The ovariectomized rat model, known to represent the most important clinical features of post-menopausal osteoporosis, was established and thoroughly characterized.

The bone regeneration process was evaluated by routine radiographic, microtomography and histological evaluations were conducted at adequate time points.

Reported data converge to substantiate a detrimental effect of the osteoporotic condition in the process the biological response to biomaterial’s implantation, and the guided bone regeneration process, in the assayed animal model, in the absence and presence of a biomechanical load.

The stated biological hindrances and the data trend substantiating the impaired biomaterial-mediated bone regeneration process in osteoporotic conditions suggest that care should be taken on pre-operative preparation and a selective choice of biomaterials should be undertaken when facing bone regeneration in osteoporotic-
compromised conditions. Moreover, there is an imperative need for new and optimized clinical interventions for the treatment of bone-related conditions, in osteoporotic patients.
RESUMO

A osteoporose é uma patologia caracterizada por uma redução da massa óssea e perda da sua arquitectura, resultando num aumento da fragilidade e risco de fractura óssea. Estas fracturas são vastamente reconhecidas como um grave problema de saúde na população idosa. A osteoporose tem maior impacto nas mulheres na pós-menopausa devido à falta de produção de estrogénio. O desequilíbrio hormonal é conhecido por favorecer a reabsorção óssea, o que leva à fragilidade e aumento do risco de fractura. Adicionalmente há estudos que sugerem que o processo de regeneração óssea, em condições osteoporóticas, possa estar alterado devido a uma diminuição do processo de formação óssea. No entanto, os mecanismos específicos associados a esta condição não estão totalmente esclarecidos.

Desta forma, este trabalho experimental tem como objectivo o estudo do processo de regeneração óssea mediada por um biomaterial num modelo animal osteoporótico em duas condições experimentais: na ausência de carga biomecânica funcional (implantação na calote craniana), e na presença de carga biomecânica funcional (implantação na mandíbula).

O modelo do rato ovarectomizado, conhecido por apresentar as características clínicas mais preponderantes da osteoporose pós-menopausa, foi estabelecido e detalhadamente caracterizado.

O processo de regeneração foi avaliado através de técnicas imagiológicas, micro-tomográficas e histológicas, nos tempos estipulados.

Os resultados obtidos demonstram um efeito negativo da osteoporose no processo de regeneração óssea, guiada por biomateriais, no modelo animal estabelecido. Verificou-se um atraso no processo regenerativo dos animais osteoporóticos, na ausência e na presença de carga biomecânica funcional.

As alterações biológicas observadas na regeneração óssea em condições osteoporóticas sugerem que a preparação pré-operatória cuidada e a selecção criteriosa dos biomateriais a utilizar devem ser consideradas no planeamento de
procedimentos ósseos regenerativos em pacientes osteoporóticos. De forma adicional, é essencial o desenvolvimento de novas terapêuticas regenerativas dirigidas a pacientes afectados por condições sistémicas que interfiram com o processo de regeneração óssea.
Systemic impaired conditions may affect the homeostasis of oral tissues, increase their susceptibility to other diseases, interfere with healing and in addition, may be treated with drugs and/or other therapeutic interventions that can potentially affect tissue remodeling and healing. The increase in life expectancy, raise the number of patients submitted to surgical interventions of osteosynthesis and joint replacement affected by age related, post menopausal and other secondary osteoporosis and systemic diseases. This is of particularly importance due to demand of surgical interventions with the requirement of biomaterials to enhance the outcome of the life expectancy and life quality. The systemic impaired conditions may affect the bone tissue and modify the bone regeneration process.

Since the beginning of oral implant surgery, some therapeutic approaches have been contraindicated for certain patients with systemic impaired conditions, namely those including bone regeneration procedures.

Several factors have been described to inhibit the osseointegration. These include: osteoporosis[1, 2], rheumatoid arthritis[3], radiation therapy[4, 5], smoking, advanced age, nutritional deficiency and renal insufficiency, pharmacological agents such as cyclosporine A[6], methotrexate and cis-platinum[7].

Osteoporosis is of major concern due to its high prevalence, being an important health problem, reporting an elevated rate of mortality and morbidity, in several countries. Global epidemiological data report that 1 in every 3 women and 1 in every 50 men over the age of 50 years old have osteoporosis.[8] In Europe alone, it is estimated that 179,000 men and 611,000 women will suffer a hip fracture each year and that the cost of all osteoporotic fractures in Europe is provisionally €25 billion.[9]

National indexes vary but reported data from the International Osteoporosis Foundation (IOF) show that in 2003, 7.8 million Germans (6.5 million women) were affected by osteoporosis[10], while in Denmark, the estimated prevalence of osteoporosis in persons aged 50 years or more was about 41% among women and 18%
among men, in 2005.[11] In Spain, approximately 2 million women have been shown to report osteoporosis and its prevalence was shown to be 26.1% of women who are 50 years of age and older.[12]

In Portugal, the prevalence of osteoporosis was established to be around 5%, in 2003.[13] The prevalence increased with age, and has been shown to be higher in women. Another report, based on the reevaluation of the population-based cohort EPIPorto (2003-2005) revealed that 3.3% men and 28.1% women had the medical diagnosis of osteoporosis[14], while a telephone-based enquiry, randomly assigned to 1800 women resident in Portugal, in 2004, revealed that 15.4% declared to have osteoporosis, of whom 96.8% referred to have an exam confirming the diagnosis.[15]

In the population attending the appointment of Oral Surgery, at Faculty of Dental Medicine, University of Porto (FMDUP), we performed a 5-year retrospective study (between September 2003 and July 2008) of all patients who underwent surgical consultation. The sample included 1423 patients, of whom 773 were women (54.3%) and 650 were men (45.7%). We documented that 2.7% of patients above 50 years reported to have osteoporosis. The major percentages of those were female (83.3%) and only 16.7% were male patients.

The appraisal of the osteoporotic condition in the Dental Medicine field is of particular relevance and broadly disregarded, mainly due to the reported influence of the disease in the alveolar bone metabolism.[16] In fact osteoporosis has been found to be associated with periodontal bone loss and temporomandibular joint bone loss.[17]

Some data suggest that osteoporosis negatively influences the healing process after a fracture.[18, 19] This is of the utmost relevance since the oral/maxillofacial bone tissue is the ground support for therapeutic and restorative approaches in dentistry, supporting roughly the vast majority of oral rehabilitation procedures.

The osteointegration rate of prosthetic implants also appears to be impaired and clinical studies substantiate a partial degree of success in the establishment of bone-related procedures and oral implant placement in osteoporotic patients.[20, 21]

Nonetheless, the detailed biological issues of the bone regenerative process in osteoporotic conditions have not been adequately disclosed.
AIMS

Osteoporosis is a major health problem in the elderly population and is known to increased bone fragility and fracture risk. Bone regeneration process, in osteoporotic conditions, may be further limited essentially due to a decrease of new bone formation, nevertheless specific mechanisms have not been stated.

Thereby, this experimental work aimed the establish and characterization of a representative animal model of the human condition of osteoporosis, i.e., the ovariectomized rat, due to functional similarity to the osteoporotic condition. Further, the biomaterial-mediated bone regeneration process was evaluated in two experimental models: in the calvarial bone (in the absence of functional biomechanical loading) and in the mandibular (in the presence of functional biomechanical loading). In each bone, a critical size bone defect was established and following filled with a biomaterial of known biocompatibility. The bone regeneration process was adequately characterized by histological, histomorphometric and microtomographic methodologies.
I. LITERATURE OVERVIEW
A. BONE BIOLOGY
**BONE TISSUE STRUCTURE**

Bone is a living and dynamic tissue that is able to provide strength and stiffness to the skeleton and yet be flexible enough to absorb energy and therefore reduce fracture risks. It is only able to perform in such a remarkable way due to its structural and compositional properties.[22] Bone is a highly vascular and mineralized connective tissue, which comprises several specialized cells in a fibrous organic matrix, permeated by inorganic constituents.

Mature bone may be classified into cortical or compact (Figure 1), and trabecular or cancellous (Figure 2).[23] Cortical bone is found on the outer surface of individual bones and surrounds the trabecular bone. Approximately 80% of the skeleton corresponds to cortical bone. The architecture and relative amount of the cortical tissue, at any given anatomical location, are related to its function at that specific area. Cortical bone is porous, but its density is considerably higher than that reported for trabecular bone – around 80% against 20%, respectively.[24] Cortical bone is composed of Haversian canals, which are a collection of cylindrical units oriented parallel to the length of the bone and enclose a neurovascular bundle. Each canal is surrounded by concentric lamellae containing small voids designated lacunae in which osteocytes may be found. The canaliculi are small channels that allow for nutritional support and oxygenation to the cellular constituents of the lacunae.[23] They are also responsible for the removal of the waste products originated from metabolic actions. The canaliculi connect to the Haversian systems which, in turn, anastomose with obliquely orientated vascular branches, designated Volkmann’s canals, which establish communication between the periosteum and the endosteum.[24] The gaps between the Haversian systems are made up of interstitial bone, which consists of similar tissue elements, but in a less organized pattern. Haversian systems are separated from one another by cement lines which are strongly basophilic and have a high content of inorganic matrix.[24]
Trabecular bone is predominant in vertebrae, pelvis, and other flat bones, as well as at the epiphyses of long bones. It is essentially constituted by calcified *trabeculae*, which result from the fragmentation of Haversian systems. The void spaces between *trabeculae* - *lacunae* - are filled with active bone marrow, responsible for nutritional support.[24] Reports indicate that trabecular bone has a high area-to-volume ratio and the capacity to respond quicker than cortical bone to biomechanical inputs. Moreover, it presents a high metabolic rate, in fact significantly greater that of cortical bone.[23]
Two distinct mechanisms determine how the bone tissue is formed during development, either by endochondral or intramembranous ossification. Most of the skeleton is crafted by endochondral ossification, a process that occurs via an intermediate step of cartilage, and involves a coordinated set of interactions requiring the transient expression of specific genes and various cell – cell and cell – matrix interactions, which results in the eventual replacement of cartilage with bone. Alternatively, new bone formation can occur via intramembranous ossification, in which *de novo* synthesis occurs. Mesenchymal cells migrate and condensate into clusters within the fibrous tissue, and differentiate directly into osteoblasts. Such a cluster is known as an ossification center, and the osteoblasts start to secrete matrix, which subsequently mineralize.[26]

In both processes, the bone tissue initially formed is characterized as primary or woven. Primary bone is a temporary tissue, soon to be replaced by the definitive lamellar or secondary bone and it may also be found during tissue healing. This tissue has an increased metabolic activity and is characterized by a scattered and irregular structure, while secondary bone portrays an ordered arrangement of cells and
matrix.[27] These mechanisms of developmental bone growth and modeling cease with the completion of adolescent growth and mark a transition to adult bone remodeling, characterized by the focusing on maintenance and repair.[26]

**BONE TISSUE REMODELING**

Bone remodeling is the process by which bone is renewed to maintain bone strength and mineral homeostasis. The main recognized functions of bone remodeling include preservation of bone mechanical strength by replacing older, micro damaged bone with newer, healthier bone and calcium and phosphate homeostasis.

Bone surfaces may be undergoing formation or resorption, or they may be inactive. These processes occur throughout life in both cortical and trabecular bone. Bone remodeling is a surface phenomenon, and it occurs on periosteal, endosteal, Haversian canal, and trabecular surfaces. The rate of cortical bone remodeling, which may be as high as 50% per year in the midshaft of the femur during the first 2 years of life, eventually declines to a rate of 2–5% per year in the elderly. Rates of remodeling in trabecular bone are proportionally higher throughout life and may normally be 5–10 times higher than cortical bone remodeling rates in the adult. [28]

Resorption and formation are closely linked by temporary anatomic structures called basic multicellular units (BMU).[29] The BMU of bone comprises the osteocytes, osteoclasts, bone lining cells and osteoblasts, this activity is regulated by mechanical forces, bone cell turnover, hormones (e.g. parathyroid hormone (PTH), growth hormone (GH)), cytokines and local factors. The osteocytes detect mechanical stress and respond to mechanical stimuli taking apart for the activation process.[30]

The cellular cycle of such units begins with the recruitment of precursors from osteoclasts, their differentiation and activation. Following, mature multinucleated osteoclasts are responsible for the resorption of the anatomical location on the surface of trabecular bone, creating the Howship's *lacunae*, or cutting cones, in cortical bone. These are identical processes - in trabecular bone the BMU may be looked upon as a sagittal section of a cortical BMU. Resorption is then followed by a reversal phase, during which a cement line is deposited. Subsequently, osteoblast precursor cells are
recruited and, following differentiation and activation, fill the cavity with a volume of new bone that undergoes rapid primary, and then slower secondary mineralization.

The rate of trabecular bone turnover is higher, more than required for maintenance of mechanical strength, indicating that trabecular bone turnover is more important for mineral metabolism. Increased demand for calcium or phosphorus may require increased bone remodeling units, but, in many cases, this demand may be met by increased activity of existing osteoclasts. Increased demand for skeletal calcium and phosphorus is met partially by osteoclastic resorption and partly by nonosteoclastic calcium influx and efflux. Ongoing bone remodeling activity ensures a continuous supply of newly formed bone that has relatively low mineral content and is able to exchange ions more easily with the extracellular fluid. Bone remodeling units seem to be mostly randomly distributed throughout the skeleton but may be triggered by microcrack formation or osteocyte apoptosis. The bone remodeling space represents the sum of all of the active bone remodeling units in the skeleton at a given time.[31]

The volume of bone resorbed and produced within each focal remodeling unit is the same, proving that no net bone loss or structural damage occurs.[22]

Figure 3 – Mechanism of bone remodeling.[32]

Proper bone remodeling relies on a regulated balance between the four principal cells which constitute the bone tissue: osteoblasts, osteocytes, bone lining cells and osteoclasts (Figure 3).[26]
BONE TISSUE CELLULAR COMPOSITION

Osteoblasts

These cells derive from undifferentiated mesenchymal cells and are responsible for the deposition of the extracellular matrix (ECM) and its subsequent mineralization. They are highly differentiated columnar shaped cells (20–30 µm in diameter), usually found in a single cell layer, intimately apposed to areas of bone formation or remodeling (Figure 4). They are metabolically active secretory cells that are characterized for having a round nucleus opposing the bone surface, small amounts of basophilic cytoplasm, and the presence of prominent Golgi complex and endoplasmic reticulum. They secrete proteins of the extracellular matrix, such as alkaline phosphatase, osteocalcin, osteopontin, osteonectin and proteoglycans, as well as soluble signaling factors (e.g., bone morphogenetic proteins (BMPs), transforming growth factor-β (TGF-β), insulin-like growth factor-I (IGF I), IGF-II, interleukin-1 (IL-1) and platelet-derived growth factor (PGDF)).[33]

Populations of osteoblasts are heterogeneous, with different osteoblasts expressing different gene repertoires that may explain the heterogeneity of trabecular microarchitecture at different skeletal sites, anatomic site-specific differences in disease states, and regional variation in the ability of osteoblasts to respond to agents used to treat bone disease.[31]

In due course, osteoblasts, following their biological role, can commit to one of three pathways: remain metabolically active cells; become entrenched by the mineralized extracellular matrix and turn into osteocytes; or, become relatively inactive and originate bone lining cells.[34]

Osteocytes

Osteocytes comprise the vast majority of bone cells of the adult skeleton. They are the result of osteoblasts entrapment by their own bone matrix-secreting activity. As they become more isolated from the bone-forming surface, osteocytes’ protein-synthesizing activity declines, the size of the endoplasmic reticulum and Golgi apparatus decreases, and the mitochondrial content falls.[35] Osteocytes
communicate with each other via cytoplasmic processes that pass through the bone canaliculi. This network is also considered to substantiate metabolic exchange and coordinate the response of bone to biomechanical challenges. [23] Osteocytes are involved in the biomechanical regulation of bone mass and structure, probably by sensing bone deformation, pressure, fluid flows and streaming potentials, which consequently affects the release of signaling molecules and growth factors that seem to regulate cell proliferation and differentiation. [36]

Osteocytes do not divide and their turnover is only assured through the remodeling process.

**Bone lining cells**

While several osteoblasts are active in bone formation, flatter and more inactive cells are found in the bone surface of the mature skeleton, i.e. bone lining cells. They are closely associated with each other via cellular adhesion complexes, and in contact with the osteocytes network. [23] They present less cytoplasmic organelles than osteoblasts and their biological function has not been definitely established: they seem to be responsible for removing the thin layer of osteoid which coats the bone surface, thus exposing the bone for osteoclastic resorption; and also enroll osteoblast-like functions following proper activation. [34]

**Osteoclasts**

Osteoclasts are derived from the fusion of mononuclear hematopoietic stem cells. These multinucleated giant cells are responsible for bone resorption and seem to attach to the bone surface, secreting hydrolytic enzymes in a acidic microenvironment. [26] They are typically large cells and are characterized for having an average of 10 to 12 nuclei (Figure 4). These cells show cellular polarity, and resorption occurs along the folds and finger-like projections of the plasma membrane, characterized as a “ruffled border”, along the bone surface. The cytoplasm adjacent to this surface is devoid of organelles, but is rich in actin filaments and other microfilament-associated proteins, suggesting that this area supports the osteoclastic bone adhesion. [23] These cells mediate the resorption process via the release of
powerful lysosomal enzymes and acids which digest protein and mineral components of the bone matrix.[37] The key family of proteinases involved in osteoclastic bone degradation are cathepsins (i.e., cysteine proteinases) and matrix metalloproteinases. [38]

Figure 4 – Cellular composition of the bone tissue. (A- osteoclasts; B- osteoblasts; in the middle of the trabeculae the osteocytes) [39]
OSTEOBLASTS-OSTEOCLASTS INTERACTION

Central to the bone remodeling activity, namely in which refers to the interaction between osteoblasts and osteoclasts, is the recognition that novel members of the tumor necrosis factor receptor family influence directly osteoclast function (Figure 5).[40] RANKL, the ligand of the receptor activator for nuclear factor κB, activates the NFκB transcriptional pathway on cells that bear RANK; such RANK-expressing cells include cells of the monocyte/macrophage lineage, and thus, osteoclasts.[38]

RANKL is synthesized and expressed by bone stromal cells and osteoblasts, that also produce macrophage colony-stimulating factor (M-CSF) that attaches to a distinct macrophage cell surface receptor.[38] Together, RANKL and M-CSF converge to differentiate/activate osteoclasts-precursor cells into their functional effector cells.[24] RANK activation is therefore a major stimulus for bone resorption. Osteoblasts also produce and secrete osteoprotegerin (OPG), a decoy for the receptor activator of nuclear factor kappa B ligand (RANKL). By binding RANKL, OPG inhibits the transcription factor nuclear kappa B (NF-κB).[41] In which relates to bone metabolism, OPG can reduce the production of osteoclasts by inhibiting the differentiation of osteoclast precursors into osteoclasts and also regulating the resorptive activity of osteoclasts.[38] OPG levels increase with age, and it is possible that OPG production rises as a homeostatic response to limit the bone loss that occurs with an increase in other bone-resorbing factors.[42, 43]
**REGULATION OF BONE CELLS ACTIVITY**

Overall, bone metabolism is under constant control by three main systemic hormonal systems i.e., calcitonin, vitamin D, and parathyroid hormone (PTH), that adjust calcium homeostasis. Briefly, the principal action of calcitonin, which is secreted by the thyroid gland in answer to an increasing of calcium plasma level, is to inhibit bone resorption.[24] Parathyroid hormone produces changes in the calcium pool, by favoring osteoclastic bone resorption and inducing calcium resorption by the kidneys.[45] 1,25-dihydroxyvitamin D$_3$, the active form of vitamin D, seems to have an effect both on osteoblasts – it increases the production of osteocalcin and alkaline phosphatase by these cells – and osteoclasts – it stimulates osteoclastic differentiation and multinucleation, depending on the stage of the remodeling cycle it acts on.[38]

Additional hormones can also influence bone cell function and bone metabolism. For instance, estrogen has an important action in preserving bone. Estrogen deficiency increases the rate of bone remodeling, as well as the amount of bone loss within each remodeling cycle. Experimental reports suggest that there are
multiple sites for estrogen action, not only on the cells of the bone remodeling unit, but also on other marrow cells. The mechanism of action is not yet known, but potential modulatory actions include effects on T cell cytokine production, on the RANKL and OPG production by stromal or osteoblastic cells, inhibition of differentiated osteoclasts, and on the enhancement of bone formation, in response to mechanical forces sensed by osteoblasts or osteocytes.[46]

**BONE TISSUE EXTRACELLULAR MATRIX**

The extracellular mineralized matrix consists of around 35% of organic and around 65% of inorganic content. The principal organic component is type I collagen, which comprises 80–90% of the organic matrix, although others types of collagen are present in small amounts, i.e., type V, VI, VIII and XII. Collagen type I is a heteropolymer of two α1 chains and one α2 chain, wound together in a triple helix. Several non-collagenous proteins are also present, such as alkaline phosphatase, osteocalcin, osteonectin, osteopontin, and several proteoglycans.[24]

Serum osteocalcin synthesized by osteoblasts was previously thought to function as a promoter or initiator of calcium deposition at the nidus between the ends of collagen fibrils and therefore regarded as a marker of bone formation. The observation that the osteocalcin knockout mouse has a high bone mass phenotype suggests that osteocalcin normally inhibits bone formation. Because serum osteocalcin is derived from both matrix releases by osteoclast activity and osteoblast synthesis, it is currently regarded as a marker of bone turnover rather than a specific marker of bone formation. The main glycosylated protein present in bone is alkaline phosphatase, this is bound to osteoblast cell surfaces via a phosphoinositol linkage and also is found free within mineralized matrix. Alkaline phosphatase plays an as-yet-undefined role in mineralization of bone.[47] The most prevalent noncollagenous protein in bone is osteonectin, accounting for approximately 2% of total protein in developing bone. Osteonectin is thought to affect osteoblast growth and/or proliferation and matrix mineralization.
The inorganic matrix contains important ionic components such as calcium, phosphate, magnesium, carbonate, hydroxyl, fluoride, citrate, and chloride. The most important crystalline component of bone is hydroxyapatite - $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, found as needle-shaped crystals with 20–40 nm in length and 3–6 nm in width, generally lying with their long axes parallel to the collagen fibers.[24] Other bone mineral ions are found in association with the surface of the hydroxyapatite crystals, or they may replace phosphate ions within the crystals composition.[23]
BONE TISSUE HEALING

Fracture healing is a unique response to bone injury, which in contrast to repair of many other types of tissues, aims to restore the anatomy, structure and function of the tissue, to its original characteristics. In general, fracture healing is completed in 6 to 8 weeks after initial injury. Fracture healing may occur both through direct (intramembranous) or indirect (endochondral) bone formation.

Fracture healing can be divided in four more or less overlapping stages (inflammation, soft callus, hard callus and bone remodeling) (Figure 6). At the cellular level, inflammatory cells, vascular cells, osteochondral progenitors, and osteoclasts are key players in the repair process. At the molecular level, fracture repair is driven by the 3 main classes of factors: pro-inflammatory cytokines and growth factors, pro-osteogenic factors, and angiogenic factors, recruiting cells and stimulating growth and/or differentiation. Thereafter, the damaged soft tissues are repaired and the fracture is bridged by soft callus and later hard callus. The bridging hard callus is eventually remodeled to re-establish the original anatomy and function of the fractured bone.

This process begins with the formation of a hematoma at the fracture site, through the release of cytokines and recruitment of inflammatory cells (granulocytes, lymphocytes and monocytes), which secrete cytokines/growth factors and advance formation of a stable fibrinous clot, along with fibroblasts, pericytes and endothelial cells. Subsequently the clot is reorganized into granulation tissue with extensive ingrowth of capillaries, allowing macrophages and other phagocytic cells to clear degenerated cells and debris. These factors contribute to further attraction of inflammatory cells, as well as multipotent mesenchymal stem cells.[48] Hypoxia and vascular disruption at the fracture site are also stimuli for cell recruitment. These cells initiate the deposition of extracellular matrix rich in fibronectin and collagen, which results in the formation of granulation tissue. Following, fibrous-vascular tissue is deposited in the areas in which the hematoma is being resorbed. Simultaneously, recruited osteoblasts and chondrocytes initiate the deposition of osteoid and cartilage, respectively, contributing to soft callus formation. Hard callus formation (primary bone
formation) represents the most active period of osteogenesis, with intense osteoblast activity and formation of mineralized bone matrix, which arises directly in the peripheral callus in areas of stability bridging the bone fragments. The soft callus is replaced with woven bone, which becomes revascularized. Sufficient vascularization yielding increased local oxygen tension and thus promoting osteoblast differentiation is critical to formation of hard callus.

The woven bone is remodeled over time to originate lamellar bone with adequate structure and anatomy.[26]
BONE MECHANICS

Bone provides mechanical support related to protection and locomotion; it functions as a system of complex metabolic mineral homeostasis and shows mechanical properties of anisotropy, nonlinearity, and viscoelasticity. These properties, along with its ability to respond to changes in its physiological and mechanical environment, make it more difficult to establish universal constants related to the physical properties of bone.[24]

Based on biomechanical principles, bone responds to forces in nature, including gravity, ground reaction, and muscle contraction. When a force or a load is applied to bone, an internal resistance develops (i.e., stress). Stress is the force per unit area and is equal in magnitude but opposite in direction to the applied load. Stress can be categorized as tensile, when too forces act along a straight line in opposite directions; compressive, when too forces act along a straight line in the same direction; or shear, when too forces are acting parallel to each other but not in the same line. Most forces applied to bone are a combination of the three stresses, resulting in a bending or torsion. The resulting deformation of the applied force is known as strain, which is equal to the change in length divided by the original length.[50]

At low levels of stress, a linear relationship exists between stress and strain. The ratio of the stress divided by the strain is known as the modulus of elasticity or Young’s modulus. This relationship or modulus relates to the overall stiffness or rigidity of bone. The linear portion of the stress-strain curve is known as the elastic region, where removal of the load results in no permanent strain or deformation. The point at which the curve becomes nonlinear, the plastic region, a permanent deformation occurs even after the load is removed. This occurs at the elastic limit or yield point. Stressing a bone beyond the plastic region will result in failure, such as a fracture. The ultimate strength of a bone is determined by calculating the maximum stress at the point of failure (Figure 7).[50]

Bone, like many other biological tissues, demonstrates the property of anisotropy; it responds differently depending on the type of load applied. Cortical bone has been shown to resist compressive forces better than tensile forces.
Compared with cortical bone, cancellous bone has a lower modulus of elasticity due to its greater porosity. Cancellous bone demonstrates the greatest strength when a compressive force is applied parallel to the trabecular system, such as a vertical force to a vertebral bone. Therefore, the strength and rigidity of bone are greatest in the direction of normal loading. Bone also demonstrates the property of viscoelasticity, which indicates that materials will demonstrate different properties according to the rate of force application. At low rates of loading, bone demonstrates a lower modulus of elasticity, and behaves like a viscous material. At higher rates of loading, bone behaves as a brittle material.[24]

Mechanical loads applied to bone are thought to be communicated through the bone by way of a mechanical signal detected by either bone lining cells or osteocytes, or both. It is believed that these mechanical signals lead to the generation of chemical signals involved in the regulation of bone formation and remodeling. The osteocytes, in particular, have received much attention in this regard. Osteocytes are connected to each other and to osteoblasts by way of cellular processes within canaliculi and are linked by gap junctions. This network allows for the possibility of electrical coupling as well as intracellular and extracellular molecular transport in cells deep within bone tissue.[51]

Figure 7 – A standard stress/strain curve of bone loaded in bending. [52]
B. OSTEOPOROSIS
Osteoporosis is by far the most common bone disease. Osteoporosis is a skeletal disorder characterized by compromised bone strength, predisposing to an increased risk of fracture.[24] The composition of the mineral and matrix, the fine structure of the trabecular bone, the porosity of the cortical bone, and the presence of micro-fractures and other forms of damage in bone are all important in determining bone strength. Changes in the fine structure or micro-architecture of trabecular bone are particularly important since the most common fractures in osteoporosis occur at sites where trabecular bone predominates.

Osteoporosis is classified as primary and secondary. Primary osteoporosis, by far the most common variety, has been also designated as postmenopausal, involutional, senile, and idiopathic osteoporosis, and seems to involve multiple pathogenetic mechanisms, many of which have not yet been adequately defined. In addition, there are many disorders that can lead to skeletal fragility with a defined cause - secondary osteoporosis - including genetic disorders (e.g., osteogenesis imperfecta and osteoporosis-pseudoglioma syndrome); endocrine disorders (e.g., Cushing syndrome, hyperparathyroidism, hyperthyroidism and hypogonadism); and inflammatory and nutritional disorders (e.g., rheumatoid arthritis, Crohn’s disease and celiac disease).[24]

**EPIDEMIOLOGY**

Osteoporosis reports a high rate of mortality and morbidity, not only in the Western countries, but also in Asia, Latin America, the Middle East, and Africa. It has been estimated that these regions will account for more than 70% of the estimated 6.26 million bone fractures, expected by the year 2050.[53] Osteoporosis affects both men and women, nonetheless the cumulative lifetime risk of having an osteoporotic fracture is 2–4 times greater in women.[23]

An estimated 40% of women and 13% of men aged 50 years old and older will sustain an osteoporotic fracture in their lifetime.[54] Taking into account future mortality trends, these figures rise to 47% for women and 22% for men.[55]
The three most common sites for osteoporotic fracture are the distal radius, the vertebral body and the upper femur.[23] Nonetheless, other bones are affected by osteoporosis, and yield a particular relevance regarding osteoporosis-associated morbidity and mortality. For instance, hip bone is of particular clinical relevance. Approximately 20–27% of women who sustain hip fracture, are expected to die within a year.[56] About half of the women who have had a hip fracture will experience long-term pain and disability,[57] and 20% will be expected to have severely impaired mobility within a year.[56]

In 2010, the number of deaths causally related to osteoporotic fractures was estimated at 43,000 in the European Union. Approximately 50% of fracture-related deaths in women were due to hip fractures, 28% to clinical vertebral and 22% to other fractures.[58]

**PATHOPHYSIOLOGY**

In adults and in physiological conditions, there is equilibrium between bone formation and resorption, in a dynamic process of bone remodeling. Osteoporosis occurs when the balance is disrupted and tilts in favor of resorption. Osteoporosis is a multifactorial disorder and any model for pathogenesis has to recognize that a different set of mechanisms may be operative in any given individual. Although a complete understanding of the underlying control mechanisms is not yet known, there are a number of features that have been reported to be associated with the development of skeletal fragility.

- **Age-related changes.** With the increase of age, osteoblasts replicate and synthesize extracellular matrix with progressively reestablished defects. The various growth factors deposited within the ECM also tend to become less effective with time.[59] Unfortunately, while new bone synthesis wanes with advancing age, the resorptive activity mediated by osteoclasts seems to remain essentially unaltered;
• **Hormonal influences.** The decline in estrogen levels associated with menopause correlates to an annual decline of as much as 2% of cortical bone and 9% of cancellous bone.[60] This can add up to 35% of cortical bone and 50% of trabecular bone within 30-40 years. It is therefore not surprising that roughly half of post-menopausal women are expected to suffer an osteoporosis-associated fractures, compared to 2-3% of men of the same age group.[60]

Bone loss accelerates in women at menopause because estrogen withdrawal increases the rate of bone remodeling, creating many foci of bone resorption on the endosteal surfaces. Estrogen deficiency increases the activity of osteoclasts, probably by suppressing their apoptosis.[42] The hypoestrogenic effects are attributable in part to augmented cytokine production (especially interleukin-1 and tumour necrosis factor). Moreover, estrogen regulates bone resorption by limiting the release of RANKL from osteoblasts, among other cells.[61] Loss of estrogen leads to significantly increased RANKL expression, which in turn result in excess formation and activity of osteoclasts.[62] At the same time, OPG levels increase with age, and it is possible that OPG production rises as a homeostatic response to limit the bone loss that occurs with an increase in other bone-resorbing factors.[42] Nonetheless, despite some simultaneous compensatory osteoblastic activity, the anabolic response seems to be inadequate to keep pace with the higher osteoclast-mediated bone resorption.[62]

Increased osteoclastic activity induces an augmented depth of erosion of bone by these cells, contributing to the trabecular penetration and disruption of bone architecture.[59] In each remodeling site, more bone is resorbed than replaced, producing a net negative BMU balance, which is the basis of bone loss. Moreover, estrogen deficiency increases the prevalence of osteocyte apoptosis, which might impair the ability of the osteocyte mechanosensory network to repair microdamage, and thus contribute further to bone fragility.[63] The initial rapid fall in bone mineral density (BMD) is the result of the increase in bone porosity of many BMUs, as remodeling moves
from a low rate before menopause to a high rate after menopause.[42] The rapid fall in BMD is the result of the normal delay in initiation of bone formation and its slower completion, within the many resorption cavities. The microarchitectural deterioration probably reflects the intensity and duration of osteoclast activity as well as the number of osteoclasts formed.[64]

The increased remodeling and negative bone balance produce bone loss, trabecular thinning and loss of connectivity, cortical thinning, and porosity.[59] Older, more mineralized interstitial bone, distant from surface remodeling, accumulates microdamage, whereas more superficial bone is replaced with younger and less mineralized bone, reducing stiffness.[65] Bone modeling by periosteal apposition reduces compressive stress by distributing loads on a larger area, and partly maintains bending strength.[66] Moreover, it may be impaired due to abnormalities in periosteal osteoblast function or osteocyte signaling.[59]

- **Lifestyle.** Smoking is associated with a reduced peak bone mass,[67] earlier menopause [68] and thinness[69], all of which are risk factors for osteoporotic fracture. Smoking appears to reduce bone mineral density by a mechanism that is independent of its effect on weight or estrogen metabolism, and may act by reducing calcium absorption.[70]

  Framingham cohort demonstrated that an alcohol intake, at around 200 ml or more per week, is a risk factor for bone loss, but concluded that weight, estrogen use, and cigarette smoking are also important predictors of bone health.[71] It is not known whether the lower bone mineral density found in alcoholics is mainly due to inadequate dietary intake, poor exercise, or a direct effect of alcohol in reducing osteoblastic activity;[23] however it is likely to be a combination of these factors.

  Because mechanical forces stimulate bone remodeling, reduced physical activity increases bone loss.[72] This effect is usually observed in an immobilized limb, but also occurs diffusely in a gravity-free environment.[73] Decreased physical activity in older individuals also contributes to senile
Because the magnitude of skeletal loading influences bone density more than the number of load cycles, the type of physical activity is important.\[74\]

- **Genetic factors.** Several population and genetic-based variations seem to play a role in peak bone mass and BMD variations. For instance, peak bone mass is greater in men than in women, and in Blacks than in Whites or Asians.\[23\] Also there is a higher concordance of peak bone mass in monozygotic than in dizygotic twins.\[22\] Moreover, women of reproductive age, whose mothers have postmenopausal osteoporosis, exhibit a lower BMD than do women of the general population.\[59\]

In fact, genetic variations explain high percentage of the variance in BMD.\[23\] Vitamin D receptor polymorphisms may account for approximately 75% of variation in the maximal peak bone mass achieved in any given individual.\[44\] Additional genetic variables can influence calcium uptake or PTH synthesis and, accordingly, influence related biological responses.

- **Calcium nutritional state.** Dietary calcium intake can majorly determine the status of bone metabolism. It is of particular relevance in worldwide population, that the majority of adolescent girls have insufficient dietary calcium intake in a period of rapid bone growth. As a result, they do not achieve the maximal peak bone mass that could be otherwise expected, and are therefore likely to develop clinically significant osteoporosis at an earlier age.\[23\]

1,25-Dihydroxyvitamin D, the active metabolite of vitamin D, has important physiologic activities, including the absorption of calcium from the gastrointestinal tract, the helping on the regulation of calcium handling in the kidney, the regulation of the homeostatic control of bone remodeling, and the regulation of the synthesis of the parathyroid hormone – functions important to the homeostatic control of calcium metabolism.\[46\] Any abnormality in vitamin D formation, metabolism or action could result in an increased bone
resorption and consequently accelerate bone loss.[24, 75] Subclinical vitamin D deficiencies have been verified in osteoporotic women.[76] Vitamin D may also have a direct effect on muscle strength and dexterity.[77] There appears to be a relationship between low serum vitamin D and age-related muscle weakness, increased body sway, increased risk of fall, and fall-related fractures.[78]

Overall, the reported mechanisms can be embraced in three summarizing features: 1) failure to achieve optimal peak bone mass and strength - largely determined by genetic background,[79] but which can also be substantially affected by lifestyle;[46] 2) accelerated bone loss due to resorption; 3) impaired bone formation response during remodeling – which physiologically occurs shortly after the achievement of peak bone mass, but may be greatly augmented by pathologic mechanisms.[80]

Osteoporosis may also develop in association with many other conditions – secondary osteoporosis. Causes include genetic disorders, hypogonadal states, endocrine disorders, gastrointestinal diseases, hematological disorders, rheumatic and autoimmune diseases, immobilization, and several other conditions, as portrayed in Table 1.
### Table 1 – Causes of Secondary osteoporosis.[81]

<table>
<thead>
<tr>
<th>Genetic Disorders</th>
<th>Hypogonadal Status</th>
<th>Endocrine Disorders</th>
<th>Gastrointestinal Diseases</th>
<th>Hematologic Disorders</th>
<th>Rheumatic and Auto-immune diseases</th>
<th>Miscellaneous</th>
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</thead>
<tbody>
<tr>
<td>Cystic Fibrosis</td>
<td>Androgen insensitivity</td>
<td>Acromegaly</td>
<td>Gastrectomy</td>
<td>Hemophilia</td>
<td>Ankylosing spondylitis</td>
<td>Alcoholism</td>
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<tr>
<td>Ehlers-Danlos syndrome</td>
<td>Anorexia nervosa</td>
<td>Adrenal insufficiency</td>
<td>Inflammatory bowel disease</td>
<td>Leukemias and Lymphomas</td>
<td>Lupus</td>
<td>Amyloidosis</td>
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<tr>
<td>Glycogen Storage Diseases</td>
<td>Athletic amenorrhea</td>
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<td>Malabsorption</td>
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<td>Chronic metabolic acidosis</td>
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<td>Gaucher’s Disease</td>
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<td></td>
<td>Celiac disease</td>
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<td></td>
<td>Congestive heart failure</td>
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<td>Hemochromatosis</td>
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<td>Primary biliary cirrhosis</td>
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<td>Depression</td>
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<td>End stage renal disease</td>
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<td>Epilepsy</td>
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<td>Idiopathic scoliosis</td>
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<td>Immobilization</td>
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Osteoporosis can also be a side effect of particular medical therapies as reported in the Table 2.
Glucocorticoid-Induced osteoporosis is by far the most common form of osteoporosis produced by drug treatment. While it has been known for many years that excessive production of the adrenal hormone cortisol can cause thinning of the bone and fractures, this condition, a form of Cushing’s syndrome, remains uncommon. With the increased use of prednisone and other drugs that act like cortisol for the treatment of many inflammatory and autoimmune diseases, this form of bone loss has become a major clinical concern. The concern is greatest for those diseases in which the inflammation itself and/or the immobilization caused by the illness also caused increased bone loss and fracture risk. Glucocorticoids, which are used to treat a wide variety of inflammatory conditions (e.g., rheumatoid arthritis, asthma, emphysema, chronic lung disease), can cause profound reductions in bone formation and may, to a lesser extent, increase bone resorption, leading to loss of trabecular bone at the spine and hip, especially in postmenopausal women and older men.[83] The most rapid bone loss occurs early in the course of treatment, and even small doses (equivalent to 2.5–7.5 mg prednisone per day) are associated with an increase in fractures.[84] The risk of fractures increases rapidly in patients treated with glucocorticoids, even before much bone has been lost. This rapid increase in fracture risk is attributed to damage to the

### Table 2 – Medications associated with secondary osteoporosis.[82]

<table>
<thead>
<tr>
<th>Medications associated with secondary osteoporosis</th>
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<tr>
<td>Glucocorticoids (and ACTH)</td>
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<td>Anticoagulants (heparin)</td>
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<tr>
<td>Anticonvulsants</td>
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<td>Cyclosporine A and Tacrolimus</td>
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<td>Cancer chemotherapeutic drugs</td>
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<td>Gonadotropin-releasing hormone agonists</td>
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<td>Lithium</td>
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<td>Methotrexate</td>
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<td>Parental nutrition</td>
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<tr>
<td>Thyroxine</td>
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</table>
bone cells, which results in less healthy bone tissue. To avoid this problem, health care providers are urged to use the lowest possible dose of glucocorticoids for as short a time as possible. For some diseases, providers should also consider giving glucocorticoids locally (e.g., asthma patients can inhale them), which results in much less damage to the bone.

Cyclosporine A and tacrolimus are widely used in conjunction with glucocorticoids to prevent rejection after organ transplantation, and high doses of these drugs are associated with a particularly severe form of osteoporosis.[85]

Bone disease has also been reported with several frequently prescribed anticonvulsants, including diphenylhydantoin, phenobarbital, sodium valproate, and carbamazepine.[81] Patients who are most at risk of developing this type of bone disease include those on long-term therapy, high medication doses, multiple anticonvulsants, and/or simultaneous therapy with medications that raise liver enzyme levels. Low vitamin D intake, restricted sun exposure, and the presence of other chronic illnesses increase the risk, particularly among elderly and institutionalized individuals. In contrast, high intakes of vitamin A (retinal) may increase fracture risk.[86]

Methotrexate, a folate antagonist used to treat malignancies and (in lower doses) inflammatory diseases such as rheumatoid arthritis, may also cause bone loss, although research findings are not consistent.

In addition, gonadotropin-releasing hormone (GnRH) agonists, which are used to treat endometriosis in women and prostate cancer in men, reduce both estrogen and testosterone levels, which may cause significant bone loss and fragility fractures.[87]
DIAGNOSIS

In the past, osteoporosis was often diagnosed on the basis of radiologic changes and in the presence of a low trauma fracture, for example a fall from standing and generally in postmenopausal women and older men.[23] More recently, an inverse relationship between bone mineral density and fracture risk was established and the diagnostic start to be based on BMD measurements, allowing the diagnose of osteoporosis and osteopenia before fractures occur, as well as the confirmation of the diagnosis in patients with fragility fractures.[88, 89]

**Radiogrammetry**

Radiogrammetry uses measurements derived from standard x-rays of the hand to determine an index that compares cortical thickness with the total bone width in the mid-shaft of at least two metacarpal bones.[82]

This technique has been used to assess bone mineral density of the peripheral skeleton, usually at the metacarpals. The metacarpal cortical thickness was used for many years to diagnose and predict the risk of osteoporosis. However, the sensitivity of this radiographic technique is poor,[90] and the results of metacarpal measurement do not reflect bone mineral density at more important sites such as the hip and spine.[91]

**Radiographic absorptiometry**

Radiographic absorptiometry (RA), also termed photodensitometry, uses a standard x-ray of the hand to measure density in the middle bones on the second, third and fourth fingers. Specialized equipment is used to calcite bone volume and bone density. The cortical thickness of the bones also can be measured.[82]

Radiographic analysis reveals recognizable bone loss only when 25–30% of bone density has been lost, at which time osteoporosis is generally considered to have developed. Although there is a correlation between bone mineral density in the peripheral and central skeleton,[91] the association is not strong enough to predict
central bone mineral density from peripheral measurements in a given subject.[91, 92] At present, the main role of radiography is in the diagnosis of fractures secondary to osteoporosis.

**BMD MEASUREMENT**

The most common method for measuring bone mass is Dual-Energy X-Ray Absorptiometry (DXA). Beside this one, Quantitative Computed Tomography (QCT), Peripheral Quantitative Computed Tomography (pQCT) and Quantitative Ultrasound (QUS) are also used.

- **Dual-Energy X-Ray Absorptiometry**

  Dual-energy X-ray absorptiometry (DXA) measures bone mineral density by determining the absorption of two beams of photons at two different energies.[93] This technique is accurate and involves low doses of x-rays. DXA is able to measure bone mineral density (as mass/area) in the proximal femur and lumbar spine, as well as the mineral density of the entire body. However, it cannot differentiate between the measurement of the cortical and trabecular bone, which are important determinants of bone strength and loss at different rates.[94] The cortical-to-trabecular ratio is 1:2 in the spine and 3:1 in the femoral neck.[23]

  DXA enables bone mineral density to be measured at the hip or spine, with greater precision than that described for other methods (precision error: 0.5–2%). The scanning time is around 5 min at each site and the radiation dose is low.[95, 96] Additional to the assessment of the classic anatomical sites, lateral views can now be used to give morphometric evaluations of vertebrae, to determine vertebral deformities and fractures.[23]

  The results are reported as a density measurement in g/cm² and T and Z scores. T scores correspond to the number of standard deviations (SDs) from the mean bone density values in normal sexmatched young adults. This is used to make a diagnosis of normal bone density, osteoporosis, or osteopenia in postmenopausal women and in men age 50 years and older.[97] Z scores represent the number of SDs from the normal mean value for age- and sex-matched control subjects. Z scores are used
preferentially to assess bone loss in premenopausal females and in men younger than the age 50 years.

The World Health Organization (WHO) defines osteoporosis as a result on BMD assessment, 2.5 SDs or more, below the mean value for young adults (a T score < −2.5), and severe osteoporosis, as a BMD below this cut-off and the concomitant occurrence of one or more fragility fractures.[89] The WHO defines osteopenia as a BMD T score between −1.0 and −2.5. It should be remembered that at the same time as osteoporotic fracture incidence is highest in those with the most pronounced osteoporosis, a substantial number of fractures occur in women who do not have very low bone density measurements.[98] A new algorithm from the WHO, for the definition of osteoporosis treatment thresholds, which includes other factors such as age, is currently awaited.

- **Quantitative Computed Tomography**

Quantitative computed tomography (QCT), with a suitable software package, enables the determination of the absorption of different calcified tissues, so that areas of particular interest, such as the vertebral body (which has a cortical-to-trabecular ratio of approximately 5:95), may be studied. The technique measures true densities with the results being expressed in g/cm³.[99]

An advantage of QCT is that trabecular bone is distinguished from cortical bone, and extra osseous calcium, which artificially increases the bone density measured by DXA, is readily identified.[100] Trabecular diameter and intertrabecular spaces can be measured using high-resolution CT, and abnormal trabecular architecture can be identified. The recent development of three-dimensional (3-D) CT allows the assessment of 3-D trabecular structural characteristics and may improve the ability to estimate bone biomechanical properties.[23]

Dual-energy scanning (with double radiation dose) may improve the accuracy, but worsens precision. Moreover is more expensive and involves a higher radiation dose than with the conventional QCT.
• **Peripheral Quantitative Computed Tomography**

Peripheral quantitative computed tomography (pQCT) uses specialized equipment to measure cortical bone (the outer, more solid shell of bone) and cancellous bone (the inner, honeycomb-like bone) in the forearm. This technique is used primarily for research.[82]

• **Quantitative Ultrasound**

Quantitative ultrasound (QUS) uses sound waves to assess bone mass and thus does not use radiation. This technique provides some information on the structural organization of bone, in addition to bone mass. The attenuation of ultrasound signals during their passage through bone may be measured by determining the reduction in ultrasound signal amplitude. Several ultrasound parameters used to characterize bone have been proposed, including broadband ultrasound attenuation, speed of sound, combined index, amplitude-dependent speed of sound and others.[101-103] For example the attenuation of the signal may reflect both the density and the architecture of bone, and the velocity of the signal reflects the density as the biomechanical properties (elasticity).

Ultrasound instruments have theoretical advantages over DXA in that they are radiation-free, portable, and inexpensive. However, at present, clinical use of ultrasound is difficult because of the absence of clear diagnostic criteria and the use of a variety of instruments. Moreover, because of the technological differences between devices, results cannot be extrapolated from one device to another.[104] Using a combination of both bone mineral density and broadband ultrasonic attenuation measurements, one may prove to have higher sensitivity and specificity for predicting fracture risk than the use of each method alone. However, for now QUS alone cannot be used for the diagnosis of osteoporosis or for monitoring the effects of treatment.[105] QUS is currently used only in research.
BIOCHEMICAL MARKERS OF BONE TURNOVER

Biochemical markers of bone turnover have been used widely in clinical research and represent the products of bone formation and resorption that are released into the circulation, and changes in these values reflect the dynamic process of bone metabolism. (Table 3)

Markers of bone-formation are released from osteoblasts and typically are measured in serum.[106] Largely because of their tissue specificity and assay sensitivity, the most useful markers are bone-specific alkaline phosphatase (BSAP) and osteocalcin.[107] Although type I collagen is the major product synthesized and secreted by osteoblasts, it also is produced by other tissues and current assays lack selectivity for bone derived type I collagen.[108] In addition, current assays for quantitating BSAP and osteocalcin are more effective at differentiating between normal and disease states compared with those for type I collagen.[108]

Bone-resorption markers are secreted during osteoclastic activity and include the collagen breakdown products pyridinoline, deoxypyridinoline, and cross-linked C- and N-telopeptides. Multiple assays are now available that can measure these products relatively quickly and inexpensively. [108] Tartrate-resistant acid phosphatase, which is a lysosomal enzyme present in cells, until recently was limited as a bone-resorption marker because early assays lacked specificity for the osteoclast-derived enzyme (TRACP) and because of its instability in assay samples.[108] Newer assays are now available that are selective for TRACP 5b, the osteoclast-specific isoform that is considered to be a promising marker for predicting vertebral fractures.[109] Indeed, in large prospective studies, biochemical markers of bone resorption have been associated with increased vertebral and nonvertebral fractures independently of BMD. However, their use in predicting fracture risk in specific patients has not been defined clearly. The value of these markers in the assessment of fracture risk therefore is likely to be in combination with other important risk factors, including BMD.[106]

Other potential uses of turnover markers include the ability to monitor drug efficacy, to predict increases in bone mass, and to assist in the selection of patients for
treatment. This makes them more suitable for treatment monitoring than bone density, as changes in density are often not apparent for two years.

Table 3 – Currently available bone turnover markers. [108]

<table>
<thead>
<tr>
<th>Bone formation markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
</tr>
<tr>
<td>Bone-specific alkaline phosphatase</td>
</tr>
<tr>
<td>Osteocalcin</td>
</tr>
<tr>
<td>Carboxyterminal propeptide of type I collagen</td>
</tr>
<tr>
<td>Aminoterminal propeptide of type I collagen</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bone-resorption markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
</tr>
<tr>
<td>Cross-linked C-telopeptide of type I collagen</td>
</tr>
<tr>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>N-telopeptide of collagen cross-links</td>
</tr>
<tr>
<td>C-telopeptide of collagen cross-links</td>
</tr>
<tr>
<td>Urine</td>
</tr>
<tr>
<td>Hydroxyproline</td>
</tr>
<tr>
<td>Pyridinolines</td>
</tr>
<tr>
<td>Deoxypyridinolines</td>
</tr>
<tr>
<td>N-telopeptide of collagen cross-links</td>
</tr>
<tr>
<td>C-telopeptide of collagen cross-links</td>
</tr>
</tbody>
</table>
LABORATORY TESTING FOR SECONDARY CAUSES OF OSTEOPOROSIS

General consensus sustain that a minimum screening laboratory profile should be considered for all patients who are diagnosed as having osteoporosis, since many diseases are associated with bone loss, being secondary causes of osteoporosis. Nonetheless, no consensus exists regarding which tests should be done.[97]

Institute for Clinical Systems Improvement (ICSI) proposes a rationale approach for the biochemical assessments of patients with newly diagnosis osteoporosis, as reported on Table 4.
Table 4 – ICSI Guidelines for Laboratory testing in patients with newly diagnosed osteoporosis.[97]

<table>
<thead>
<tr>
<th>Laboratory Test</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine</td>
<td>Renal failure is associated with secondary hyperparathyroidism.</td>
</tr>
<tr>
<td>Liver function tests</td>
<td>Intrinsic liver disease and cholestatic disorders are associated with multifactorial causes of increased risk of osteoporosis.</td>
</tr>
<tr>
<td>Serum calcium</td>
<td>Increased in patients with hyperparathyroidism and decreased in those with malabsorption or vitamin D deficiency.</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Increased in patients with Paget disease of bone, prolonged immobilization, acute fractures, and other bone diseases.</td>
</tr>
<tr>
<td>Serum phosphorus</td>
<td>Decreased in patients with osteomalacia.</td>
</tr>
<tr>
<td>Thyroid studies (thyrotropin and thyroxine)</td>
<td>Hyperthyroidism-associated bone loss.</td>
</tr>
<tr>
<td>Sedimentation rate or C-reactive protein</td>
<td>May indicate an inflammatory process or monoclonal gammopathy associated with bone loss.</td>
</tr>
<tr>
<td>Complete blood cell count</td>
<td>To evaluate for bone marrow malignancy, infiltrative processes (anemia, low white blood cells, or low platelets), or malabsorption (anemia, microcytosis, or macrocytosis).</td>
</tr>
<tr>
<td>Urinary calcium excretion</td>
<td>24-hour urinary calcium excretion on a high calcium intake diet screens for malabsorption and hypercalciuria—a correctable cause of bone loss; low 24-hour urinary calcium excretion suggests vitamin D deficiency, osteomalacia, or malabsorption due to small bowel disease such as celiac sprue.</td>
</tr>
<tr>
<td>Serum 25-hydroxyvitamin D</td>
<td>To identify vitamin D deficiency.</td>
</tr>
<tr>
<td>Serum intact (whole-molecule) PTH</td>
<td>Screening for hyperparathyroidism.</td>
</tr>
<tr>
<td>Serum testosterone (total and free)</td>
<td>Screening for hypogonadism in men; if abnormal, Luteinizing Hormone (LH), Follicle-stimulating hormone (FSH), and prolactin measurements may be indicated to determine the cause of the hypogonadism.</td>
</tr>
<tr>
<td>Serum estradiol</td>
<td>Screening for hypogonadism in premenopausal or perimenopausal women; if abnormal, LH, FSH, and prolactin measurements may be indicated to determine the cause of the hypogonadism.</td>
</tr>
<tr>
<td>Tissue transglutaminase antibodies</td>
<td>If gluten enteropathy is suspected clinically.</td>
</tr>
<tr>
<td>24-hour urinary free cortisol and overnight dexamethasone suppression test</td>
<td>If hypercortisolemia is suspected.</td>
</tr>
<tr>
<td>Serum and urine protein electrophoresis with immunoelectrophoresis as indicated</td>
<td>If monoclonal gammopathy is suspected.</td>
</tr>
</tbody>
</table>
ASSESSMENT OF RISK OF OSTEOPOROSIS OR FRACTURE: ASSESSMENT MODELS

FRAX approach

FRAX® (fracture risk) is a computer-based algorithm that calculates the 10-year probability of a major fracture (hip, clinical spine, humerus or wrist fracture) and the 10-year probability of hip fracture.[110] Fracture risk is calculated from age, body mass index and dichotomized risk factors comprising prior fragility fracture, parental history of hip fracture, current tobacco smoking, ever use of long-term oral glucocorticoids, rheumatoid arthritis, other causes of secondary osteoporosis and alcohol consumption. The use of clinical risk factors in conjunction with BMD and age improves sensitivity of fracture prediction without adverse effects on specificity. [110]

The models were developed from large population-based cohorts from Europe, North America, Asia and Australia. FRAX is a better risk tool for older women after age 60 years who are at risk for hip fractures, Spine DXA cannot be used in the FRAX analysis because elderly people with osteoarthritis show an artifactual increase in spine BMD by 10% and this limits the use of FRAX in predicting which women in the age group 50–60 years will develop spine osteoporosis in the next 10–15 years.[111]

QFractureTM

The QFractureTM tool is based on a UK prospective open cohort study of routinely collected data from 357 general practices on over 2 million men and women aged 30–85 years (www.qfracture.org). Like the FRAX tool, it takes into account history of smoking, alcohol, corticosteroid use, parental history (of hip fracture or osteoporosis) and several secondary causes of osteoporosis. Unlike FRAX, it also includes a history of falls (yes/no only over an unspecified time frame) and excludes previous fracture history and BMD. It has been internally validated (i.e. from a stratum of the same population) and also externally validated in the UK.[112]
BONE REGENERATION IN OSTEOPOROTIC CONDITIONS

The bone regeneration process around implanted biomaterials are reported as very similar to the principle mechanism occurring during fracture repair and involve a cascade of cellular and extracellular events.[113] Bone is complex and is mainly composed by cells, mineralized extracellular matrix and organic extracellular matrix. In the organic extracellular matrix are local factors such as cytokines and growth factors that are released in the lesion site, that play an interesting role in this process. The further studied local factors included the interleukin 1 (IL-1), interleukin 6 (IL-6), tumor necrosis factor alfa (TNF-α), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β1) and bone morphogenic proteins (BMP). In the initial phases to reaction to trauma PDGF and TGF-1 are released by activated platelets. Then macrophages and other inflammatory cells release FGF, PDGF, TGF-β1 and IL-1 and IL-6. These factors are present during the first hours following the trauma to achieve the regeneration and act on the bone, periosteal, medullar and adjacent soft tissue cells. Mesenchymal staminal cells are present 3 days after the trauma and remain for days, playing an important role in this process.

Osteoporosis can modify cell proliferation, cell synthetic activity, cell reactivity to local factors and mesenchymal staminal cell number. The bone multi-cellular units are also important for the remodeling of bone. These units are altered on osteoporosis, on the activation frequency and the formation and resorption phases. Decreased ability of osteoblasts to fill resorption lacunae will, contribute to unbalanced remodeling and loss of bone, but this component varies much less than activation frequencies in the healthy and diseased skeleton.[114] These biological differences, associated with the biomechanical and microarchitectural bone properties, are involved in a reported increased risk of bone remodelling in osteoporotic bone.[115]
ORAL IMPLICATIONS OF OSTEOPOROSIS

It is considered that osteoporosis could be the cause behind mineral density and mass reduction of jawbones, due to the influence of estrogen. Some studies report an association of post-menopausal tooth loss and metacarpal bone mass, and others consider a relationship between general low skeletal bone mineral densities to tooth loss.[116, 117] Others studies sustain that the thickness of the mandibular inferior border tends to reduce in subjects with osteoporosis.[118, 119] Nonetheless, others refute this association and have found no relationship between skeletal and mandibular BMD.[120-122]

Alveolar bone can undergo the same metabolic changes as limb bones since osteoporosis is a systemic disease that affects all the skeletal bones. Particularly, the similarity in microstructures and composition between the jawbones and the limb bones may support such assumption. Although, recently, it has been suggested that the embryologic origin of the maxillofacial bones changes their response to osteoporosis.[123] Jawbones are derived from cells of the anterior neural crest while the limb bones are developed from the lateral plate mesoderm, and these developmental stages of the jaws are complex processes being coordinated by a variety of genes and transcription factors not yet clearly understood.[124]

Consequently, it is not surprising to know that limb bones and the bones of oral cavity show regionally different gene expression resulting in site-specific bone cell behavior and activity as well as matrix composition. This might explain why jawbones can respond differently to osteoporosis.[124]

Osteoporosis has also been found to be associated with periodontal and alveolar bone loss and temporomandibular joint bone loss. Osteoporosis in the jaws may present a risk for alveolar bone loss following the use of full dentures.[16] Conflicting results highlight the controversy of whether a causal relationship between systemic bone loss and various oral conditions is present or not.

Several authors report the association of osteoporosis in postmenopausal women with periodontitis, attachment loss and gingival recession. [125-127] Various studies have indicated that reduced bone mineral density was associated with
increased clinical attachment loss. [128, 129] Nonetheless, others have found weak or no significant associations between systemic bone mineral density and clinical attachment loss.[130, 131]

As far as dental implants and bone grafts are concerned, because of increasing life expectancy and popularity of these treatments, surgeons face a larger amount of osteoporotic patients who require bone augmentation and implant placement. The main concern of performing oral surgery in patients with osteoporosis is the premise of a metabolic disease affecting the bones of the maxilla and mandible in the same way it impairs other bones, reducing the bone mass.[20] In surgical procedures, such as alveolar bone augmentation or implant rehabilitation, the bone healing and the incorporation of the graft or an implant, include a course of events involving osteogenesis, osteoclastic resorption, osteoinduction and osteoconduction.[132] In osteoporosis the success of these interventions may be altered due to the impaired bone formation and resorption activity, the changes in the bone density and micro-architectural of the patient’s bone.[20] The estimated lower implant fixation in osteoporosis is probable due to the structural and mechanical bone changes, decreased number of bone formation (osteoblasts) cells, increased activity of osteoclasts cells, and an imbalanced bone remodeling at the site of attempted osseointegration.[133] Some authors have already anticipated that in osteoporotic induced animal models, before, after or simultaneously with the placement of implants, changes the process of bone regeneration, particularly in trabecular bone, and causes a significant decrease in the percentage of bone–implant contact and in the mechanical strength of the bone-implant interfaces. [115, 134] The suggestion that osteoporosis is a risk factor for dental implant failure has been proposed by several studies. [135-137]

However, osteoporosis does not imply that the systemic bone is unable to achieve osseointegration; there is a correlation between systemic bone loss and loss of density and quantity in the jaw but osteoporosis is not an absolute contraindication for bone augmentation and implant therapy. Though, modifiable risk factors for osteoporosis should be eliminated before surgery.[137-141]
The diagnosis of jaw osteoporosis is of major importance. It requires assessment of the bone mineral content (BMC)/density (BMD) using specially constructed jaw bone scanners and development of a corresponding gender-related set of normal BMC/BMD. Nowadays, the available techniques for in vivo bone mass measurement techniques in the craniofacial area are expensive and not cost effective to screen the general population.[142] Recent studies have suggested that because dental panoramic radiographs are frequently made during the general practice of dentistry, the findings on such exams may help dentists to identify patients with undetected low BMD and refer them to medical professionals for bone densitometry.[142, 143]

Some authors report that mandibular bone density may be indicative of systemic bone mineral density and, in most cases, osteoporosis may be associated with a severe decrease in BMC of the jaws.[138] These kind of analyses would not be used as a diagnostic, but rather to refer patients for appropriate evaluation and treatment as necessary.[144] Adding to that, the only site of the jaws which fulfills the criteria for a reference site is the basal area of the mandible, posterior to the premolar region.[138]

Systemic treatment involving bone metabolism affects BMC/BMD in the skeleton, including the jaws, either negatively (long-term high dose glucocorticosteroids) or positively (calcium + vitamin D3, estrogen replacement therapy).[145] Nevertheless, it still remains to be tested to what extent this effective preventive therapy against general osteoporosis may prevent osteoporosis in the jaws.

On the other side, the effect of bisphosphonates on the jaw BMC and alveolar bone loss has not been full analyzed. These drugs are still under evaluation and future trials ought to comprise analyses of possible adverse effects of arrested bone resorption in relation to periodontal and surgical diseases and treatments or, in case of abrupt changes, in the function of the jaws. Further studies on the effect of implant treatment with and without bone transplantation in relation to the BMC/BMD changes in jaws and the systemic bone metabolic changes will also be of special interest.[16]
BMD in the mandible has proven, in a number of studies, to be positively correlated with that in lumbar spine, femoral neck, and forearm, which are important sites in osteoporosis.[146]

Another indicator to aid in the diagnosis of osteoporosis and osteopenia referred by Reddy et al. consists in the analysis of salivary parameters such as calcium, phosphorus and alkaline phosphatase, along with some oral signs like periodontitis and number of missing teeth.[147] The authors state that they could be indicators to aid in the diagnosis of osteoporosis and osteopenia in postmenopausal women.[147]
MANAGEMENT OF OSTEOPOROSIS

Several non pharmacological and pharmacological interventions are recommended to an osteoporotic condition, depending on the fracture risk assessment, that can be measured for example by the FRAX system.[111]

NON-PHARMOLOGICAL MANAGEMENT

Preventive treatments have the intention to increase peak bone mass and reduce the subsequent rate of bone loss. All patients with osteoporosis and fractures should be given advice on lifestyle measures that decrease bone loss. These include eating a balanced diet rich in calcium and vitamin D, moderating tobacco and alcohol consumption, maintaining regular physical activity, and exposure to sunlight.

• Immobilization Vs. Exercise

Immobilization is an important cause of bone loss, as we can see in immobilized patients that lose as much bone in a week when confined to bed than they would otherwise lose in a year. For this reason, immobility should, wherever possible, be avoided. [58]

A regular and well structured exercise can be a significant factor in osteoporosis prevention, as it increases muscle mass and strength, improves balance and coordination and reports a reduce risk of falls about 25%, in elderly persons.[148] Weight-bearing exercise appears to be effective in maintaining or increasing bone density at the lumbar spine and hip, in postmenopausal women.[149]

• Fall Prevention

Elderly patients have high risk factors associated with fall prevention that were observed in randomized clinical trials, allowing the accurate measures to prevent them.[150] Risk factors consist of visual impairment, impaired cognition, poor balance,
muscle weakness, joint deformities due to osteoarthritis, postural hypotension, multiple medications, and environmental hazards.

The following measures can be implanted to reduce falls risk such as: physical therapy to improve strength and balance, correcting decreased visual acuity, reducing consumption of medication that alters alertness and balance, prevention or treatment of hypotension, elimination of environmental hazards and use of appropriate footwear or clothing.[58, 151]

Several randomized trials have shown that wearing hip protectors can distinctly reduce hip fracture risk, mostly in the elderly living in nursing homes. However a meta analysis of randomized controlled trials has, cast some doubt about the anti-fracture efficacy of this preventive measure.[58]
PHARMACOLOGICAL INTERVENTIONS

The methods of pharmacological intervention of osteoporosis may be broadly divided into antiresorptive agents that prevent the bone resorption (such as hormone replacement therapy (HRT), selective estrogen receptor modulators (SERMs), calcitonin, bisphosphonates, denosumab) and anabolic agents that stimulate bone formation (such as teriparatide and strontium ranelate - which also has some antiresorptive activity).[97, 111, 152] Antiresorptive agents decrease bone resorption and, due to transient uncoupling of bone turnover, result in a small increase in BMD of between 5 and 10%, usually in the first or second year of treatment. In contrast, anabolic agents can increase BMD by up to 20%. Recently, combined regimens have been under investigation.[97]

A. Antiresorptive agents

• **Hormone replacement therapy (HRT)**
  Estrogens reduce the accelerated bone turnover induced by menopause and prevent bone loss at all skeletal sites regardless of age and duration of therapy. Therapeutic approach may consist of estrogens administration alone or in combination with progestin. HRT slows bone turnover and increases BMD at all skeletal sites in early and late postmenopausal women, and is an appropriate method of treatment and prevention of osteoporosis and which effects, both beneficial and adverse, have been well studied.[57, 153-155] A majority of literature reports show that HRT decreases fragility fracture risk by 20 to 35%, and interruption of the HRT results in acceleration of bone turnover, decrease in BMD and eventual loss of anti-fracture efficacy. Although, HRT use has declined as a result of patient concerns about breast and endometrial cancer, and increased incidence of cardiovascular events (unstable angina, thromboembolic stroke, venous thromboembolism), and also as a slight increase in the risk of dementia.[155-157]

  HRT should remain a mainstay of treatment, particularly for prevention of the disease in younger postmenopausal women. Some countries chose to recommend HRT
only for climacteric symptoms, at a dose as small as possible and for a limited period of time.[58]

It remains to be seen whether several years of therapy around the time of menopause can result in future fracture prevention for years to come.[23]

- **Selective estrogen receptor modulators (SERMs)**

  SERMs are synthetic molecules, non-steroidal agents, which bind to the estrogen receptor and act as estrogen agonists or antagonists, depending on the target tissue. The concept of SERMs was triggered by the observation that tamoxifen, which is an estrogen antagonist in breast tissue, is a partial agonist on bone, reducing the rate of bone loss in postmenopausal women.[58, 158-160]

  Raloxifene is the only SERM broadly available for the prevention and treatment of postmenopausal osteoporosis. Raloxifene prevents bone loss and reduces the risk of vertebral fractures by 30–50 % in postmenopausal women with low bone mass and with osteoporosis with or without prior vertebral fractures. Raloxifene reports the benefits of cardiovascular prevention, although its common adverse effects include increase risk of venous thromboembolism and increased vasomotor symptoms.[159] RUTH (Raloxifene Use for The Heart) study[159] confirmed the reduction in vertebral fractures, and the reduction of breast cancer, but demonstrated conclusively that raloxifene does not reduce hip fracture incidence therefore its use is not appropriate for women who are at high risk of non-vertebral fractures. [58, 159, 161]

  Lasofoxifene is another third generation SERM with excellent oral bioavailability. It has a high affinity for both ERα and ERβ, approximately the same as estradiol, and about 10-fold higher than the other SERMs raloxifene, tamoxifen and droloxifene. Two doses of lasofoxifene 0.25 and 0.5mg were evaluated in a randomized trial (PEARL study). In the three-year analysis lasofoxifene 0.25 mg and 0.5 mg daily increased lumbar spine BMD by 3.0% and 3.1% respectively, increased femoral neck BMD by 2.9%-3.0% respectively relative to placebo. Lasofoxifene 0.25 mg and 0.5 mg/d reduced the risk of vertebral fractures by 31% and 42%, respectively (P < 0.002), while nonvertebral fractures were significantly reduced by 22% with the 0.5
mg/d dose. The higher dose of lasofoxifene reduced breast cancer by 81% and the lower dose by 49%. [162]

Bazedoxifene is a selective estrogen receptor modulator that has been approved in Europe but is only available in Spain and Germany and recently approved in United States. Bazedoxifene was shown to significantly reduce the risk of new vertebral fracture, with favorable effects on bone mineral density, bone turnover markers and the lipid profile.[163] In a subgroup of women at increased risk of fracture, bazedoxifene significantly decreased non-vertebral fracture risk. In contrast to raloxifene, the efficacy of bazedoxifene is dependent on the level of fracture risk. However like with raloxifene, venous thromboembolic events, primarily deep vein thrombosis, leg cramps and hot flushes were more frequently reported in the active treatment groups compared with the placebo group.[164]

- **Bisphosphonates (BPs)**

  BPs are stable analogs of pyrophosphate, which bind to the bone surface and inhibit osteoclastic activity. They have a strong affinity for bone apatite, both in vitro and in vivo, which is the basis for their clinical use. They are potent inhibitors of bone resorption and produce their effect by reducing the recruitment and activity of osteoclasts and increasing their apoptosis. The potency and chemical affinity to bone of bisphosphonates determines their effect to inhibit bone resorption and varies greatly from compound to compound.

  Bisphosphonates are available in oral and intravenous formulations, with weekly, monthly and annual dosing schedules. They persist in the skeleton for many months or years and their duration of action is prolonged, beyond the period of administration. Bisphosphonates have been shown to reduce vertebral fracture risk and increase BMD, whereas some have demonstrated reductions in non-vertebral and hip fracture risk as well.[97, 152]

- **Alendronate** - has been reported to preserve bone mineral density, reduce approximately by half the vertebral, wrist and hip fracture risk in women
with prevalent vertebral fractures and to be well tolerated in women with low bone mineral density, in large prospective studies.[165, 166]

- **Risedronate** - decreases the incidence of new vertebral and peripheral fractures by the same extent as alendronate in women with low BMD and in women with prevalent vertebral fractures.[167]

- **Ibandronate** - is a potent nitrogen-containing bisphosphonate available as a once-monthly oral formulation for the treatment and prevention of osteoporosis. It has been showed that it can induced a rapid, pronounced and persistent decrease in bone turnover, increase BMD and reduce the incidence of vertebral fractures by at least 50%.[168, 169] It has been reported that significantly more women with postmenopausal osteoporosis preferred once-monthly ibandronate therapy to once-weekly alendronate therapy.

- **Zoledronic acid** - is a bisphosphonate which principal advantage is its extended dosing interval, allowing a once-a-year administration. There is evidence of a clinically significant effect on the increase in bone density and reduce fracture risk in the hip, spine and total body in women with osteoporosis.[170, 171]

Several apprehensions have been discussed such as the low oral absorption, this is less than 1 percent of the dose and must be taken on an empty stomach for maximal absorption with the patient sited upright. Particular side effects with systemic repercussions have been reported included the gastrointestinal intolerability, ranging from mild acid reflux to serious esophageal ulcers. Musculoskeletal pain is a significant side effect, like hypocalcaemia, atrial fibrillation, among others.

Some possible side effects of the bisphosphonates have been raised, because they are potent suppressors of bone resorption and such extreme inhibition of bone remodeling may lead to an accumulation of microdamage, which might compromise bone strength and increase the risk of low trauma fracture or delay fracture healing.[172-175]
Lately there have been long-term safety concerns about bisphosphonates that include a possible increased risk of atypical femoral fractures and osteonecrosis of the jaw. It is possible that both are very rare adverse events and are time related. It has been estimated that these risks in osteoporotic are 1 in 50,000 to 1 in 150,000. These events are more common in patients with cancer but these patients are given much higher doses of bisphosphonates for metastatic bone disease as well as taking chemotherapy that is toxic to bone cells. [176]

• **Calcitonin**

Calcitonin is an endogenous polypeptide hormone that directly suppresses the activity of osteoclasts and also inhibits their recruitment. Salmon calcitonin is approximately 40–50 times more potent than human calcitonin, and the majority of clinical trials have been performed with salmon calcitonin.[58] Calcitonin prevents bone loss and vertebral fractures, but it has not been shown to reduce nonvertebral or hip fractures.[177] Calcitonin as a short-term effect on acute pain relief and may be useful in the management of the pain associated with acute osteoporotic fractures.[178] Calcitonin is approved in the United States for administration by the subcutaneous, intramuscular, and intranasal routes. However some concerns were reported about the use of a nasal spray formulation of the peptide hormone salmon calcitonin can be associated with an increased risk of cancer. [111]

Since other medications with improved efficacy in fracture reduction are available, and the downsides of repeated injections and the high costs of the nasal formulation, the use of calcitonin as a first-line treatment for osteoporosis was excluded.[58, 177-179]

• **Denosumab**

Denosumab is a human monoclonal antibody that specifically binds RANKL, blocks the binding of RANKL to RANK thereby reducing bone resorption and increasing bone density. The FDA has approved the anti-fracture efficacy of denosumab using 60
mg subcutaneously every 6 months for treatment of osteoporosis. Nevertheless, it is not yet approved for prevention (pending long-term safety data). Denosumab is shown to improve bone mineral density (BMD) in postmenopausal women with low BMD.[111]

In the FREEDOM trial, 7868 postmenopausal women (60 to 90 years of age) with osteoporosis were randomly assigned to subcutaneous denosumab (60 mg every six months) or placebo for three years; denosumab increased BMD of the lumbar spine and total hip compared with placebo and significantly reduced the risk of new vertebral fractures by 68%, hip fractures by 40%, and non-vertebral fractures by 20%.[180] In FREEDOM extension trial of 4550 postmenopausal women, the FREEDOM denosumab group is continued on 3 more years of denosumab for a total of 6 years and women from the FREEDOM placebo group are given 3 years of denosumab.[181] Lumbar spine and hip BMD increased significantly at the end of 6 years (15.2% and 7.2% respectively). In the cross over group lumbar spine and total hip BMD increased by 9.4% and 4.8% respectively. The effects of denosumab on fracture risk are particularly marked in patients at high fracture probability.

In the sixth year of the extension trial, six cases of osteonecrosis of jaw were observed in the long-term group. Atypical fracture was reported in one participant. The most common side effects of denosumab include urinary and respiratory tracts infections, cataracts, constipation, rashes and joint pain. [58, 111]

Denosumab, by targeting the RANKL pathway, has a fundamentally different mechanism of action from that of bisphosphonates. Denosumab prevents RANKL from binding to its receptor, RANK, thereby inhibiting the development, activation, and survival of osteoclasts. This is different from the mechanism of action of bisphosphonates, which bind to bone mineral and probably inhibit osteoclast function mainly by being taken up by osteoclasts at sites of bone resorption (Figure 8).[182]

This may explain differences in the degree and rapidity of reduction of bone resorption, their potential differential effects on trabecular and cortical bone, and the reversibility of their actions. Denosumab has shown efficacy against vertebral, nonvertebral, and hip fractures compared with placebo.[58, 111]
LITERATURE OVERVIEW

Bisphosphonates

Denosumab

BPs bind to bone mineral and are taken up by mature osteoclasts at sites of bone resorption

Denosumab blocks RANKL

BPs cause loss of resorptive function, but 'disabled' osteoclasts may persist

Denosumab blocks osteoclast formation, function and survival

Figure 8 – Osteoclast inhibition with denosumab vs. bisphosphonates (BPs).[182]

B. Anabolic agents

- **Strontium ranelate**

Strontium ranelate appears to both stimulate bone formation and inhibit bone resorption.[183] It leads to the differentiation of pre-osteoblast to osteoblast, which increases the bone formation and also stimulates osteoblasts to secrete osteoprotegerin leading to the decrease of bone resorption. The vertebral and hip fractures decreases by about 40%, in some studies.[183, 184]

Strontium ranelate is an orally active drug recommended at a daily dose of 2g, between meals, due to its poor absorption when in presence of food. This drug is not recommended for patients with severe renal impairment. The most common adverse events are nausea and diarrhea, an increase in the incidence of venous
thromboembolism (VTE), and some cases of drug reaction with eosinophilia and systemic symptoms syndrome have been reported.[185, 186]

- **Teriparatide**
  Is the name given to the human recombinant parathyroid hormone (PTH) that has an identical sequence to the 34 N-terminal amino acids (the biologically active region) of the 84-amino acid human parathyroid hormone. Teriparatide increases serum calcium by reducing renal excretion and mobilizing bone calcium through increased osteoclastic resorption. It also indirectly increases intestinal calcium absorption. Moreover, its effect on bone is also anabolic, since it increases bone formation.[187] Reductions in vertebral fractures of around 65% were reported and significant reductions in all non-vertebral fractures have been observed, although a significant reduction in hip fracture has not yet been established.[188] The recommended dose is 20 μg of teriparatide daily, given as a subcutaneous injection. Beneficial effects on non-vertebral fracture with teriparatide have been shown to persist for up to 30 months after stopping the medication.[189]

  The most common reported adverse events in patients treated with teriparatide are nausea, pain in the limbs, headache and dizziness. Small and passing elevations of serum calcium concentrations have been observed following the injection teriparatide. The use of peptides of the PTH family is contra-indicated in conditions characterized by abnormally increased bone turnover (e.g. pre-existing hypercalcaemia; metabolic bone diseases other than primary osteoporosis, including hyperparathyroidism and Paget's disease of the bone; unexplained elevation of alkaline phosphatase; prior external beam or implant radiation therapy to the skeleton or in patients with skeletal malignancies or bone metastasis). Severe renal impairment is also a contraindication. Studies in rats have indicated an increased incidence of osteosarcoma, with long-term administration of very high doses of teriparatide from the time of weaning. These findings have not been considered relevant for patients treated with very much smaller doses of teriparatide.[58]
A comparative analysis of the efficacy on drug-based fracture prevention therapies is presented and safety of osteoporosis treatments in Table 5.

Table 5 - Antifracture efficacy of the most frequently used treatments and safety of postmenopausal osteoporosis. (+ evidence; x no evidence; 0-no effects). Adapted from[94]

<table>
<thead>
<tr>
<th>Drug</th>
<th>Route</th>
<th>Vertebral fractures</th>
<th>Non-vertebral fractures (hip)</th>
<th>Side-effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bisphosphonates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>. Alendronate</td>
<td>Oral</td>
<td>+</td>
<td>+</td>
<td>Osteonecrosis of the jaw, subtrochanteric femur fractures</td>
</tr>
<tr>
<td>. Risedronate</td>
<td>Oral</td>
<td>+</td>
<td>+</td>
<td>Oesophageal irritation</td>
</tr>
<tr>
<td>. Ibandronate</td>
<td>Oral / IV</td>
<td>+ / x</td>
<td>x / x</td>
<td>Oesophageal irritation, acute phase reaction, potential renal toxic effects</td>
</tr>
<tr>
<td>. Zoledronic acid</td>
<td>IV</td>
<td>+</td>
<td>+</td>
<td>Osteonecrosis of the jaw, subtrochanteric femur fractures</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>Oral</td>
<td>+</td>
<td>0</td>
<td>Thromboembolic disease</td>
</tr>
<tr>
<td>Strontium ranelate</td>
<td>Oral</td>
<td>+</td>
<td>+</td>
<td>Thromboembolic disease, drug rash with eosinophilia systemic syndrome, abdominal discomfort</td>
</tr>
<tr>
<td>Teriparatide</td>
<td>Subcutaneous</td>
<td>+</td>
<td>0</td>
<td>Hypercalcaemia, nausea, diarrhea</td>
</tr>
</tbody>
</table>
New compounds are in development and in advanced clinical trials, and the number of available drugs will increase considerably in the coming years. Present antiresorptive treatments are effective, but some are limited by side effects, concurrent comorbidities, and inadequate long-term compliance. Many of the new drugs combine efficacy with convenient administration that might translate into better adherence. Patients with severe osteoporosis may benefit from the sequential use of one drug to stimulate bone formation in association with another to prevent resorption. The effectiveness of such combinations has not been studied extensively, and must be supervised by a physician who has experience of this method of treatment.

Still, conventional antiresorptive such as amino bisphosphonates and denosumab can profoundly suppress bone resorption and formation, which might contribute to the pathogenesis of osteonecrosis of the jaw. Some of these issues are still under debate, and long-term clinical data are needed.

New therapies, as the odanacatib and saracatinib represent a distinct class of antiresorptive that inhibit osteoclast activity rather than impairing osteoclast viability, however if these compounds have an advantage over conventional antiresorptive remains to be seen.[94, 190] Calcilytic drugs and antagonist of Wnt inhibitors are encouraging developments.[94] Moreover it is probable that new bisphosphonates will emerge and, new forms of administration of parathyroid hormone (PTH) peptides are being developed.[191, 192] Another form of HRT is under development, comprising the combination of estrogen with a SERM.

Although the ideal osteoporosis treatment has not yet been developed it should take into account these parameters:

- Anti-fracture efficacy at various skeletal sites, including spine, non-vertebral sites, and hip
- High safety margin, both skeletal and extra-skeletal
- Mode of administration and treatment interval translate into patient’s adherence
- Compatibility with drugs prescribed for other medical conditions
- Inexpensive cost. [94]
ANIMAL MODELS OF OSTEOPOROSIS

In vivo research reaching hand of animal models has been a preferred experimental methodology since, by approximation, provides pertinent data gathering regarding physiological and pathological conditions that could be of hand to establish more effective clinical interventions. Accordingly, experimental in vivo research establishes a bridge connection between in vitro experimentation and clinical trials.

Experimental animal models are essential to provide an adequate approximation to translational applications and clinical settings. Accordingly, the selection of the appropriate investigative design and animal model relies, in the very end, in the therapeutic modality that is being tested. Furthermore, the selection of an appropriate animal model relies on: 1) appropriateness as an analog, 2) transferability of information, 3) genetic uniformity of organisms where applicable, 4) background knowledge of biological properties, 5) cost and availability, 6) generalizability of the results, 7) ease and adaptability to experimental manipulation, 8) ecological considerations and 9) ethical and societal implications.[113, 193]

ANIMAL MODELS OF OSTEOPOROSIS

A proper experimental animal model for the study of osteoporosis reduces bioethics problematic and minimizes the limitations associated with studying the disease in humans, namely time and behavioral variability among test subjects.[194] Since 1994, the United States Food and Drug Administration (FDA) requires data from both the rat and a well validated large animal model for preclinical evaluation of new experimental anti-osteoporosis drug therapies.[195]

Animal models play a crucial role in osteoporosis - they allow the research of biological and biomechanical characteristics of bone metabolism/remodeling, orthopedic implants placement, bone graft substitutes and prosthetic devices. Experimental designs can be tested on large numbers of animals and maintained within
a high confidence level of experimental control.[196] These models have the advantage of skirting the elevated cost, long time frame and biosafety of the clinical testing.[197]

Several mammalian species have been used in bone-related fundamental, feasibility and bioactive research, such as the mouse, rat, rabbit, sheep, dog, goat and, less frequently, others such as nonhuman primates.

Some large animals could be used as models of osteoporosis, but the most suitable are the dog, the pig, the sheep, and the nonhuman primates (Table 6).[198]

The dog as a model for estrogen depletion was extensive reviewed. This model offers numerous advantages. The most relevant feature is that dogs possess haversian systems of cortical bone and internal bone remodeling of cortical and trabecular bone similar to that of the human.[199] They are less expensive than the primate, and are in general easier to work with. There are, however, disadvantages, available reports on the nature of the skeletal response of the dog skeleton to ovariectomy are conflicting, probably because these animals unlike humans and the primate models which are polyestrous, dogs are diestrus, with ovulation occurring twice a year. The removal of ovaries and uterus does not appear to be sufficient to create significant bone loss showing that cessation of ovarian function in the dog does not predispose it to osteoporosis as it does in the human.[200] Furthermore there is an emotional attachment to the dog as "man's best friend" contributes to difficulty along with housing regulations.

The pigs have also well-developed Haversian systems as the dogs. The pigs experience a continuous estrous cycle lasting about 20 days, in a way similar to women. They are omnivorous and experimental serial biopsies can be easily obtained. Miniature laboratory pigs are also available. However, there are some disadvantages, information on ovariectomized pigs used for bone studies is limited, commercial farm pigs are large in size and are difficult to manage and minipigs are quite expensive.[198, 201]

The sheep is a promising large animal model for bone and cardiovascular systems. This is a seasonally polyestrous animal, experiencing several estrous cycles during their breeding season. A 7 months old sheep has a well developed Haversian
systems, an aged sheep display bone loss although has a lack of natural menopause and a limitation of normal estrus cycles to fall and winter. The sheep is docile, relatively inexpensive and available in large numbers.[198] Moreover, due to their large body size, they require large spaces to be housed. Reviewing this topic, Oheim et al. conclude that although different ewe models for osteoporosis have been successfully established, a large animal model that perfectly mimics the human disease is still to be found.[202]

Nonhuman primates include a wide range of diverse species, however only the “man-like monkeys” is used for studying human diseases. The Old World Monkeys, such as rhesus are the most frequently used. The use of these animals is popular because the FDA demands a large animal model data for the osteoporosis therapeutics, in addition to the data from rodents, genetically they are very close to humans and have menstrual cycles and a menopause similar to human females, although their menopause occurs much later chronologically.[203] There are some of them that sustain an upright body posture with a bone biomechanical loading pattern similar to that of humans, they lose bone mass at an advanced age and their immune system is similar to humans. The nonhuman primate is the most widely used large animal model to evaluate the effects on bone of new drug entities, especially with the increase in testing new biologic agents.[204] However, they also have certain disadvantages because they must be ovariectomized to induce ovarian hormone deficiency and bone loss of postmenopausal type. A large number of animals are necessary to achieve adequate statistical significance and special facilities are required with high costs.[198, 203]
Table 6 - Overview of initial practical aspects of animals commonly used for osteoporosis research.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Cost</th>
<th>Availability</th>
<th>Handling</th>
<th>Life span (years)</th>
<th>Social and ethical issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>Low</td>
<td>Readily</td>
<td>Easy</td>
<td>2.5-3</td>
<td>Minimal</td>
</tr>
<tr>
<td>Dogs</td>
<td>Moderate</td>
<td>Readily</td>
<td>With care</td>
<td>10-12</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Domestic minipigs</td>
<td>Moderate</td>
<td>Readily</td>
<td>Loud and noisy, can be aggressive</td>
<td>10-15</td>
<td>Less critical</td>
</tr>
<tr>
<td>Sheep</td>
<td>Moderate</td>
<td>Readily</td>
<td>Easy</td>
<td>10-15</td>
<td>Less critical</td>
</tr>
<tr>
<td>Primates</td>
<td>High</td>
<td>Difficult (especially in wild state)</td>
<td>Difficult Veterinary</td>
<td>30-40</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

Rat model

Rats are considered the preferred animal model for studying the bone changes associated with loss of reproductive function and has been the animal model of choice, being selected for 38% of bone repair-related publications.[205] In fact, rat reports to be of high level of experimental reproducibility due to the detailed definition of the model in a biological, genetic and immunological point of view.[206]

The rat when compared to larger mammals, reports some limitations which include a different loading pattern, a small blood volume for multiple biochemical measurements, a reduced amount of total bone tissue available, minimal intra-cortical bone remodeling, and size-related difficulties in the performance of surgical procedures.[207] Moreover, a number of differences can be designated when comparing rodent bone tissue with the one of humans: for example, the skeleton of rodents continues to model during their life, and growth plates remain open until adulthood; rodents report limited trabecular bone content and haversian remodeling.
does not occur, although cancellous remodeling is established; the rat skeleton has proportionally less bone and a smaller contribution of cancellous bone to the total bone mass.[208, 209]

On the other hand, compared to large animal models, rodents are inexpensive to purchase and maintain, are widely available, easy to manipulate, and report minimal social concern. Rats have lamellar bone, cancellous bone remodeling, it is easy to perform biomechanical tests on bones under standardized conditions and the skeleton has any similarities with the human skeleton.[209] Additionally, they have reproductive cycles resembling humans. The rat maintained continuously under laboratory conditions will have estrus cycles (polyestrus) of 4 to 5 days duration. Therefore, the rat skeleton will be affected by regular fluctuations in gonadal steroids. This makes rats more sensitive to the loss of ovarian hormones compared to species having only one estrus (monoestrus) cycle per year such as dog.[210]

Furthermore, if data that can be validated and that are trustworthy can be acquired, animals placed at the lower end of the phylogenetic scale should be preferably used – in this case; rodents are a first choice option over large mammals. Due to their widespread utilization, much is known regarding the biological processes associated with bone turnover, diet modifications and drug administration. A large quantity of biological tools – such as PCR primers, microarrays, probes and antibodies – is available and simplifies the investigative process. Furthermore, the reduced lifespan of these animals allows for the study of the influence of ageing in the bone metabolism and regeneration processes; while the fast turnover of individuals and the rapid occurrence of biological processes substantiates the usefulness of rats in bone-related research.[211]

**Rat models of osteoporosis**

There are several methods to induce osteopenia and osteoporosis in the rat as reported in Figure 9. Immobilization, dietary manipulations, and hormonal deprivation or administration are the methods of study that are usually chosen. The rate of loss of
bone mass in male and female rats is highly dependent on the method used to induce osteoporosis. All experimental osteoporosis protocols can be applied in skeletally immature or mature rats. Although rats reach sexual maturity at the age of 2.5 months, their skeleton is considered mature after the age of 10 months.[206]

The ovariectomized (Ovx) rat is the most commonly used model for the study of osteoporosis pathophysiology, diagnosis and therapy.[18] In this context, 2 different animal types can be used: the aged rat and the mature rat. The mature rat is an animal whose skeletal properties have been stabilized and no longer subject to age changes, that is, similar to the skeletal characteristics of the women who are susceptible to postmenopausal osteoporosis. The use of this type of rat brings with it sufficient confidence that the bone changes observed after Ovx are really due to estrogen deficiency and are not complicated by continued rapid bone growth as occurs in
younger animals or by age-related bone loss and diseases that can occur later in the life of the rat. The aged rat is usually sacrificed several months after OVX to permit the loss of a significant amount of bone. As a consequence, the aged rat is expensive and its availability is limited, so that the less expensive and more easily available mature rat is preferentially used. The mature rat is an approximately 2.5 months old and capable of responding appropriately to the sex hormone deficiency induced by Ovx, whose effects, in fact, become manifest in a month or less.[212] The bone changes that occur in the two types of animals are similar.

The Ovx induced bone loss in the rat and postmenopausal bone loss share many features such as: the increased rate of bone turnover with resorption exceeding formation; Initial rapid phase of bone loss following by a much slower phase; superior loss of cancellous than cortical bone; reduced intestinal absorption of calcium; particular protection against bone loss by obesity; similar skeletal response to therapy with estrogen, tamoxifen, bisphosphonates, parathyroid hormone, calcitonin, and exercise.[213]

There is extensive literature on the histomorphometric changes, biochemical markers, methodology for bone densitometry and evaluation of bone fragility in the Ovx rat.[213-215] The observation that acute ovarian hormone deficiency leads to elevated cancellous bone turnover have led to the wide scale adoption of this model.[214, 216] The rat loses cancellous bone following the ovariectomy procedure by the imbalance in bone turnover, where bone resorption exceeds bone formation.[195]
C. BONE REGENERATION
Bone tissue is responsible for proper functional properties, such as mechanical support and protection of the organs. Bone has the ability to regenerate in response to injury and it’s continuously being remodeled, changing the equilibrium between osteogenesis and resorption. Because the capacity of bone to regenerate and self-repair is limited to small fractures, therapeutic solutions need to be applied to promote bone healing in case of defects of crucial size.

There are many bone diseases that can alter the function, such as bone loss to removal of tumors, fractures and joint malfunction, cosmetic procedures and degenerative disorders that affect many patients.

In order to improve the quality of life of a large amount of patients who otherwise had a limited and a painful life, orthopedic materials including grafts and synthetic materials are available.

To date mainly transplantation of autogeneic and/or allogeneic bone grafts, or implantation of graft materials with osteoconductive and osteoinductive properties have been used in orthopedic applications.

Bone properties as a material have been extensively investigated and, although depending of many factors like age and site, it is evident that its mechanical properties are quite different from the ones of materials currently used as orthopedic implants. Bone is much more complex than a simple material and bone substitute cannot be restricted to a purely mechanical problem, leading to medium-to-long term implant failure and substitution, especially in patients with poor bone quality, associated for example with degenerative disorders. Although major advancements occurred in the field of bone regenerative medicine, the current therapies, such bone grafts still have several limitations and the search of an ideal bone implant material is still going on.
BIOMATERIALS

Biomaterials is the definition of materials that are part of medical implants, extracorporeal devices and disposables that have been used in medicine, surgery, dentistry and veterinary medicine as well as in every aspect of patient health care.[217]

The National Institutes of Health Consensus Development Conference defined a biomaterial as “any substance (other than a drug) or combination of substances, synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a systemic which treats, augments, or replaces any tissue, organ or function of the body”. [218] Biocompatibility is a common feature that all the biomaterials need. Synthetic materials used in biomedical applications include metals and alloys, polymers and ceramics among others. They present different structures and properties therefore are used in different forms. The selection of a biomaterial is based on the specific applications and founded in the physicochemical properties, durability of the material, nature of the physiological environment of the organ/tissue level, adverse effects in case of failure, cost, mechanical strength, stiffness, fatigue endurance, wear resistance and dimensional stability.

GUIDED BONE REGENERATION (GBR)

The guided bone regeneration (GBR) is a surgical approach in order to attain bone regeneration through the use of barrier membranes.[219]

The membranes should follow a series of criteria’s: biocompatibility, cell occlusiveness, integration by the host tissues, clinical manageability, space making function and limited susceptibility to complications.[220]

Further criteria’s should be achieved in the biodegradable and bioresorbable membranes: the inflammatory response of the tissue to the resorption of the membrane should be insignificant, reversible and be harmless to the regeneration of the tissues on target.[221]
General Aspects

BIOCOMPABILITY

This is defined by the capacity of a material to function in a specific application in the presence of an appropriate host response, and biocompatible materials should offer safety to the patient.[222] All the medical devices should be biocompatibles.

These parameters are measure by the cytotoxicity, histocompatibility, genotoxicity, mutagenicity and microbial effects.[222]

One parameter that must be taken into account is the fact that inert materials often present a less complex safety situation than degrade materials, since those release breakdown products into the surrounding host tissue that can result in a local and systemic adverse reaction.[223]

CELL OCLUSION

This principle was to exclude all the connective tissue cells that could fill the space for the new bone. Tatakis et al. studied this concept thoroughly where the exclusion of connective tissue and the dentogengival epithelium was proposed to enable periodontal regeneration around the root surface.[224]

However, the awareness of total cell occlusion has been contested because of the need of nutrients transfer across the membrane to a fruitful regeneration process.[222] Zellin et al. and Wikesjo et al. claim a better outcome in bone regeneration with the use of macroporous membranes than occlusive barrier. [225, 226]

SPACE MAKING AND SPACE MAINTENCE

The space making capacity is define as the capacity to create and maintain volume and geometry of space without collapsing allowing the bone regeneration.[222] This feature is determined by the stiffness of the material.

The bioresorbable membranes due to the degradable materials often lose the stiffness and begin to collapse; a way to overcome this problem is the simultaneous use of supporting materials (bone grafts).[227, 228]
CLINICAL HANDLING

Membranes should be easy to handle to simplify the surgical procedure by allowing to be trimmed, cut or shaped to fit and cover the bone defect.

Non-resorbable membranes require screws and pins to sustain the shape [229, 230], while the bioresorbable membranes, especially the collagen, due to their hydrophilicity adhere to the surrounding bone and grafting materials as soon as they soaked with blood.[231]

SUSCEPTIBILITY TO COMPLICATIONS

Non-resorbable membranes have a higher risk of complications since they are more likely to a premature exposure, leading to a wound infection.

Bioresorbable membranes are easier to handle and present lower risk of exposure[232], although an inflammatory and foreign body reaction can be encountered during the resorption period when synthetic materials are used.[233]
TYPES OF MEMBRANES

A. NON-RESORBABLE MEMBRANES

1. POLYTETRAFLUOROETHYLENE

The GBR technique was first introduced in 1982 and the expanded polytetrafluoroethylene (e-PTFE) membrane has been considered the gold standard for barrier function materials.[234]

Expanded PTFE is a synthetic fluoropolymer that relies on an exceptionally strong bond between carbon and fluorine for its nondegradable, biologically inert properties. There is no known enzyme in the body capable of cleaving carbon-fluorine bonds polymer providing high stability in biological systems that resists breakdown by host tissues and doesn’t stimulate immunologic reactions.[235, 236]

This membrane is non-resorbable, biocompatible and allows good coagulum stability by covering the defect.[237]

However they have the disadvantage of a second surgery to remove the bioinert membrane, which causes discomfort and increased the costs for the patient, as long the risk of losing some of the regenerated bone due to the flap elevation.[238, 239] One of frequent complications reported is the exposure and subsequent infection.[240-243] These exposure can lead to a premature retrieval of the membrane[244, 245] and an impair bone regeneration is associated with this complication, in a number of animals experiments[246-248] and clinical studies.[249-252]

2. TITANIUM REINFORCED ePTFE

When rehabilitating a large defect, conventional ePTFE membranes cannot support and maintain the space unless they are filled with grafting materials. To overcome this inconvenient the membranes have a double layer of ePTFE with a titanium framework interposed, that provides a stable form.[253] Vertical ridge augmentation demonstrated successful results with this membrane.[254, 255]
B. BIORESORBABLE MEMBRANES

Since non-resorbable membranes need a second surgical intervention for their retrieval, this leads to patient morbidity and physiological stress, risk of tissue damage and cost versus benefits, the clinicians and researchers encourage the use of bioresorbable membranes.

Beside the advantage of the absence for a second surgery to remove the membrane, the bioresorbable membranes offer a range of benefits that include a simplified surgical procedure, decreased patient morbidity, improved soft tissue healing and greatest cost-effectiveness. [220, 236, 256]

On the other hand, the choice of the materials are critical since they have distinctive features and biological effects, such as a different inflammatory reaction in the tissue that vary from mild to severe.[257, 258] These features guide the choice of the membranes by the clinicians depending on the longevity of the barrier function, tissue response and membrane breakdown.

1. SYNTHETIC MEMBRANES

Synthetic polyester membranes are made from polyglycolides (PGAs), polylactides (PLAs), copolymers, polydioxanones or trimethylene carbonates.[259, 260]

These membranes have as advantage the capacity of been produce under controlled conditions and produce in a large scale, unlike collagen membranes. Another feature of these polymeres is the total biodegradation into carbon dioxide and water by the Krebs cycle.[261]

The degradation of biodegradable polymers in vitro and in vivo is affected by several factors, like their structure, chemical composition, molecular weight, shape, processing conditions, sterilizing process, physicochemical factors and mechanism of hydrolysis.[262, 263] In maxillofacial and orthopedic surgery some of these polymers had to be debrieded or removed since it triggered an inflammatory and foreign-body reaction.[233, 264, 265]
Several studies tried to define the time of reabsorption of these membranes. Robert et al. affirm that changing the polymer concentration, the membranes hold out for 4 months.[266] Laurell et al. featured the timeline of reabsorption between 6 to 12 months, and that the hydrolyses of the membrane caused little inflammation.[267] Regardless that, many studies have the consensus in the time of reabsorption between the 6 months.[268-270]

Nowadays there are new polymers and copolymers being used, such as the poly DTE-carbonate that shows promising features since it induces low immunological reaction and high ability to stimulate bone regeneration.[271]

2. COLLAGEN MEMBRANES

The majority of the membranes are from type I collagen or a combination of type I and III collagen. There source of collagen varies from bovine tendons, bovine dermis, calf skin, porcine dermis among others.[272]

Collagen material appears to be an ideal choice for a bioresorbable GTR (Guided Tissue Regeneration) or GBR barrier. Some of the advantages are: provides a good hemostasis, chemotaxis for periodontal ligament fibroblasts and gingival fibroblasts, weak immunogenicity, easy manipulation, a direct effect on bone formation, and ability to augment tissue thickness.[273-275]

The reabsorption of the collagen membranes occurs due to the action of collagenases that cleave the collagen in two molecules which are denaturized at 37°C and decomposed into oligopeptides and aminoacids by the gelatinase and proteinase.[237]

To prolong the barrier function of collagen membranes we can modify by cross-linked treatment. Several treatments have been used such as ultraviolet radiation, glutaraldehyde, diphenyphosphoryl-azide and hexamethylene diisocyanate.[276] The most broadly used of these chemical cross-linking techniques is the glutaraldehyde technique, that reduces the inflammatory response and prevent degradation of the membrane since 30 days, providing a prolonged presence of a mechanical barrier for new bone formation.[277] Although some authors affirm that some cytotoxic residues are left during the process.[278]


**EVOLUTION®**

The membrane is obtained from mesenchymal tissue (heterologous pericardium) and is completely resorbable. Its structure is made of dense collagen fibers of high consistency and of high resistance that offer: the maximum adaptability to bone tissue and soft tissues, an easy and secure suture ability to nearby tissues, the best membrane/bone and membrane/periosteum interface, stability and prolonged protection of the underlying graft.

This membrane can be shaped with sterile scissors until the desired size is reached; it must then be rehydrated with lukewarm physiological solution. Once it acquires the desired plasticity, it must be adapted to the grafting site. The membrane offers one smooth side and one micro-rough side to improve the fitting to the tissues. The rough side is faced to the bone to optimize integration and positioning. [279]

In case of accidental exposure, the dense collagen matrix of the membrane protects the graft from infection. The membrane itself will also not be infected, allowing second intention healing.

The average reported values are indicative and subject to a variability range depending on the composition of the tissues of origin.

The estimated resorption time is about 4 months (standard thickness; 0.5-0.7mm) and 3 months (fine thickness; 0.3-0.5mm), although the reported values are estimates and purely indicative: these values can therefore vary depending on the patient and grafting site.

The membranes have several clinical indications such as in oral surgery and traumatology, implantology and periodontology. They have been used in lateral access in the sinus lift procedure to cover de antrostomy and protect the sinus membrane from cutting risk due to graft pressure.[280, 281] Many authors also described there valued outcome in protecting peri-implant regenerations and periodontal grafts.[279, 282, 283]
BONE GRAFTS

Bone graft is the second most employed transplantation tissue, being blood the first.[284] Bone grafts are often necessary to provide support, fill voids, and enhance biologic repair of skeletal defects. It is estimated that more than 500,000 bone grafting procedures are performed annually, in the United States.[285] Bone grafting procedures are broadly used in complicated fractures that fail to heal or severely atrophied regions (like the mandibula after teeth extraction) that need augmentation. In addition, they are often applied in the case of fractures with a high risk of nonunion, when extensive regions of bone have been removed, and to ease fusion of bones after extensive reconstructive procedures that are performed in orthopedic and maxillofacial surgery.[207]

According to their origin, bone grafts can be classified in autogenic, when the tissue is harvested from the same individual; allogenic, if the graft originates from another individual of the same specie; xenogenic, if the graft is obtained from a donor of another specie; and alloplastic, if it is a synthetic material.

The exact mechanisms by which bone graft incorporation occurs are not completely understood, although several distinct bone formation mechanisms have been determined. As so, osteoconduction is a property attributable to grafts that allows deposition of bone tissue and subsequent integration within the surrounding bone. Osteoinduction is the property of grafted materials that can induce surrounding precursor cells to differentiate and produce new bone tissue. Osteogenesis is the process by which vital osteoblasts originating from the bone graft contribute to new bone growth.

AUTOGENIC GRAFTS

These bone grafts are considered the gold standard application for bone regenerative clinical applications, because they possess osteoconductive, osteoinductive and osteogenic capabilities[207]. The tissue is obtained from the patient to himself. Nonetheless, autogenic grafts have significant limitations: the
harvesting prolongs the time of the surgery; causes donor site morbidity (e.g., pain, blood loss and infections); may originate tissue in inadequate amount and/or inappropriate form; may present limited mechanical strength for specific applications; among others.[286] These limitations have prompted the increasing interest in alternative grafts.

**ALLOGENIC GRAFTS**

Allograft is an alternative and offers the same characteristics as autograft with the exclusion of osteogenic cells. Bone allografts, by nature, are derived from different human donors and may be cancellous, cortical, or a combination of both.[287] These grafts are easily available, report predictable results and eliminate the need for surgical harvesting of the graft tissue. Although they are an attractive source, there are several problems encountered in their clinical use, including the risk of disease transmission, immunological rejection, loss of biologic and mechanical properties secondary to its processing, increased cost, and limited availability due to financial and religious concerns.[288] Due to the potential risk of disease transmission allografts undergo several treatments. The grafts are devitalized by freezing, and often additional procedures like sterilization with ethylene oxide and/or high-dosage of $\gamma$ irradiation are applied. These sterilization steps, however, can significantly reduce the osteoinductivity of the grafts.[289]

A table comparing the main properties of autogenic and allogenic grafts is shown in Table 7.
Table 7 – Comparative properties of bone grafts.[287]

<table>
<thead>
<tr>
<th>Bone graft</th>
<th>Strength</th>
<th>Osteoconducton</th>
<th>Osteoinduction</th>
<th>Osteogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autogenic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancellous</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Cortical</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Allogenic</td>
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**XENOGENIC GRAFTS**

Bone xenografts for human application are essentially derived from pig, bovine and equine origin, among others. This material is widely available and can be combined with autologous material to augment graft quantity and control the eventual resorption of the tissues.[290] Clinical outcomes are variable, with moderate to good clinical results in some oral/maxillofacial applications.[291-293] Biosafety issues have been raised regarding antigenicity and possibility of disease transmission.[294, 295]

**ALLOPLASTIC MATERIALS**

Synthetic bone grafts such as ceramics, polymers and composites have different characteristics regarding biomechanical, biochemical and biological properties. Specifically, they present a wide range of structural strength, rate of resorption or replacement by host, mechanism of action, osteoinductive potential, osteoconductive properties and handling capability. Some disadvantages of these graft materials include cost, poor handling and variable resorbility.[285]
ORAL/MAXILLOFACIAL APPLICATIONS OF BONE GRAFTS

With the advances in biomaterials and surgical techniques that allow predictable bone regeneration, the needs of the patients for better oral rehabilitations can be satisfied. Frequently, these patients have soft and hard tissue defects, resulting from a variety of causes, such as infection, trauma, and tooth loss that causes continuous resorption of the alveolar ridge. These defects create an anatomically less favorable foundation for ideal implant placement.[296]

For prosthetic dental implant therapy, bone augmentation of the alveolar bone through a variety of regenerative surgical procedures has become predictable. Regenerative procedures are used for socket preservation, sinus augmentation, and horizontal and vertical ridge augmentation.[297, 298]

There are several techniques that allow a predictable bone regeneration procedure in oral application, reaching hand of a wide range of bone grafts, from autografts to alloplastic materials, xenografts or allografts.

Augmentation techniques can be realized by means of a local bone graft (autograft) taken from: the symphysis area of the chin, maxillary tuberosity, cranial vault (calvarium), iliac wing bone or by rib grafts, among others. Materials derived from other individuals (allografts), from different species (xenografts) or even from synthetic origin (alloplastic materials), have also been widely used.

These techniques are useful in trauma situations, forehead defects and oral rehabilitations.[207] Another augmentation technique, which can be used in vertically regeneration, is the distraction osteogenesis.[299] In the maxilla, another surgical technique that allows bone augmentation is the sinus lift procedure. Reported used materials include grafts from several origins, such as: autografts (iliac crest bone [300], mandibular symphysis [301], maxillary tuberosity[301]), allografts (freeze-dried demineralized bone[302, 303]), xenografts (Hydroxyapatite of bovine origin[304]) and alloplastic materials (Beta-tricalcium phosphate (β-TCP)[305]). In socket preservation, different materials have also been reported to be used, such as: porcine bone [293], Beta-tricalcium phosphate (β-TCP) [306], nanocrystalline hydroxyapatite paste [307], Bio-Oss Collagen®[308] among others.
BIO-OSS®

Bio-oss® is an inorganic mineral product, of bovine origin, that is chemically processed at low temperatures, in order to assure the extraction of the organic matter. According to the manufacturers, the processing assures the complete removal of all organic components from bovine bone, resulting in an almost unaltered natural mineral structure.[309] Nonetheless, it has been reported the presence of a notable content of organic waste that can be harmful to the biological response.[310, 311]

Bio-oss® presents physical and chemical characteristics similar to the mineral matrix of human bone.[312] This material has a porous structure, with an average pore size of 100µm, that facilitates the proliferation of blood vessels and bone cells, providing a three-dimensional frame for the growth of new bone.[313] Due to the crosslink of pores and small crystals, the inner surface of Bio-oss® covers more than 90m²/g, which is a specific volume similar to the one of human cancellous bone. The crosslinking of micro and macro pores also favors the stabilization of the clot. This compound has a porosity of around 60% and crystal size between 10-60nm.

Bio-oss® is slowly resorbed and integrated into the natural remodeling process of bone filling - the requirements of an osteoconductive material. The mineral component of the small crystals of hydroxyapatite, with less hydroxyl ions and more carbon than the synthetic hydroxyapatite, seems to facilitate the process of natural bone remodeling.[314] The proportion of calcium and phosphate is 2:1, similar to the one of human bone.

Bio-oss® has been widely employed in clinical dentistry as a xenograft material for implantation and sinus lift procedures.[236, 315-318] Zitzmann et al. affirmed that the long-term implant survival rate in Bio-oss® grafet sites is similar to the survival rate of implants placed in native bone (112 implants, 5 years follow-up).[318] Schekegel et al. showed that after sinus floor augmentation with a mixture of Bio-Oss® and autologous bone, the percentage bone volume was preserved throughout the study period, while the height of a purely autologous bone graft diminished markedly.[319] Bio-oss®, in maxillary sinus augmentation, was shown to not to interfere with normal healing process and to promote new bone formation, as assessed by light, scanning and transmission electron microscopy.[320] Hämmerle et al., performed twelve lateral
ridge augmentations in patients, with Bio-oss® and a commercial collagen membrane to improve the alveolar ridge thickness, to implant therapy. The authors found that this treatment is a valid option for horizontal bone augmentation with a decreased morbidity.[235] Felice et al., evaluated vertical ridge augmentation procedures and compared the results using Bio-oss® or bone blocs from the iliac crest.[321] The authors established that both procedures achieved good results, but the use of bovine blocs was less invasive.

There are several indications for the use of Bio-oss® in dentistry such as: horizontal augmentation, vertical augmentation, ridge reconstruction, ridge preservation, sinus floor elevation and correction of intraosseous defects. These procedures have been shown to be predictable, with good results and a valid alternative to the use of other grafts.

**PUTTY®**

Putty is an antigen-free bone paste composed by a mix of cancellous and cortical porcine bone, and the collagen tissue is preserved. It’s physical form as a plastic consistency composed of 80% micronized heterologous bone (granulometry ≤ 300 µm) and 20% pure collagen gel. It is made with a particular process that provides the product with special malleability and plasticity, making it easy to apply in sockets and peri-implant defects with walls. Thanks to its collagen component, the product facilitates blood clotting and the subsequent invasion of repairing and regenerative cells. This product has an average resorption time of less than 4 months.

This product as been applied in Implantology because of its versatile alveolar filler aiming to preserve the crestal volume and in immediate post-extraction implants to facilited the primary stability.[322] Authors have described its use in treatment of periimplantitis and in split crests.[323] In oral surgery its recommended to preserve the dental extraction socket and bone defects in result of granulomas or dentigenous cysts.[324]
ANIMAL MODEL OF BONE REGENERATION

Currently, several animal models are used to evaluate the bone regeneration process, the bone–biomaterial interaction and the physiological or pathological evidence of the modulation of the ossification pathway. Experimental models can be classified as heterotopic or orthotopic, based on the vicinity to the autologous bone tissue. Accordingly, the rat heterotopic model has been used successfully to show the sequence of events involved in the ossification process and most common implantation sites include the subcutaneous tissue or intramuscular anatomical locations.[207] Alternatively, orthotopic models within intraosseous locations have been used to evaluate the bone regeneration process in the repair of discontinuous and weight-bearing lesions. The selection of orthotopic models in bone research relies broadly on one of the following options: calvarial, long bone or mandible segmental, partial cortical and cancellous bone defects.[207]

CALVARIAL DEFECT MODEL

The calvarial model stands out as an adequate model for evaluating the bone regeneration process, as well as the biocompatibility and adequacy of complex materials and tissue engineering constructs aiming bone regeneration.

The calvarial model is very popular and appropriate for the following reasons:[325-327]

• The calvarial bone is a plate which allows the creation of a uniform circular defect that enables convenient radiographical and histological analysis;
• Craniotomies are moderately easy to perform, have low morbidity rates, and are highly reproducible if performed with a circular trephine;
• The calvarial bone has an adequate size for the surgical procedure and specimen handling;
• No fixation is required because of the good support given by the dura and the overlying skin;
The model has been thoroughly used and studied, and is well reproduced;

calvaria, which develops from a membranous precursors, has a poor blood supply and relatively little bone marrow;

materials and tissue engineering constructs of all kinds can be tested in that location, which is particularly well suited for assaying granular or paste-like materials;

Laterally performed craniotomies have the advantage of allowing paired design and minimized morbidity while avoiding accidental damage to the midsagittal sinus.

This model has been established in an extensive range of animal species counting, mice, rats, rabbits, dogs, sheeps, goats, pigs and nonhuman primates, although rats and rabbits are the most commonly used animals.

The selection of the calvarial model implies the determination of the size of the defect. According to experimental design objectives and the regenerative behavior, two types of defects can be surgically created: critical size and sub critical size defects, which vary according to the animal species, gender, age, strain, anatomic site, shape, size and micro-mechanical environment.[326, 327]

A critical size defect (CSD) by definition means that the defect will not heal spontaneously during the expected lifetime of the animal. This is a essential quality that permits to conclude that, in case of success, the therapy healed a defect instead of only enhance its spontaneous repair.[207]

Critical size defects cannot rely on the physiological repair mechanisms of the organism to fully heal the defect with bone tissue, and fibrous connective tissues is broadly developed within the residual defect.[326, 327] It has not been known why a small defect can be repaired but a large defect cannot. Furthermore, little information is available on how bone formation ceases during the repair of a critical size defect. A variety of studies have looked into the repair of critical size bone defects with growth factors, biomaterials, cell and tissue implantation, although the biological mechanism that regulates the healing of bone defects has not been fully understood.[328]
Bosc *et al.*, reported that the defects that satisfied the criteria for a critical size in Wistar rats calvarial bone were full thickness 5mm in diameter, since no signs of spontaneous bone regeneration was verified 12 months after surgery, apart from residual bone formation on the defects’ margins.[329-331]
MANDIBULAR BONE DEFECT

There are many factors, which can alter the regeneration on the created defects such as the anatomical location, cortical involvement, and the presence of periosteum, dura, or both. The model of critical-size calvarial defects have presented a respectable bone remodeling, although Kaban & Glowacki describe the mandible as a unique bone that is subjected to continuous motion and significant compressive and shearing forces; therefore bone defects created in other craniofacial bones or in long bones may not apply to the mandible.[332]

In the maxillofacial skeleton, a frequently used animal model is the mandibular critical size defect in the rat.[332] This model consists of a circular through and through defect of a diameter varying from 4 to 7 mm drilled into the mandibular ramus.[332] The term critical size, as cited previously, implies that the defect will not heal spontaneously, so that healing, if obtained, is caused by the experimental intervention.[326, 333] The rat mandibular defect model has been used to evaluate bone regeneration of biomaterials and osteoconductive properties of membranes with or without growth-stimulatory factors.

The experimental mandibular model was based on Dahlin et al. article and clearly illustrates the potential for bone regeneration. This mandibular model is widely used and suitable for the following reasons:[219, 334]

- The surgical procedures on the rat mandibular bone are relatively simple;
- Spontaneous healing would not occur in the control site;
- Observations can be focused on the healing process of bone, since there are not any major nerves or blood vessels around the rat mandibular angles;
- The preparation of tissue specimen is easy;
- The parameters can be simply and accurately measured in each specimen.
II. MATERIALS AND METHODS
A. EXPERIMENTAL DESIGN

This work, aiming to evaluate the bone regeneration process in a valid animal model of the human condition of osteoporosis, was conducted during the period between January 2009 and December 2013.

The experimental design was divided in three phases: establishment and characterization of an experimental osteoporotic model; the study of the calvarial bone regeneration and the mandibular bone regeneration. The study was performed under the authorization of the Direção Geral de Veterinária for the project “Avaliação do processo de regeneração em condições osteoporóticas”, according to the ordinance nº 49 paragraph b). The technical standards of protection of experimental animals were observed, according to the Portuguese (Decree No. 1005/92) and European (Directive 2010/63) legislation.

1. ESTABLISHMENT OF THE OSTEOPOROTIC MODEL

Establishment and characterization of an experimental osteoporotic model 82 adult female albino Wistar rats (Rattus norvegicus), 7-8 weeks old, were used. At 2 months of age, animals were randomly divided into two groups: Ovx and Sham. The Ovx group of animals underwent a bilateral ovariectomy surgery while the Sham group of animals was submitted to a control surgical procedure. Following ovariectomy or sham surgery, the osteoporotic condition was assessed in all the animals by several procedures.

Uterus, femur, tibiae, vertebra, maxilla, calvarial bone and the right mandible were resected and adequately stored for posterior processment.

The uteri were dissected, collected and weighted in three time periods (month 3, 5 and 8).

Blood was collected to address plasmatic parameters as the alkaline phosphatase activity, calcium, phosphorous concentrations and estrogen levels in the
three time points (month 3, 5 and 8). Albumin, Creatine kinase, Total protein and Triglycerides, in Sham and Ovx animal groups, were evaluated in two time periods (month 3 and 5).

The resected bones such as the calvaria, mandible, tibiae, femur, maxilla and vertebra were analyzed radiographically and these images were evaluated with an image software program ImageJ® to evaluate the densitometric values in two time periods (month 3 and 5).

Proximal tibial bone specimens from both 5 and 8 months were scanned by microcomputed tomography and microstructural measures included bone volume per total volume (BV/TV), connective density (CD), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp).

The femur and mandible were subject to biomechanical evaluation at month 3.

Micro-CT evaluation of the mandible and maxilla were performed in a circumscribed area in the space between roots of the 1st molar, at month 3 and 5.

Harvested samples were evaluated by routine radiographic, micro-tomography and histological techniques, at the described time points (Figure 10).

**STUDY DESIGN:**

**T1** — 3 month following ovariectomy (Ovx) and Sham operation (Sham)

**T2** — 5 months following ovariectomy (Ovx) and Sham operation (Sham)

**T3** — 8 months following ovariectomy (Ovx) and Sham operation (Sham)

![Figure 10](image-url) – Study timeline of the ovariectomy (Ovx) and Sham operation (Sham).
2. ASSESSMENT OF THE CALVARIAL BONE REGENERATION

In the calvarial bone regeneration assay, 82 adult female albino Wistar rats (Rattus norvegicus), 7-8 weeks old, were used. The animals used to within the establishment and characterization of an experimental osteoporotic model were simultaneously enrolled for the assessment of the calvarial bone regeneration. Following 2 months (at 4 months of age), a single 5mm in diameter bicortical defect (CSD- Critical Size Defect) was created in the skull (parietal bone) of Ovx and Sham animals, previously to the implantation of Bio-Oss® (Geistlich) . Defects left untreated were used as negative controls. Animals were euthanized at 1, 3, and 6 months following the craniotomy procedure (at 3, 5 and 8 months after the ovariectomy procedure i.e., at 5, 7 and 10 months of age respectively).

Harvested samples were evaluated by routine radiographic, micro-tomography and histological techniques, at the described time points (Figure 11).

STUDY DESIGN:

T1 — 1 month healing following craniotomy
T2 — 3 months healing following craniotomy
T3 — 6 months healing following craniotomy

![Figure 11 – Study timeline of the calvarial experiment.](image-url)
Animals were randomized according to the following group organization:

**Figure 12** – Schematic representation of the number of animals used for experimentation.
3. ASSESSMENT OF THE MANDIBULAR BONE REGENERATION

In the study for the mandibular bone regeneration, 68 adult female albino Wistar rats (Rattus norvegicus), 7-8 weeks old, were used. At 2 months of age, animals were randomly divided into two groups: Ovx and Sham. The Ovx group of animals underwent a bilateral ovariectomy surgery while the Sham group of animals was submitted to a control surgical procedure. Following 2 months (at 4 months of age), animals of each group were assigned to 3 sub-groups: Membrane (M), Membrane and Biomaterial (MB) and Control (C).

The animals were subject to a surgery of a 4mm in diameter bicortical circular defects on the right-half ramus of the mandible of both Sham and Ovx animals, in order to address the guided bone regeneration process. The membrane used in this study was Evolution® (Osteobiol), and its composed by 100% pericardium of equine origin with a thickness of 0,5-0,7mm. The used biomaterial to assess bone regeneration was Putty® (Osteobiol), a mix of 80% of granulated cancellous and cortical porcine bone with a granulometry ≤300µm and 20% of collagen gel (type I and III).

In the Membrane group (M), the membranes were applied with the rough side faced to bone to optimize integration and positioning. The defect was covered with a barrier membrane on the bucal and lingual side. In the Membrane and Biomaterial group (MB) the defect was filled with the Putty® and the membrane was following applied as in the M group. In the Control group the mandibular defect was left untreated. The wound was closed in layers using 4-0 resorbable sutures (Vicryl Rapid 4-0, Ethicon, Johnson & Johnson).

Animals were euthanized 1 and 3 months following the mandibular bone defect procedure (at 3 and 5 months after ovariectomy, i.e at 5 and 7 months of age respectively).

Harvested samples were evaluated by routine radiographic, micro-tomography and histological techniques, at the described time points (Figure 13).
MATERIALS AND METHODS

STUDY DESIGN:

**TE 1** – 1 month healing following mandibular bone defect

**TE 2** – 3 months healing following mandibular bone defect

*Figure 13* – Study timeline of the mandible experiment.
Animals were randomized according to the following group organization:

**MANDIBULAR BONE DEFECTS**

![Diagram](image)

*Figure 14 – Schematic representation of the number of animals used for experimentation.*
MATERIALS AND METHODS
B. ANIMAL CARE

After assessing the general health of the animals, they were housed in groups, in conventional type II cages, on a controlled environment of temperature and humidity, in a 12h light/dark cycle, for a quarantine period of two weeks. All animals were clearly identified using nicks on the ears (Figure 15). Dry feed (Mucedola S.R.L., Italy) and water was supplied ad libitum.

During the experimental period animals were housed in similar conditions as previously described.
C. SURGICAL PROCEDURES

1. ESTABLISHMENT OF OSTEOPOROTIC CONDITION

- Surgical ovariectomy (Ovx) and Sham operation (Sham)

The animals assigned to ovariectomy were weighted and anesthetized by the intraperitoneal (IP) injection of xylazine (10mg/kg) (Rompun® 2%, Bayer) and ketamine (90mg/kg) (Imalgene® 1000, Merial) (Figures 9). After assessment of the anesthetic plane an IP injection of tramadol (10mg/Kg), for post-operative analgesia was given, and the skin around the back midline was shaved. Animals were following transferred onto a heating pad (maintained at 37 ºC) in ventral recumbency and the operating field was disinfected with iodopovidone solution (Figures 16-19).

![Figure 16 – Weighing of the animals.](image)

![Figure 17– Animal placement in ventral recumbency. The red line indicates the incision site.](image)
A 2-3 cm dorsal midline skin incision was made halfway between the caudal edge of the ribcage and the base of the tail. (Figures 20-21).

A single incision of about 15 mm long was made into the muscle wall on both the right and left sides approximately 1/3 of the distance between the spinal cord and the ventral midline.

The ovaries are small organs, around 0.5 cm in diameter, with a pink-red irregular surface, connected to each oviduct, which then connects to the uterus (Figure 22).
Figure 22 – Left – Anatomical representation of the ovaries and uterus following exposure of the abdominal organs. Right – Location of the sutures and area of cutting for the ablation of the ovaries.[335]

Figure 23 – Uterus and its irrigation system.

Figure 24 – Placement of ligatures.

Figure 25 – Placement of ligatures.
The ovary and the oviduct were exteriorized through the muscle wall (Figures 23-25).

The ovary, surrounded by a considerable amount of fat, was identified in the cranial end of the uterus. The fat tissue was grasped and the ovary pulled out through the incision. Ligatures were placed, with absorbable 4-0 sutures (Vicryl Rapide®, polyglatin 910), around the uterine horns together with some fat tissue: one between the ovary and the uterus and the other one in the cranial end, blocking the ovarian artery just below the Fallopian tube.

Each ovary and part of the oviduct was removed with single cuts through the oviducts near the ovary (Figures 26-27).

Figure 26 – Surgical removal of the ovary.

Figure 27 – Assessment of hemostasis.

Figure 28 – Ovary removed.
After the removal of the two ovaries and inspection for hemorrhage, the remaining tissue was replaced into the peritoneal cavity. The muscle tissue and skin were sutured, with absorbable 4-0 sutures (Vicryl Rapide®, polyglatin 910), and a liquid bandage was placed over the incision (Figures 29-32). After weighted, the animals were placed into a heat recovering chamber, until full recovery from anesthesia.

The same procedure was performed to all animals assigned to the Sham operated group, with the exception of placement of the ligatures and the ovaries removal.

Figure 29 – Suture of the muscle.

Figure 30 – Suture of the skin.

Figure 31 – Suture of the skin.

Figure 32 – Disinfection of the skin.
2. ESTABLISHMENT OF CALVARIAL DEFECT

Surgical craniotomies were performed and aimed to establish a critical size defect (CSD) with 5 mm in diameter, in which the biomaterial bone-mediated regeneration process was accessed. Both Ovx and Sham groups of animals where submitted to craniotomy procedures.

Prior to the intervention, all animals were weighted and anesthetized by the intraperitoneal (IP) injection of xylazine (10mg/kg) (Rompun® 2%, Bayer) and ketamine (90mg/kg) (Imalgene® 1000, Merial)(Figures 9). After assessment of the anesthetic plane an IP injection of tramadol (10mg/Kg) (Tramal®, Pfizer), for post-operative analgesia was given. A subcutaneous injection of 0,3-0,4 ml of 1% lidocaine (Lidocaina 1%, Braun®), was conducted for enhanced local anesthesia. After assessment of the anesthetic plane, the skin around the incision was shaved and disinfected with iodopovidone solution (Fig.33-34).

Animals were following transferred onto a heating pad (maintained at 37 ºC) in ventral recumbency and the surgical field was placed (Figures 35-36).

Figure 33 – Anesthetized animal in ventral recumbent.

Figure 34 – Pre-operative preparation of the calvarial region - tricotomy.
Following, a midline incision through the skin allowed access to the calvarial bone. The skin was then reflected bilaterally and a midline periosteal incision (around 3 cm long), allowed the division of the subcutaneous fascia and the bilateral reflection of the periosteal flaps, following blunt dissection, to expose the calvarial bone surface (Figures 37-38).

A trephine bur (external diameter of 5mm, Komet® ref.050/5) was used to establish one CSD, in the mid-portion of each parietal bone (Figures 39-42).
The trephines were mounted on a low-speed dental handpiece (WH® model WS 56E, Implantmed) and trephination was conducted with permanent irrigation with sterile saline solution. Careful drilling aimed to prevent the damage the dura mater or the underlying blood vessels and sinus.

CSD defects were then washed with saline solution and, according to the protocol, left unfilled (negative control) or filled with Bio-Oss® (with a granulometry of 0.25 to 1mm) (Figure 43).
The surgical wound was then closed in layers with 4-0 resorbable suture (Vicryl Rapide®, polyglatin 910). Finally, the wound closed area was cleaned with iodo povidone solution (Figures 44-45).

Following surgical intervention, the animals were given a subcutaneous injection of sterile saline (10 mL/kg/h of surgery). The rats were placed in soft-bedded plastic cages and housed individually after the procedure. Each animal received a subcutaneous injection of tramadol (10 mg/kg) (Tramal®, Pfizer) at 12, 24 and 36 h
after surgery for continued postoperative analgesia. Animals were given free access to food and water and were monitored daily, in the postoperative period until euthanasia, for any complications or abnormal behavior. Seven days following craniotomy procedure, animals were housed in groups.

**EUTHANASIA**

The animals were euthanized at the determined timepoints (Figure 11). Animals were weighted and anesthetized by intraperitoneal injection of 10mg/kg xylazine (Rompun® 2%, Bayer) and 90mg/kg ketamine(Imalgene® 1000, Merial) and euthanized by exsanguination, following the assessment of the anesthetic plane.
3. ESTABLISHMENT OF MANDIBULAR DEFECT

The mandibular bone defects were performed and aimed to establish a bone defect with 4 mm in diameter, aiming to evaluate the guided bone regeneration process. Both Ovx and Sham groups of animals were submitted to the mandibular bone defect procedures.

The animals were subject to a surgery to perform a bicortical circular defect on the right-half ramus of the mandible with a 4 mm in diameter. The membrane used in this study was an Evolution®, composed by 100% pericardium of equine origen with a thickness of 0,5-0,7mm. The biomaterial to assess bone regeneration was Putty®, a mix of cancellous and cortical porcine bone with a granulometry ≤300µm (80%) 20% of collagen gel (type I and III).

In the Membrane group (M), the membranes were applied with the rough side faced to bone to optimize integration and positioning. The defect was covered with a barrier membrane on the bucal and lingual side. In the Membrane and Biomaterial group (MB) the defect was filled with the Putty® and the membrane was applied as in the M group. In the Control group the mandibular defect was left untreated.
Prior to the intervention, all animals were weighted and anesthetized using intraperitoneal (IP) injection of xylazine (10mg/kg) (Rompun® 2%, Bayer) and ketamine (90mg/kg) (Imalgene® 1000, Merial). An IP injection of tramadol (10mg/Kg) (Tramal®, Pfizer) for post-operative analgesia was given, and animals were then transferred onto a heating pad, maintained at 37 °C, in the operating field. A subcutaneous injection of 0,3-0,4 ml of 1% lidocaine (Lidocaina 1%, Braun®), was conducted for enhanced local anesthesia. After assessment of the anesthetic plane, the skin around the incision was shaved and disinfected with iodopovidone solution (Fig.46-47).

Following, a submandibular skin incision (around 2 cm long) was made, which incised the masseter muscle and periosteum. The skin and muscle was then reflected bilaterally and following blunt dissection, the mandibular bone surface was exposed (Figures 48-49).
With a trephine bur (external diameter of 4 mm, Komet® ref.040/2.9), a standardized ‘through-and-through’ was created in the right mandible.

The trephines were mounted on a low-speed dental handpiece (WH® model WS 56E, Implantmed) and trephination was conducted with permanent irrigation with sterile saline solution. Careful drilling aimed to prevent the damage the submandibular structures as the underlying blood vessels and nerves.

The mandibular bone defects were then washed with saline solution and, according to the protocol, the negative control (C) was left unfilled, in the Membrane...
group (M), the membranes were applied around the mandibular bone defect and in the Membrane and Biomaterial group (MB) the defect was filled with the Putty® and finally was surrounded by the membrane (Figure 52-56).

Figure 52 – Trephine bur with 4 mm of external diameter.

Figure 53 – Establishment of the defect on the right mandible.

Figure 54 – The membrane was placed on the lingual side of the mandible.

Figure 55 – Mandibular bone defect implanted with Putty®.

Figure 56 – The membrane placed around the mandibular defect.
The membrane was placed on the lingual and buccal side of the mandible, surrounding the defect. A 4-0 resorbable suture (Vicryl Rapide®, polyglatin 910) was placed around the membrane that allowed to secure it.

The muscular layer was then closed in layers with 4-0 resorbable suture (Vicryl Rapide®, polyglatin 910) and the skin was closed with 4-0 non-resorbable suture (Silkam® natural non-absorbable suture made of silk). Finally, the wound closed area was cleaned with iodopovidone solution (Figures 57-60).
Following surgical intervention, the animals were given a subcutaneous injection of sterile saline (10 mL/kg/h of surgery). The rats were placed in soft-bedded plastic cages and housed individually after the procedure. Each animal received a subcutaneous injection of tramadol (10 mg/kg) (Tramal®, Pfizer) at 12, 24 and 36 h after surgery for continued postoperative analgesia. Animals were given free access to food and water and were monitored daily, in the postoperative period until euthanasia, for any complications or abnormal behavior. Seven days following the mandibular bone defect procedure, animals were housed in groups.

EUTHANASIA

The animals were sacrificed after 1 and 3 months of the mandibular bone defect procedure, according to the time line procedure (Figure 13). Animals were weighted and anesthetized by intraperitoneal injection of xylazine 10mg/kg (Rompun® 2%, Bayer) and ketamine 90mg/kg (Imalgene® 1000, Merial) and euthanized by exsanguination, following the assessment of the anesthetic plane.
D. SAMPLE COLLECTION

The animals from both bone regeneration experiments were subject to sample and data collection to evaluate the osteoporotic condition as described below:

BLOOD COLLECTION AND PLASMA SEPARATION

Cardiac puncture aimed to collect blood for determination of plasmatic markers of bone remodeling and estrogen levels.

Blood was collected into a heparinized tube previously to centrifugation. Centrifugation protocol (5000 rpm/15 minutes at room temperature) aimed to separate plasma from the cellular component of the blood (Figures 61-62).

Figure 61 – Collection of the blood from the left ventricle.

Figure 62 – Plasma separation by centrifugation.
NECROPSY

Internal cavities were assessed for pathological organ alterations and presence of abnormal fluids. Uterus, femur, tibiae, vertebra, maxilla, calvarial bone and the mandible were resected and adequately stored for posterior processment.

COLLECTION OF THE UTERUS

The uterus were dissected, collected and weighted.

Figure 63 – Uterus from an Ovx group animal, at month 6.  
Figure 64 – Uterus from a Sham group animal, at month 6.
COLLECTION OF BONES (FEMURS, TIBIAS, MAXILLA, MANDIBLE, CALVARIA AND VERTEBRAE)

Femur, tibiae, maxilla, left mandible, right mandible, calvarial and vertebrae were removed, cleaned for soft-tissue and stored at 4°C in buffered natural formaldehyde 10% to address evaluation by x-ray, Micro-CT and histological analysis (Figures 65–75).

Some of the femurs and mandibles were forthwith stored at -80°C, to perform biomechanical tests.
MATERIALS AND METHODS

MODULATION OF THE BONE REGENERATION PROCESS IN SYSTEMIC IMPAIRED CONDITIONS

Figure 69 – Top view of the calvaria bone of the CSD without Bio-oss®.

Figure 70 – Calvarial bone block of the CSD without Bio-oss®.

Figure 71 – Top view of the calvaria bone of the CSD with Bio-oss®.

Figure 72 – Calvarial bone block of the CSD with Bio-oss®.

Figure 73 – Buccal view of the right mandible.

Figure 74 – Lingual view of the right mandible.
Figure 75 – View of the left mandible, without intervention.
E. DATA COLLECTION

1. CHARACTERIZATION OF THE OSTEOPOROTIC CONDITION

PLASMA ANALYSIS

The attained plasma was pipetted and stored at -80 °C until the determination of plasmatic parameters. Alkaline phosphatase activity, calcium, phosphorous, albumin, total protein, triglycerides and creatine cinase concentrations were determined in an autoanalyzer (PRESTIGE 24i, Cormay) and estrogen levels were assessed by an ELISA kit (Mouse/Rat Estradiol (E2) ELISA Kit – Calbriotech, USA) – Sensitivity <3pg/mL.

X-RAY DENSITOMETRIC ANALYSIS OF THE BONES

The resected bones such as the calvaria, mandible, tibiae, femur, maxilla and vertebra were analyzed radiographically.

Imaging protocol and computer image analysis was achieved with a RVG intraoral sensor (KODAK® RVG 5100, USA) and processed with software KODAK® Dental Imaging Software 6.8.6.0. As a source of X-ray, a conventional X-ray tube (Trophy, type 708, long cone, 8mA, 70kV) was used. The relative position of the sensor and exposure time was constant for all specimens (film-focus distance of 20cm and exposure time of 0.2seg).

These images were evaluated with an image software program ImageJ®. This is a Java-based image processing program developed at the National Institutes of Health that allows processing of digital images. [336, 337] This software can calculate area and pixel value statistics in determined regions of interest (ROIs) and create density
histograms and line profile plots. The density or gray scale calibration measured is also available and brightness values of images are calculated, that allow us to retrieved the intensity statistics (Mean, Modal, Median, Min. & Max. Gray Value, Standard Deviation and Integrated Density) that were performed on the definied ROI for each bone, to assess the bone regeneration process in the calvaria and mandible and to adress te densitometric values in the bone collected to appraise the osteoporotic condition.

![Figure 76 - Placement of the bones for x-ray collection.](image1)

![Figure 77 – X-ray acquirement.](image2)

For the densitometric analysis, regions of interest (ROIs) were defined. These regions for the assessment of tibiae, femur, maxilla, mandible, calvarial and vertebra bones, were established as seen in the images to evaluate the densitometric values (Figures 78-83).

Intensity of signal was evaluated in unprocessed TIFF files and normalized by the intensity of aluminium references.
MATERIALS AND METHODS

MODULATION OF THE BONE REGENERATION PROCESS IN SYSTEMIC IMPAIRED CONDITIONS

Figure 78 – ROI of the tibiae.

Figure 79 – ROI of the femur.

Figure 80 – ROI of the vertebra.

Figure 81 – ROI of the calvaria.
Figure 82 – ROI of the maxilla. Interradicular space in the 2nd molar.

Figure 83 – ROI of the mandible. Interradicular space in the distal root of the 1st molar.
MICRO-CT ANALYSIS OF THE TIBIAE

Proximal tibial bone specimens from both 8 months Sham and Ovx animals were scanned by microcomputed tomography (μCT). The μCT was performed using a desktop scanner (μCT 35; Scanco Medical, Bruttisellen, Switzerland) with a voxel size of 12 mm, an X-ray tube voltage of 70 kVp, current intensity of 114 mA, and an integration time of 600 ms. A volume of interest was manually drawn on each specimen to include the trabecular structure of the proximal tibia. Structural evaluation was carried out using the version 6.0 of the Scanco Medical software. Microstructural measures included bone volume per total volume (BV/TV), connective density (CD), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp). The computation of these structural measures has previously been described in detail elsewhere.[338]

BIOMECHANICAL TEST

The femur and mandible were subject to biomechanical evaluation by using an Instron 1186 Materials Testing System (Instron Corp.) and a JJ Instruments force cell with 5000N. Force records were collected using LabView 2.0 data acquisition software (National Instruments).

The femurs and mandible were stored at -80°C. The samples were defrosted to room temperature, before the analysis. These were kept moist during the preparation and testing using physiological saline solution. The maximum load (N), ultimate stress (mpa) and young’s module (gpa) were evaluated, on the diaphysis of the femur and mandible from each group. Both groups Sham and Ovx were assessed at month 3 of the osteoporotic condition. Each femur were placed on two supports spaced 12 mm apart, and load was applied to the bone midway between the supports at a deformation rate of 1 mm/minute until failure. The femurs were positioned such that bending occurred about the medial-lateral axis.
2. CHARACTERIZATION OF THE CALVARIAL AND MANDIBULAR BONE REGENERATION MODELS

X-RAY DENSITOMETRIC ANALYSIS

The resected bones of the calvaria of the CSD calvarial bone regeneration and the right mandible with the bone regeneration procedure were analyzed radiographically.

Imaging protocol and computer image analysis was achieved with a RVG intraoral sensor (KODAK® RVG 5100, USA) and processed with software KODAK®. These images were evaluated with an image software program ImageJ® as seen previously in the protocol for evaluation of the osteoporotic condition.

In the assessment of the bone regeneration of CSD of the calvarial bone defect, circular ROIs with 5 mm in diameter were established, as the original bone defect, as seen in the image (Figure 84). To assess the bone regeneration of CSD of the mandibular bone defect, circular ROIs with 4 mm in diameter were established, as the original bone defect, as seen in the image (Figure 85). Intensity of signal was evaluated in unprocessed TIFF files and normalized by the intensity of aluminium or gutta-percha cone fragment as references.
MATERIALS AND METHODS

MODULATION OF THE BONE REGENERATION PROCESS IN SYSTEMIC IMPAIRED CONDITIONS

Figure 84 – ROI of the CSD of the calvaria.

Figure 85 – ROI of the mandible. Circular region with 4mm of diameter such as the original bone defect.
MATERIALS AND METHODS

MICRO-CT ANALYSIS

Micro-CT imaging of the calvarias from both 6 months Sham and Ovx animals were performed. The mandibles were evaluated at month 1 and 3, both the Sham and Ovx animals. The μCT was performed with a desktop scanner (μCT 35; Scanco Medical, Bruttisellen, Switzerland) with a voxel size of 12 mm, an X-ray tube voltage of 70 kVp, current intensity of 114 mA, and an integration time of 600 ms. The volume of interest (VOI) was manually selected involving the area of the defect. Quantification of bone volume was performed using MicroView software (GE Healthcare).

HISTOLOGIC ANALYSIS

The area of the original surgical defect and the surrounding tissues were removed in one piece. In the cut of the calvaria and mandible, a bone saw with mechanical cooling was used, ensuring the integrity of all margins of the defects. All the specimens were prepared for non-decalcified histological study, according to an established protocol.[339]

Briefly, the blocks were fixed in 10% neutral formalin, pH 7.4. After the fixation period, the process of continuous agitation in a dehydration and infiltration system (Exakt 510) was established. The samples were immersed in a growing series of ethanol concentrations followed by the infiltration in growing series of methacrylate in ethanol and, finally, two passages in 100% of resin (Figure 86).
The subsequent stage was to embed the samples in a resin (EXAKT Technovit 7200 VLC Embedding media) and its photopolimerization in a light polymerization unit (EXAKT 520) (Figure 87).

![Figure 87](image-url) – Light Polymerization Unit (EXAKT 520) and embedding media (Technovit 7200 VLC).

After the polymerization, the blocs were set up in an acrylic sheet (50x100x1, 5 mm) with a specific methacrylate resin (Technovit 4000).

This allowed the blocks to maintain with parallel surfaces, including the areas of interest (Figures 88-89).

![Figure 88](image-url) – Block with the sample of the mandibles.

![Figure 89](image-url) – Cut of the block with the sample.

The samples were cut in a microtome for hard tissues (Exakt 310 CP). This is a precision cutting system with regulator speed and irrigation, using a band saw (Figure 90). Following, the samples were grinded in a polishing turntable, with adjustable speed and permanent irrigation (Exakt 400 CS) (Figure 91). The control of the thickness
was carried out at a constant weight of 50 grams. The slides were prepared in the final sandwich technique using a light-cured resin (Technovit 7210 VLC) as glue between layers. The final thickness of around 50 µm was obtained by cutting with a saw band, followed by polishing with calibrated disks, decreasing in grain size. The final thickness control was made with digital micrometer (Mitutoyo 2093).

The sections were stained with Toluidine blue for analysis by light microscope Olympus® CX31 with DP-25 Camera (Imaging Software Cell^B) (Fig. 92) and by magnifying glass a Nikon® SMZ800 (Stereoscopic Zoom Microscopes) (Fig. 93).
HISTOMORPHOMETRIC ANALYSIS

The most central portion of each defect of the calvarial bone defect and mandibular bone defect from both groups, Sham and Ovx, at all the time points of the study, were identified and subjected to histomorphometric analysis. The sections were viewed and evaluated for new bone formation by two calibrated and blinded examiners using a light microscope, for high magnifications.

Histomorphometric analyses were performed with appropriate software (Image-Pro Plus® (version 6.0.0.260), Media Cybernetics, Berkshire, UK) which allowed calibrated calculation of the area of tissue sections.

The total bone area, newly formed bone area, and bone substitute (biomaterial) area were manually delimited. Following, bone volume per tissue volume (BV/TV; i.e. the percentage of newly formed bone in the region of interest) and bone substitute volume per tissue volume (BSV/TV; i.e. the percentage of bone substitute in the region of interest) were determined.

QUANTITATIVE REAL-TIME PCR EVALUATION

Regenerating bone samples were harvested from calvarial tissue, at month 6 following the craniotomy from both groups Sham and Ovx. These sections enclosed a bone volume exceeding the defect region by 2mm. The samples were frozen in liquid nitrogen and grinded to powder in a mortar. Following, total RNA was isolated using the TRIzol reagent (Invitrogen, Germany) according to the manufacturer’s protocol. The concentration of the total RNA was determined using a spectrophotometer. cDNA was synthesized from 0.5 μg of total RNA using a commercial first-strand cDNA synthesis kit (QIAGEN, Germany). Reverse transcription PCR was performed in triplicate for each sample, using primers specific for amplification of rat alpha-1 type I collagen (Col I), osteocalcin (OC), Runt-related transcription factor 2 (RUNX2), estrogen receptor-alpha (ER-a), estrogen receptor-beta (ER-b) and peroxisome proliferator-activated receptor gamma (PPAR-g) genes. Sequences of the used primers are...
presented in Table 8. Real-time PCR reactions were performed using SYBRGreen in a 7900HT Fast Real-Time PCR System (Applied Biosystems). To discriminate specific from nonspecific PCR products, a melting curve was obtained at the end of each run. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize the data and to determine the relative expression of the target genes.

**Table 8 – Sequence of the PCR primers for amplification of expressed genes.**

| SEQUENCES OF PRIMERS                  | Col 1a1 F        | Col 1a1 R        | OC F             | OC R             | Runx2 F          | Runx2 R          | ER-alpha F       | ER-alpha R       | ER-beta F        | ER-beta R        | PPAR-γ F         | PPAR-γ R         | GAPDH F          | GAPDH R          |
|---------------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Col 1a1 F                             | TCCTTGTTGAATTGTGTTCGC | GGGATAAAAAACTGCTTTTGT | ATGCCACTGCGTATTGGTTGA | TCCGCTAGCTCGTCAAAATTG | CGAAATGCCTCTGCTGTTAT | TTCTGCTCTGCCCCTTCTTTCG | TGCACAGTGTTACGAAATGGG | TTCGCGCCCTTCCAAGTCTCTC | AAAAACTCACCGTCAGCCTT | GCTGAATACACTCATGGCGGTG | TGTGGACCTCTCTGTGATGG | CATTGGGTAGCTCCTTGTGA | GTGAGGTGACCACGCACTCTTCT | CTTGCCGTTGGTAGATGCAT |
F. DATA STATISTICAL ANALYSIS

All the data were collected and stored in a database created in Excel® software. Subsequently, analyses were performed using the statistical analysis program SPSS® v.16.0 (Statistical Package for the Social Sciences) and a significance level of 5% was considered (p<0.05).

For descriptive analyses, summary statistics were applied as appropriate. Continuous variables were described by measures of central tendency (mean) and dispersion (standard deviation). Continuous variables were also represented by box-plot graphs. Hypotheses on the distribution of continuous variables between two independent samples were tested using the Student t-test or a non-parametric test (Mann-Whitney), when appropriate. Normality distribution of variables was tested by the Shapiro-Wilk test.
III. RESULTS
A. CHARACTERIZATION OF THE OSTEOPOROTIC MODEL
In order to establish the characterization of the osteoporotic condition we addressed the animals from the calvarial experiment, since they were observed for a longer period than in the experiment evaluating the mandibular regeneration. The time points analyzed in the results refer to the time following the ovariectomy procedure, i.e. the rats at 2 months of age underwent sham or ovariectomy procedure; the TE1 corresponds to 3 months following ovariectomy or sham procedure; the TE2 corresponds to 5 months following ovariectomy or sham procedure and TE3 corresponds to 8 months following ovariectomy or sham procedure (see the materials and methods).

All the animals survived both surgical procedures without the development of major post-operative complications.
1. ANIMAL’S WEIGHT

From the observation of the weight of the animals, it was possible to perceive that at all times, the animals that underwent the ovariectomy procedure presented a mean weight higher than the animals that underwent the sham procedure (Table 9). These results were evaluated at the three time points (month 3, 5 and 8) and in all the time points the weight of the Ovx animals were significantly higher than that of Sham animals (p<0.05).

Table 9 – Comparison of weight of animals between the two groups under study of the critical size defects (CSD): animals submitted to ovariectomy (Ovx) and Sham animals.

<table>
<thead>
<tr>
<th>Time following surgery</th>
<th>Weight of animals</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Ovx</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (g)</td>
<td>Mean (g)</td>
<td>sd (g)</td>
</tr>
<tr>
<td>Month 3</td>
<td>252.71</td>
<td>377.57*</td>
<td>38.449</td>
</tr>
<tr>
<td></td>
<td>37.498</td>
<td>38.449</td>
<td></td>
</tr>
<tr>
<td>Month 5</td>
<td>297.03</td>
<td>420.43*</td>
<td>45.232</td>
</tr>
<tr>
<td></td>
<td>27.210</td>
<td>45.232</td>
<td></td>
</tr>
<tr>
<td>Month 8</td>
<td>396.29</td>
<td>450.14*</td>
<td>51.874</td>
</tr>
<tr>
<td></td>
<td>28.439</td>
<td>51.874</td>
<td></td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test,
* p<0.05 Vs. Sham group.; sd: standard deviation
2. UTERI CHARACTERIZATION

Ovariectomy was confirmed by observation of an atrophic uterus and failure to detect ovarian tissue (Figures 94-97).

MONTH 3

Figure 94 – Uterus from Ovx animal at month 3.

Figure 95 – Uterus from Sham animal at month 3.

MONTH 8

Figure 96 – Uterus from Ovx animal at month 8.

Figure 97 – Uterus from Sham animal at month 8. Note the presence of the ovaries.
The weight of the animal uterus at all time points of evaluation (month 3, 5 and 8) of those which underwent the ovariectomy procedure (Ovx) was significantly lower than for the Sham group (Table 10). These results were significantly higher than that of Sham animals (p<0.05), at all time points of observation.

Table 10 – Comparison of weight of uterus between the two groups under study of the critical size defects (CSD): animals submitted to ovariectomy (Ovx) and Sham animals.

<table>
<thead>
<tr>
<th>Time following surgery</th>
<th>Weight of uterus</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Ovx</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (g)</td>
<td>Mean (g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sd (g)</td>
<td>sd (g)</td>
<td></td>
</tr>
<tr>
<td>Month 3</td>
<td>0.492</td>
<td>0.093</td>
<td>0.080*</td>
</tr>
<tr>
<td>Month 5</td>
<td>0.550</td>
<td>0.097</td>
<td>0.087*</td>
</tr>
<tr>
<td>Month 8</td>
<td>0.574</td>
<td>0.081</td>
<td>0.088*</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test,

* p<0.05 Vs. Sham group.; sd: standard deviation
3. PLASMA LEVELS

Blood was collected to address plasmatic parameters as the alkaline phosphatase activity, calcium, phosphorous concentrations and estrogen levels in the three time points (month 3, 5 and 8). Albumin, Creatine kinase, Total protein and Triglycerides, in Sham and Ovx animal groups, were evaluated in two time periods (month 3 and 5).

In the Figure 98 the estradiol levels (pg/mL), measured at month 3, 5 and 8, for both animals’ groups: Ovx and Sham are presented. In Sham animals, mean estradiol levels were kept around 20pg/mL over time, but in the Ovx animals, levels were too low to be detected by the used measurement methods. In the Figure 98 the mean plasma values of alkaline phosphatase activity (ALP), calcium (Ca) and phosphorous (P), in Sham and Ovx animal groups, in three time periods (month 3, 5 and 8) are presented. No significant differences were verified in either groups or timepoints.
In the Figure 99 the mean plasma values of albumin (ALB), Creatine kinase (CK), Total protein (TP) and Triglycerides (TG), in Sham and Ovx animal groups, in two time periods (month 3 and 5), are presented. No significant differences were verified in ALB and CK in both timepoints. There are statistically significant differences in PT and TG plasma concentrations groups. In the evaluation of the total protein in the month 3 there are significant differences between the Sham and Ovx group. In the evaluation of the Triglycerides levels in the month 3 there are significant differences between the Sham and Ovx group.
RESULTS

Figure 99 – Graphic representation of the comparison of the levels of Albumin (g/dl), Total protein (TP), Triglycerides (TG) and Creatine kinase (CK), plasma concentrations by Sham and Ovx groups, over time. (Comparisons performed by the Mann-Whitney test; * p<0.05 Vs Sham group.)
4. EVALUATION OF TIBIAE

The tibiae were studied to examine and analyze whether differences between the animals from the Sham and Ovx group existed. To this, x-ray images were obtained and a densitometric analysis was performed. Micro-CT images of the tibiae were also taken, that allowed us to examine microstructural parameters of the trabecular structure. We also performed biomechanical tests and histological analysis to disclose, respectively, the mechanical properties and the structure of tibiae within Sham and Ovx animals.

I. X-RAY OF ANIMALS’ TIBIAE

Radiological evaluation of the tibias X-rays reveal a well-marked difference between the structure of tibiae between the Sham and Ovx groups. This was of particular relevant at the month 8 of evaluation (Figures 100-103). A substantial reduction of the trabecular structure in the Ovx group needs also to be highlighted.

Densitometric evaluations were performed using the ImageJ® software. Densitometric results are shown in Table 11. The Ovx group reported lower values at month 5 and 8 compared to the Sham group, but significant differences were only attained at the month 8 of evaluation. In both groups, a markedly decrease of the densitometry values was observed between the two evaluation points.
MONTH 5

**Figures 100A and 100B** – Radiographic image of left tibia of Sham animal, month 5, A: lateral view and B: anteroposterior view.

**Figures 101A and 101B** – Radiographic image of left tibia of Ovx animal, month 5. A: lateral view and B: anteroposterior view.
MONTH 8

Figures 102A and 102B – Radiographic image of left tibia of Sham animal, month 8. A: anteroposterior view and B: lateral view.

Figures 103A and 103B – Radiographic image of left tibia of Ovx animal, month 8. A: lateral view and B: anteroposterior view.
A progressive loss of trabecular structure was determined in which regards the Ovx group, from month 5 to month 8. In the Sham group, the decrease was not significant, as we can observe in the Table 11.

**Table 11** – Densitometry of tibiae of Sham and Ovx animals, over time.

<table>
<thead>
<tr>
<th>Time following surgery</th>
<th>Densitometry (AU)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Ovx</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>Mean</td>
<td>sd</td>
</tr>
<tr>
<td>Tibiae Month 5</td>
<td>1335.25</td>
<td>1267.50</td>
<td>123.43</td>
</tr>
<tr>
<td>Tibiae Month 8</td>
<td>1200.91</td>
<td>900.00*</td>
<td>95.47</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:

*p<0.05 Vs. Sham group; sd: standard deviation; AU: arbitrary unit

In Figures 104-105 is presented the graphical distribution of the densitometry values of tibiae at month 5 and 8, in Ovx and Sham groups.
Figure 105 – Box plot of the densitometry values of tibiae at month 8 of Sham and Ovx groups.*p<0.05 Vs Sham group.
II. MICRO-CT OF TIBIAE

The 3D rendering of the high resolution microtomographies of 8 months time point from Sham and Ovx animals shows the analyzed volume of interest in the proximal tibia. This exam revealed, as compared to Sham controls, a thinner trabecular organization, with decreased interconnectivity and increased separation, in Ovx animals (Figure 106).

![Figure 106](image-url) – Representative 3D renderings of 8 months time point, showing the analyzed volume of interest in the proximal tibia. Scale bar represents 1 mm A: Sham animal; B: Ovx animal.
Morphological analyses were confirmed by the quantification of the microstructural parameters that revealed a marked reduction in the Bone volume per total volume (BV/TV), Connective density (CD), Trabecular thickness (Tb.Th), and Trabecular number (Tb.N) and an increased Trabecular separation (Tb.Sp) in Ovx animals (Table 12).

Table 12 – Microstructural parameters of the trabecular structure of the proximal tibia in Sham and Ovx animals at the 8 months time point.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.49 (0.081)</td>
<td>317.2 (40.6)</td>
<td>7.36 (0.56)</td>
<td>0.081 (0.002)</td>
<td>0.116 (0.03)</td>
<td>903.6 (9.3)</td>
</tr>
<tr>
<td>Ovx</td>
<td>0.17 (0.052)*</td>
<td>89.2 (7.9)*</td>
<td>1.89 (0.28)*</td>
<td>0.071 (0.003)</td>
<td>0.538 (0.06)*</td>
<td>828.5 (8.5)*</td>
</tr>
<tr>
<td>8 Months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.54 (0.051)</td>
<td>300.4 (34.5)</td>
<td>8.07 (0.65)</td>
<td>0.090 (0.003)</td>
<td>0.100 (0.06)</td>
<td>883.5 (8.5)</td>
</tr>
<tr>
<td>Ovx</td>
<td>0.18 (0.043)*</td>
<td>33.7 (8.4)*</td>
<td>1.89 (0.32)*</td>
<td>0.092 (0.003)</td>
<td>0.545 (0.07)*</td>
<td>836.2 (9.5)*</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:

*p<0.05 Vs. Sham group,

III. BIOMECHANICAL TEST OF TIBIAE

The biomechanical parameters of the tibiae were evaluated at month 3 of the osteoporotic condition. By the observation of the results the Ultimate stress and Young’s module parameters were significant different between the two groups in evaluation, the Sham and Ovx animals. The Ultimate stress and Young’s module present higher values in the Sham animals. The maximum load parameter is higher in the Ovx animals although without a significant difference.

Table 13 – Biomechanical parameters of the proximal tibia in Sham and Ovx animals at month 3.

<table>
<thead>
<tr>
<th></th>
<th>Maximum Load (N)</th>
<th>sd</th>
<th>Ultimate stress (Mpa)</th>
<th>sd</th>
<th>Young’s module (GPa)</th>
<th>sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>49.62</td>
<td>6.15</td>
<td>349.05</td>
<td>143</td>
<td>5.02</td>
<td>1.74</td>
</tr>
<tr>
<td>Ovx</td>
<td>57.37</td>
<td>9.41</td>
<td>264.26*</td>
<td>49.58</td>
<td>3.43*</td>
<td>1.78</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:

*p<0.05 Vs. Sham group, sd: standard deviation
IV. HISTOLOGY

The histologic sections of the tibiae at month 8, allows us to observe in the Ovx group a loss in the number of trabecular structures and a thinner thickness of these trabeculae. The medular bone in the Sham group as denser trabeculae.

Figure 107 – View of the proximal tibia with a magnification of 40x of the Sham animal at month 8. Toluidine blue staining.

Figure 108 – View of the proximal tibia with a magnification of 40x of the Ovx animal at month 8. Toluidine blue staining.
5. EVALUATION OF THE FEMUR

The x-ray images were obtained and a densitometric analyzes was performed in order to realized if there were differences between the animals from the Sham and Ovx group. 3 point bending tests were conducted to measure femur’s biomechanical properties and histological analysis was conducted to adress the microstructure between the Sham and Ovx animals.

I. X-RAY

Radiological evaluation of the femurs X-rays reveal a well-marked difference between the structure of femur between the Sham and Ovx groups, with a substantial reduction of the trabecular structure in the Ovx group (Figures 109-113).

Densitometric evaluations were performed as previously described. Densitometric results are shown in Table 13. The Ovx group reported lower values at month 3 and 5 compared to the Sham group, and were significant differences at both times of the evaluation. In both groups, a markedly decrease of the densitometry values was observed between the two evaluation points.
RESULTS

Figure 109 – Femur from Sham animal at month 3.

Figure 110 – Femur from Ovx animal at month 3.

Figure 111 – Femur from Sham animal at month 5.

Figure 112 – Femur from Ovx animal at month 5.
Table 13 – Densitometry of femurs of Sham and Ovx animals, over time.

<table>
<thead>
<tr>
<th>Time following surgery</th>
<th>Densitometry (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Femur Month 3</td>
<td>3159.23</td>
</tr>
<tr>
<td>Femur Month 5</td>
<td>2975.71</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:
*p<0.05 Vs. Sham group,

sd: standard deviation; AU: arbitrary unit

In Figures 113-114 is presented the graphical distribution of the densitometry values of tibiae at month 3 and 5, in Ovx and Sham groups.

Figure 113 – Box plot of the densitometry values of femurs at month 3 of Sham and Ovx groups. *p<0.05 Vs Sham group.
**Figure 114** – Box plot of the densitometry values of femurs at month 5 of Sham and Ovx groups. *p<0.05 Vs Sham group.
II. BIOMECHANICAL TEST OF FEMUR

The biomechanical parameters of the femur were evaluated at month 3 of the osteoporotic condition. By the observation of the results the Ultimate stress parameter was lower in the Ovx group and was significant different than the Sham group. The Maximum load and Young’s module present higher values in the Sham animals, although without a significant difference, between the two groups.

<table>
<thead>
<tr>
<th></th>
<th>Maximum Load (N)</th>
<th>sd</th>
<th>Ultimate stress (Mpa)</th>
<th>sd</th>
<th>Young’s module (GPa)</th>
<th>sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>92.66</td>
<td>9.09</td>
<td>100.07</td>
<td>7.44</td>
<td>1.92</td>
<td>0.19</td>
</tr>
<tr>
<td>Ovx</td>
<td>91.7</td>
<td>5.53</td>
<td>86.75*</td>
<td>6.38</td>
<td>1.48</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test.
*p<0.05 Vs. Sham group, sd: standard deviation
III. HISTOLOGY

A loss of trabecular structure was observed in the histologic sections of the femur at month 3 of the osteoporotic condition in the Ovx group. It is of relevance the diminished number and decreased thickness of trabeculae in the Ovx animals.

Figure 115 – View of the femur with a magnification of 10x of the Sham animal at month 8. Toluidine blue staining.

Figure 116 – View of the femur with a magnification of 10x of the Ovx animal at month 8. Toluidine blue staining.
6. EVALUATION OF THE CALVARIA

X-ray images of the calvaria were taken to evaluate the differences on mineral density of the bone, between the Sham and Ovx animals. Densitometric evaluations of the calvarial were performed using the ImageJ® software at month 3 and 5.

I. X-RAY

Radiological evaluation of the calvaria X-rays show a variance between the structure of calvaria between the Sham and Ovx groups, with a slight reduction of the radiopacity in the Ovx group with more radiolucent images (Figures 117-120).

Figure 117 – Calvaria from Sham animal at month 3.

Figure 118 – Calvaria from Ovx animal at month 3.
RESULTS

Densitometric evaluations of the calvarial were performed using the ImageJ® software at month 3 and 5. Densitometric results are shown in Table 15. The Ovx group reported slightly lower values in all time points of evaluation, with significant differences.

Table 15 – Densitometry analyses of calvarial bones of Sham and Ovx animals, at month 3 and 5.

<table>
<thead>
<tr>
<th>Time following surgery</th>
<th>Densitometry (AU)</th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Ovx</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>sd</td>
<td>Mean</td>
</tr>
<tr>
<td>Calvaria Month 3</td>
<td>612.01</td>
<td>98.36</td>
<td>517.69*</td>
</tr>
<tr>
<td>Calvaria Month 5</td>
<td>568.27</td>
<td>72.35</td>
<td>438.08</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:

*p<0.05 Vs. Sham group,
sd: standard deviation; AU: arbitrary unit
RESULTS

Figure 121 – Box plot of the densitometry values of the calvarial bone at month 3 of Sham and Ovx group.

Figure 122 – Box plot of the densitometry values of the calvarial bone at month 5 of Sham and Ovx group.
7. EVALUATION OF THE VERTEBRA

X-ray images of the vertebra were taken to evaluate mineral density differences between the Sham and Ovx animals. Densitometric evaluations of the vertebral were performed using the ImageJ® software at month 3 and 5.

I. X-RAY

A progressive loss of trabecular structure was significantly determined in which regards the Ovx group, at month 5. Radiological evaluation of the vertebra revealed a well-marked difference between the structure of vertebra between the Sham and Ovx groups (Figures 123-126).

Figure 123 – Vertebra from Sham animal at month 3.

Figure 124 – Vertebra from Ovx animal at month 3.

Figure 125 – Vertebra from Sham animal at month 5.

Figure 126 – Vertebra from Ovx animal at month 5.
The Ovx group reported lower values at the densitometric analysis, at month 3 and 5 compared to the Sham group, but significant differences were only attained at the month 5 of evaluation. In both groups, a markedly decrease of the densitometry values was observed between the two evaluation points.

**Table 16** – Densitometry analyses of vertebrae of Sham and Ovx animals, at month 3 and 5.

<table>
<thead>
<tr>
<th>Time following surgery</th>
<th>Densitometry (AU)</th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Ovx</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>sd</td>
<td>Mean</td>
</tr>
<tr>
<td>Vertebra Month 3</td>
<td>3705.58</td>
<td>619.73</td>
<td>2236.67 *</td>
</tr>
<tr>
<td>Vertebra Month 5</td>
<td>3543.85</td>
<td>924.28</td>
<td>1955.25 *</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:

* p<0.05 Vs. Sham group,

sd: standard deviation; AU: arbitrary unit

**Figure 127** – Box plot of the densitometry values of the vertebrae at month 3 of Sham and Ovx group. *p<0.05 Vs Sham group.
**Figure 128** – Box plot of the densitometry values of the vertebrae at month 5 of Sham and Ovx group. *p<0.05 Vs Sham group.
8. EVALUATION OF THE MAXILLA

X-ray images of the maxilla were taken to compare the mineral density between the Sham and Ovx animals. Densitometric evaluations of the maxilla were then performed using the ImageJ® software at month 3 and 5. Micro-CT images of the maxilla were also acquired, that allowed us to examine microstructural parameters of the trabecular structure within the interradicular area of the 1st molar.

1. X-RAY

Radiological evaluation of the maxilla X-rays shows a slight variation between the radiopacity of the maxilla bone between the Sham and Ovx groups, with a slight dissimilarity in the reduction of the trabecular structure when observed between the roots in the Ovx group, more notorious at month 5 (Figures 129-132).

**Figure 129** – Maxilla from Sham animal at month 3.  
**Figure 130** – Maxilla from Ovx animal at month 3.  
**Figure 131** – Maxilla from Sham animal at month 5.  
**Figure 132** – Maxilla from Ovx animal at month 5.
RESULTS

Densitometric evaluations of the maxilla were performed using the ImageJ® software at month 3 and 5. Densitometric results are shown in Table 17. At month 3 no significant differences were found between groups. The Ovx group reported lower values at month 5 compared to the Sham group, despite no significant differences were observed.

Table 17 – Densitometry analyses of maxilla of Sham and Ovx animals, at month 3 and 5.

<table>
<thead>
<tr>
<th>Time following surgery</th>
<th>Densitometry (AU)</th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Ovx</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>sd</td>
<td>Mean</td>
</tr>
<tr>
<td>Maxilla Month 3</td>
<td>1289.23</td>
<td>186.79</td>
<td>1306.00</td>
</tr>
<tr>
<td>Maxilla Month 5</td>
<td>1520.00</td>
<td>514.91</td>
<td>1217.75</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:

*p<0.05 Vs. Sham group,

sd: standard deviation; AU: arbitrary unit
Figure 133 – Box plot of the densitometry values of the maxilla at month 3 of Sham and Ovx group.

Figure 134 – Box plot of the densitometry values of the maxilla at month 5 of Sham and Ovx group.
II. MICRO-CT

Micro-CT evaluation was performed in a circumscribed area in the space between roots of the 1st molar, of the maxilla, at month 3 of the study, and reported a significant decrease in the number of trabecular structures in the Ovx animals. Moreover, Ovx animals also revealed an increased dimension of the medullary cavity.

Figure 135 – Maxilla from Sham animal at month 3. Figure 136 – Maxilla from Sham animal at month 3.

Figure 137 – Maxilla from Ovx animal at month 3. Figure 138 – Maxilla from Ovx animal at month 3.
A microstructural analysis of the maxilla was performed at month 3 and 5 in both groups. The parameter bone volume per tissue volume (BV/TV), was higher in the Sham animals in both time of the evaluation, month 3 and 5. These values were only significant different on month 5. The connective density (CD) was lower in the Sham animals in both the times of the evaluation, month 3 and 5, without significant differences. The trabecular number (Tb.N) and trabecular thickness (Tb.Th) had elevated values in the Sham group in both the evaluation time points, but only the trabecular thickness (Tb.Th) had significant differences. The trabecular separation (Tb.Sp) was higher in the Ovx animals in both time points of evaluation, with significant differences in both times of evaluation, month 3 and 5. The density was higher in the Sham animals in both time points of evaluation, with significant differences in the month 5 of evaluation.

<table>
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<tbody>
<tr>
<td><strong>Month 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.88 (0.078)</td>
<td>43.5 (8.94)</td>
<td>5.72 (0.48)</td>
<td>0.255 (0.02)</td>
<td><strong>0.121 (0.02)</strong></td>
<td>1081.7 (13.2)</td>
</tr>
<tr>
<td>Ovx</td>
<td>0.79 (0.054)</td>
<td>69 (15.43)</td>
<td>5.15 (0.54)</td>
<td>0.232 (0.03)</td>
<td><strong>0.156 (0.03)</strong>*</td>
<td>1055.2 (11.5)</td>
</tr>
<tr>
<td><strong>Month 5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td><strong>0.88 (0.067)</strong></td>
<td><strong>36.7 (10.4)</strong></td>
<td><strong>5.32 (0.53)</strong></td>
<td><strong>0.297 (0.06)</strong></td>
<td><strong>0.145 (0.07)</strong></td>
<td><strong>973.2 (10.5)</strong></td>
</tr>
<tr>
<td>Ovx</td>
<td>0.75 (0.035)*</td>
<td>51.2 (8.54)</td>
<td>4.87 (0.68)</td>
<td><strong>0.255 (0.04)</strong>*</td>
<td><strong>0.159 (0.05)</strong>*</td>
<td><strong>816.3 (13.7)</strong>*</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:
*p<0.05 Vs. Sham group,
9. EVALUATION OF THE MANDIBLE

X-ray images of the mandible were taken to address the differences regarding the mineral density between the Sham and Ovx animals. Densitometric evaluations were then performed using the ImageJ® software at month 3 and 5. Micro-CT images of the mandibles were also taken, that allowed us to examine microstructural parameters of the trabecular structure. We performed biomechanical tests to measure among other values of maximum stress between the Sham and Ovx animals.

I. X-RAY

Radiological evaluation of the mandible shows a variance between the radiopacity of the mandibular bone between the Sham and Ovx groups, with a slighty reduction of the trabecular structure when observed in the alveolar bone between the roots in the Ovx group. These diffences are more perceptible at month 5 (Figures 139-142).

![Figure 139 – Mandible from Sham animal at month 3.](image1)

![Figure 140 – Mandible from Ovx animal at month 3.](image2)

![Figure 141 – Mandible from Sham animal at month 5.](image3)

![Figure 142 – Mandible from Ovx animal at month 5.](image4)
RESULTS

Densitometric evaluations of the mandible were performed using the ImageJ® software at month 3 and 5. Densitometric results are shown in Table 19. The Ovx group reported slightly lower values in all time points of evaluation, and no significant differences were observed.

<table>
<thead>
<tr>
<th>Time following surgery</th>
<th>Densitometry (AU)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Ovx</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>Mean</td>
<td>sd</td>
</tr>
<tr>
<td>Mandible Month 3</td>
<td>6794.96</td>
<td>6618.03</td>
<td>915.39</td>
</tr>
<tr>
<td>Mandible Month 5</td>
<td>7080.86</td>
<td>6661.85</td>
<td>705.64</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:
*p<0.05 Vs. Sham group,
sd: standard deviation, AU: arbitrary unit.

Figure 143 – Box plot of the densitometry values of the mandible at month 3 of Sham and Ovx group.
Figure 144 – Box plot of the densitometry values of the mandible at month 5 of Sham and Ovx group.
II. MICRO CT OF MANDIBLE

Micro-CT evaluation was performed in a circumscribed area in the space between roots of the 1\textsuperscript{st} molar, of the mandible, and at month 3 of the study, reported a significant decrease in the number of trabecular structures in the Ovx animals (Figures 145-148). Moreover, Ovx animals also revealed an increased dimension of the medullary cavity.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure145.png}
\caption{Mandible from Sham animal at month 3.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure146.png}
\caption{Mandible from Sham animal at month 3.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure147.png}
\caption{Mandible from Ovx animal at month 3.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure148.png}
\caption{Mandible from Ovx animal at month 3.}
\end{figure}
A microstructural analysis of the mandible was performed at month 3 and 5 in both groups. The parameter bone volume per tissue volume (BV/TV) was higher in the Sham animals in both time of the evaluation, month 3 and 5. These values were significant different on both time points of evaluation. The connective density (CD) was higher in the Sham animals at month 3, but lower in month 5, with significant differences in this time point. The trabecular number (Tb.N) and trabecular thickness (Tb.Th) had elevated values in the sham group on month 3. On month 5 the trabecular number (Tb.N) was lower in the Sham animals and the trabecular thickness (Tb.Th) higher. None of these values presented significant differences. The trabecular separation (Tb.Sp) was higher in the Ovx animals in both time points of evaluation, with significant differences at month 5. The density was higher in the Sham animals in both time points of evaluation, without significant differences in both months.

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<tbody>
<tr>
<td>Month 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.79 (0.072)</td>
<td>26.2 (2.8)</td>
<td>4.67 (0.41)</td>
<td>0.26 (0.03)</td>
<td>0.157 (0.05)</td>
<td>1035.5 (13.2)</td>
</tr>
<tr>
<td>Ovx</td>
<td>0.65 (0.062)*</td>
<td>25.3 (3.1)</td>
<td>4.14 (0.38)</td>
<td>0.248 (0.03)</td>
<td>0.197 (0.04)</td>
<td>1021.2 (14.5)</td>
</tr>
<tr>
<td>Month 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.83 (0.061)</td>
<td>17.2 (1.7)</td>
<td>3.94 (0.65)</td>
<td>0.28 (0.03)</td>
<td>0.129 (0.06)</td>
<td>926.7 (13.4)</td>
</tr>
<tr>
<td>Ovx</td>
<td>0.68 (0.054)*</td>
<td>24.7 (2.7)*</td>
<td>4.67 (0.32)</td>
<td>0.276 (0.04)</td>
<td>0.147 (0.03)*</td>
<td>897.3 (16.7)</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:
*p<0.05 Vs. Sham group,
III. BIOMECHANICAL TEST OF MANDIBLE

The biomechanical parameters of the mandible were evaluated at month 3 of the osteoporotic condition. The Ultimate stress parameter was lower in the Ovx group with a score of 27.5 Mpa (sd=4.5), comparing with the Sham group with 38.9 Mpa (sd=6.4). The Ultimate stress presented significant differences between these groups.
RESULTS
B. EVALUATION OF THE CALVARIAL BONE DEFECT
RESULTS

In order to address the biomaterial-mediated bone regeneration process in a bone not submitted to biomechanical loading, a critical size defect was established within the calvarial bone and implanted with a xenograft. The time points analyzed in the results refer to the time following the calvarial bone defect, i.e. the rats at 2 months of age underwent the sham or ovariectomy procedure; the TE1 corresponds to 1 month of healing following the craniotomy and 3 months following ovariectomy or sham procedure; the TE2 corresponds to 3 months of healing following the craniotomy and 5 months following ovariectomy or sham procedure and TE3 corresponds to 6 months of healing following the craniotomy and 8 months following ovariectomy or sham procedure (Figure 149).

All the animals survived the surgical procedures without the development of major post-operative complications.

![Figure 149 – Study timeline of the craniotomy procedure.](image)

**ANIMALS EUTHANASIA:**

**TE1** – Time of experiment 1 – 1 month healing following craniotomy (month 3 after ovariectomy procedure)

**TE2** – Time of experiment 2 – 3 months healing following craniotomy (month 5 after ovariectomy procedure)

**TE3** – Time of experiment 3 – 6 months healing following craniotomy (month 8 after ovariectomy procedure)
1. MACROSCOPIC EVALUATION

In the CSD filled with Bio-oss®, it is established that the vast majority of the biomaterial was well contained within the defect, apart from sporadic finding of granules outside the delimited defect area. Moreover, the vast majority of granules seemed not to have been resorpted, even after month 6 of implantation, both in Sham and Ovx animals (Figures 150-155).

The unfilled defects (negative control group) remained open with reduced evidence of new bone formation, throughout the evaluation period, both in Ovx and Sham animals (Figures 156-157).

MONTH 1

Figure 150 – Sham calvarial CSD with Bio-oss®, at month 1.

Figure 151 – Ovx calvarial CSD with Bio-oss®, at month 1.
MONTH 3

Figure 152 – Sham calvarial CSD with Bio-oss®, at month 3.

Figure 153 – Ovx calvarial CSD with Bio-oss®, at month 3.

MONTH 6

Figure 154 – Sham calvarial CSD with Bio-oss®, at month 6.

Figure 155 – Ovx calvarial CSD with Bio-oss®, at month 6.
MONTH 6 – NEGATIVE CONTROL

Figure 156 – Sham calvarial CSD without Bio-oss®, at month 6.

Figure 157 – Ovx calvarial CSD without Bio-oss®, at month 6.
2. X-RAY EVALUATION

By the evaluation of the X-rays it is recognizable that the Bio-oss® granules are present in all of the seeded CSD. No evidence of biomaterial resorption was found, nonetheless the vast majority of the graft material was confined to the calvarial defects. Apparently, new bone formation area is limited to the margin of the defect, in both Ovx and Sham groups (Figures 158-163).

CALVARIAL CRITICAL DEFECTS – BIO- OSS®

Figure 158 – Radiographic image of Sham CSD filled with Bio-Oss®, 1 month following craniotomy.

Figure 159 – Radiographic image of Ovx CSD filled with Bio-Oss®, 1 month following craniotomy.
Figure 160 – Radiographic image of Sham CSD filled with Bio-Oss®, 3 months following craniotomy.

Figure 161 – Radiographic image of Ovx CSD filled with Bio-Oss®, 3 months following craniotomy.

Figure 162 – Radiographic image of Sham CSD filled with Bio-Oss®, 6 months following craniotomy.

Figure 163 – Radiographic image of Ovx CSD filled with Bio-Oss®, 6 months following craniotomy.
CALVARIAL CRITICAL DEFECTS – NEGATIVE CONTROL GROUP

Note the dotted area with 5mm of diameter, to highlight the region of the original surgical defect.

The X-rays analysis reveals that the unfilled defects (negative control group) remained open, with residual evidence of new bone formation, throughout the evaluation period, both in Ovx and Sham animals (Figures 164-165).
3. MICRO-CT OF CALVARIAL

Micro-tomographic analysis was conducted for samples of Sham and Ovx animals, 6 months following craniotomy and biomaterial implantation. Due to density differences and the high resolution acquisition, biomaterial granules with high radiopacity (bright white) could be easily differentiated from old bone and from newly regenerated bone tissue (light grey). Overall, the amount of newly formed bone was relatively low, at the assayed time point, in both experimental groups. At the defect margin, a centripetal growth of the newly formed radiopaque tissue could be identified at the sagittal view, in both Sham and Ovx samples (Figure 166). Evidence of newly formed bone could be depicted in light grey, throughout and in close contact with the interspersed granular material. In the assessed Sham samples an evidence of a higher bone formation could be verified by the presence of denser areas of newly formed bone tissue.

![Figure 166](image-url) – Micro-Ct of the Ovx and Sham CSD filled with Bio-oss®, at month 6, sagittal view. Scale bar represents 400µm.
In the axial view (Figure 167), it is observable that the granules of Bio-oss® are uniformly distributed within the defect. In the Sham defect we can observe an increased area of newly formed bone tissue (arrows), at the margin of the defect, comparing to the Ovx group of animals. Some Bio-oss® granules seem to be surrounded by newly formed bone, in both groups (Figure 167).

![Sham & Ovx](image)

*Figure 167 – Micro-Ct of the Sham (A) and Ovx (B) CSD filled with Bio-oss®, at month 6, axial view.*

Details of the bone regeneration process in the vicinity of the defect margin were disclosed by the axial view (Figure 168). In Sham animals, an increased bone tissue regeneration could be branded, as evidenced by the greater amount of newly formed mineralized tissue engulfing the implanted granules (dotted rectangle), and increased amount of newly formed bone within the implanted granules surface (triangles). In Ovx animals, the evidence of newly formed mineralized tissue was far less scarce with no signs of biomaterial engulfment and reduced amount of formed tissue on biomaterial surface (triangles).
Quantitative analysis of the BV within the assayed TV (BV/TV), considering segmentation based on the grey scale value, was used to determine the bone formation process. At the assayed time points, values were found to be consistently lower in the Ovx group, as comparing to the time matched Sham group (Figure 169). Differences attained significance at the 3 and 6 months’ time points.
RESULTS

**Figure 169** – Graphic representation of the bone volume per tissue volume (BV/TV), of the calvarial bone defects at month 1, 3 and 6, of Sham and Ovx group. *p<0.05 Vs Sham group.
4. HISTOLOGY

Histological sections were analyzed and scored to address the tissue response at the bone–material interface and the tissue ingrowth within the grafted defects. Due to the slow regenerative process verified throughout the dimension of the defect, a special focus was set at the defect margins.

CALVARIAL CRITICAL DEFECTS – BIO-OS$	extsuperscript{S}$®

MONTH 1

At 1 month following craniotomy, at the defect margin, it was verified the typical formation of a cone with the vertex oriented toward the center of the defect, in which the centripetal tissue growth was attained. Osteogenic activity is observed with a pattern of intramembranous ossification, especially along the margins of the bone defect, for both Sham and Ovx animals. The biomaterial granules seem to be interspaced by fibrous tissue in the vast majority of the sample. In the close vicinity to the defect margin, some Bio-oss$	extsuperscript{S}$® granules seem to be partially surrounded by a thin layer of newly formed bone tissue, both in Ovx and Sham samples (Figures 170-173).
**RESULTS**

**Figure 170** – Representative image of the Sham CSD filled with Bio-oss®, at month 1. 40x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Old bone; 3 - New bone.

**Figure 171** – Representative image of the Sham CSD filled with Bio-oss®, at month 1. 100x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Old bone; 3 - New bone; 4 - Connective tissue; 5 - New blood vessels.
Figure 172 – Representative image of the Ovx CSD filled with Bio-oss®, at month 1. 40x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Old bone; 3- New bone.

Figure 173 – Representative image of the Ovx CSD filled with Bio-oss®, at month 1. 100x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Old bone; 3- New bone; 4- Connective tissue.
MONTH 3

At 3 months osteogenic activity is visualized at the margins of the defect, with the characteristic cone formation in both Ovx and Sham samples (Figures 174-178). In the representative images of the Sham group of animals, there is also a significant amount of newly formed bone tissue without contact with biomaterial particles, and apparently unrelated to the spatial top, bottom, outer and median of the defect margin (Figures 174-176). In both Ovx and Sham samples, the majority of biomaterial granules seem to be interspaced by fibrous tissue, nonetheless, some Bio-oss® granules are surrounded by new bone (Figures 174-178).

Figure 174 – Representative image of the Sham CSD filled with Bio-oss®, at month 3. 40x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Old bone; 3- New bone; 4- Fibrous tissue.
**RESULTS**

*Figure 175* – Representative image of the Sham CSD filled with Bio-oss®, at month 3. 100x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Old bone; 3 – New bone; 4 – Fibrous tissue.

*Figure 176* – Representative image of the Sham CSD filled with Bio-oss®, at month 3. 100x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Fibrous tissue; 3 – New bone.
RESULTS

Figure 177 – Representative image of the Ovx CSD filled with Bio-oss®, at month 3. 40x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Old bone; 3- New bone; 4- Fibrous tissue.

Figure 178 – Representative image of the Ovx CSD filled with Bio-oss®, at month 3. 100x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Old bone; 3- New bone; 4- Fibrous tissue.
RESULTS

MONTH 6

At month 6 of evaluation, a significant amount of bone formation was visualized at the margins of the defect, either in Sham and Ovx animals (Figures 179-183). In Sham animals, it is evident the inclusion of biomaterial granules into the newly formed bone at the margin of the defect. In the Figures 179-180, it was evident the engulfment of biomaterial granules into the newly formed bone at the margin of the defect a biomaterial granule fully surrounded and enclosed by new bone is presented.

In Ovx samples, significant newly formed bone tissue is visualized in close association with marginal Bio-oss® granules, but no evidence of biomaterial entrapment by the regenerative process is seen. Nevertheless, both in Ovx and Sham animals, most of the space between the biomaterial granules seemed to be fulfilled with fibrous tissue.

Figure 179 – Representative image of the Sham CSD filled with Bio-oss®, at month 6. 40x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Old bone; 3- New bone.
RESULTS

Figure 180 – Representative image of the Sham CSD filled with Bio-oss®, at month 6. 100x magnification. Toluidine blue staining. Bio-oss®(1) surrounded by new bone(2).

Figure 181 – Representative image of the Ovx CSD filled with Bio-oss®, at month 6. 40x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Old bone; 3 – New bone.
**Figure 182** – Representative image of the Sham CSD filled with Bio-oss®, at month 6. 40x magnification. Toluidine blue staining. 1 – Bio-oss®; 2 – Old bone; 3– New bone; 4– Fibrous tissue.

**Figure 183** – Representative image of the Ovx CSD filled with Bio-oss®, at month 6. 100x magnification. Toluidine blue staining. 1–Bio-oss®; 2– New bone; 3– Fibrous tissue.
CALVARIAL CRITICAL DEFECTS –NEGATIVE CONTROL GROUP

In the defects left unfilled, after 6 months of healing, reduced new bone formation was attained. Osteogenic activity was minimal and limited to the margins of the defects, both in Sham and Ovx groups (Figures 184-185). No significant differences were attained between groups.

Figure 184 – Representative image of the Sham animal, at month 6. 100x magnification. Toluidine blue staining. 1 – Old bone; 2 - New bone.

Figure 185 – Representative image of the Ovx animal, at month 6. 100x magnification. Toluidine blue staining. 1 – Old bone; 2 - New bone.
5. HISTOMORPHOMETRIC ANALYSIS

In order to validate the qualitative histologic findings by a quantitative approach, the relative amount of the newly formed bone was determined. The bone volume per tissue volume (BV/TV) was lower in Ovx animals, comparing to Sham animals, throughout the experimental period. Significant differences were attained at the month 3 and 6 following the craniotomy procedure. The bone substitute volume per tissue volume (BSV/TV) was also calculated and no significant differences between experimental groups were found, at any assayed time point (Table 21).

Table 21 – Morphometric analysis of histological samples. Histomorphometric analysis of the bone regeneration process in Sham and Ovx animals, throughout 6 months of healing following craniotomy.

<table>
<thead>
<tr>
<th>Time following surgery</th>
<th>BV/TV</th>
<th>BSV/TV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham (% /sd)</td>
<td>Ovx (% /sd)</td>
</tr>
<tr>
<td>Month 1</td>
<td>4,98 (3,78)</td>
<td>2,35 (1,67)</td>
</tr>
<tr>
<td>Month 3</td>
<td><strong>18,35 (5,78)</strong></td>
<td><strong>13,67 (7,65)</strong>*</td>
</tr>
<tr>
<td>Month 6</td>
<td><strong>28,45 (7,89)</strong></td>
<td><strong>16,56 (8,76)</strong>*</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:
*p<0.05 Vs Sham group; sd: standard deviation, BV/TV: bone volume per tissue volume, BSV/TV: bone substitute volume per tissue volume.
6. QUANTITATIVE PCR ANALYSIS OF GENE EXPRESSION DURING BONE HEALING

Quantitative real-time PCR was employed for quantifying bone healing related gene expression at mRNA level. Transcriptional regulation of gene expression is a key mechanism controlling cellular differentiation and function in all cell types. Osteoblasts differentiate from mesenchymal precursor cells and then produce the bone-specific extracellular matrix.

Its use can be pertinent, since the dysregulation of estrogen receptors could be involved in the underlying mechanism responsible for osteoporotic bone healing after estrogen depletion among other factors. The Col1, osteocalcin and RUNX 2 mRNA expression are associated with bone formation, as the ER-alpha.[340] Runx2 plays crucial role as a transcriptional activator of osteoblast differentiation and the serum osteocalcin is a sensitive and specific marker of osteoblastic activity and its serum level thus reflects the rate of bone formation.[341]

The PPAR-gamma is a master regulator of the adipogenic differentiation, its expression as the expression of ER-beta can be related with diminished bone formation.[342]

The expressions of osteoblast-specific genes Col I, OC and RUNX 2, as well as ER-alpha, ER-beta and PPAR-gamma genes, in the regenerating calvarial bone tissue following 6 months of graft implantation are expressed in Figure 186.

The expression of osteoblastic specific markers was significantly higher in Sham animals rather than in ovariectomized rats. ER-alpha mRNA expression levels were significantly lower in the regenerating tissue of Ovx animals, whereas the expression of ER-beta was significantly higher in Ovx rats, as comparing to Sham animals. PPAR-gamma expression was significantly higher in Ovx animals, comparing to Sham controls.
**RESULTS**

<table>
<thead>
<tr>
<th>Col 1</th>
<th>Runx 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PPAR-γ</th>
<th>ER beta</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image3.png" alt="Graph" /></td>
<td><img src="image4.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ER alpha</th>
<th>OC</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image5.png" alt="Graph" /></td>
<td><img src="image6.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

*Figure 186 – Graphic representation of Collagen type I (Col I), Runt-related transcription factor 2 (RUNX2), peroxisome proliferator-activated receptor gamma (PPAR-γ), estrogen receptor-beta, estrogen receptor-alpha and osteocalcin (OC) expression between the Sham and Ovx group ate month 6 following the craniotomy. *p<0.05 Vs Sham group.*
C. EVALUATION OF THE MANDIBULAR BONE DEFECT
In order to address the guided bone regeneration process biomaterial-mediated in a bone submitted to biomechanical loading, a critical size defect was established within the mandibular bone and implanted with a xenograft and a collagen membrane.

The ovariectomized rat model was selected and the bicortical critical sized defect was established in the mandible right angle. Three groups were formed Membrane (M), Membrane and Biomaterial (MB) and Control (C) group. In the Membrane group (M), the membranes were applied and the defect was covered on the bucal and lingual side. In the Membrane and Biomaterial group (MB) the defect was filled with the Putty® and the membrane was applied as in the M group. In the Control group the mandibular defect was left untreated. These groups were performed in the Sham and Ovx animals, as described in the Material and Methods chapter.

The time points analyzed in the results refer to the time following the mandibular bone defect (see in the Material and Methods). The TE1 corresponds to 1 month of healing following the mandibular bone defect and 3 months following ovariectomy or sham procedure and the TE2 corresponds to 3 months of healing following the mandibular bone defect and 5 months following ovariectomy or sham procedure.

All the animals survived the surgical procedures without the development of major post-operative complications.
1. MACROSCOPIC EVALUATION

In the macroscopic evaluation of the mandibles in which the bone defect was filled, the Evolution® membranes that surrounded the defects were visible. The CSD filled with Putty® and surrounded by the membrane Evolution®, allowed us to establish that the biomaterial was well contained within the defect (Figures 187-188). The integrity observed macroscopically of bone regeneration procedure suggests that the membrane was not completely resorbed in the two time points of evaluation.

The unfilled defects (negative control group) remained open with reduced evidence of new bone formation, throughout the evaluation period, both in Ovx and Sham animals (Figures 189-190).


2. X-RAY EVALUATION

The evaluation of the mandibular X-rays allow us to perceive the incomplete bone regeneration process, by detecting the margins of the original defect on the right angle of the mandible, with a radiopaque image around the edges in all the groups. The biomaterial is not perceptible assuming that this one as benn reabsorved. The Control group, with the defect left untreated, showed clearly the original defect as a radiolucent image, in both groups Sham and Ovx, assuming an absence of intense bone regeneration (Figures 191-192).

Centripetal growth of the newly formed radiopaque tissue could be observed in the margins of the original defect in the M (membrane) and MB (membrane and biomaterial) group.

The M group in the Sham animals, presents a more radiopaque image in the defect when compared with the Ovx animals (Figures 193-194). On the MB group the Sham animals present, as above, a more radiopaque image when compared with the Ovx animals (Figures 195-196).

Apparently, new bone formation area is limited to the margin of the defect, in both Ovx and Sham groups (Figures 193-196).
RESULTS

MONTH 1

CONTROL

Figure 191 – Mandibular CSD control group Sham animal at month 1.

Figure 192 – Mandibular CSD control group Ovx animal at month 1.

MEMBRANE

Figure 193 – Mandibular CSD M group, Sham animal at month 1.

Figure 194 – Mandibular CSD M group, Ovx animal at month 1.
MEMBRANE AND BIOMATERIAL

Figure 195 – Mandibular CSD MB group, Sham animal at month 1.

Figure 196 – Mandibular CSD MB group, Ovx animal at month 1.

The evaluation of the mandibular bone defect with a high magnification, allows us to calculate the bone density by the radiopacity in the x-ray. Densitometric evaluations were performed using the ImageJ® software. Densitometric results are shown in Table 22. The Ovx group reported lower values compared to the Sham group in both the groups (M and MB), but no significant differences were attained. Between all groups of evaluation, the Sham animals with the membrane and biomaterial reported a higher densitometric value.
RESULTS

Table 22 – Densitometry of mandibular bone defects of Sham and Ovx animals, at 1 month.

<table>
<thead>
<tr>
<th></th>
<th>Membrane</th>
<th>Membrane and biomaterial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (AU)</td>
<td>sd (AU)</td>
</tr>
<tr>
<td>Membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>3166.67</td>
<td>1266.107</td>
</tr>
<tr>
<td>Ovx</td>
<td>2119.57</td>
<td>1655.640</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test between the Sham and Ovx group.
sd: standard deviation; Au: Arbitrary unit

Figure 197 – High magnification of the mandibular defect. A – Sham animal M group; B – Ovx animal M group; C - Sham animal MB group; D – Ovx animal MB group, at month 1.
In Figure 198 is presented the graphical distribution of the densitometry values of mandibular bone defects in the Membrane group and the Membrane and biomaterial group at month 1, in Ovx and Sham groups.

Figure 198 – Box plot of the densitometry values of the mandibular bone defect with the Membrane (A) and Membrane and Biomaterial (B), at month 1 of Sham and Ovx groups.
MONTH 3

By the evaluation of the mandibular X-rays at month 3 it is possible to observe the center of the original defect with a radiolucent image, on the right angle of the mandible, revealing the absence of complete regeneration. The M group in the sham animals presents a more radiopaque image in the defect when compared with the M group Ovx animals (Figures 199-200). The centripetal regeneration is observed presenting a more radiopaque image in the borders of the defect. On the MB group the sham animals as above presents a more radiopaque image when compared with the ovx group (Figures 201-202). A substantial reduction of the trabecular structure in the Ovx group needs also to be highlighted, noticeable on the angle of the mandible.

MEMBRANE

Figure 199 – Mandibular CSD with an Evolution® membrane, Sham animal at month 3.
Figure 200 – Mandibular CSD with an Evolution® membrane, Ovx animal at month 3.
MEMBRANE AND BIOMATERIAL

Figure 201 – Mandibular CSD with an Evolution® membrane and Putty® Sham animal at month 3.

Figure 202 – Mandibular CSD with an Evolution® membrane and Putty® Ovx animal at month 3.

The evaluation of the mandibular bone defect with a high magnification, allows us to calculate the bone density by the radiopacity in the x-ray. Densitometric evaluations were performed using the ImageJ® software. Densitometric results are shown in Table 23. The Ovx group reported lower values compared to the Sham group in both the groups (M and MB). As noted earlier in the month 1, the sham animals in the MB group, presents the highest value for the densitometric analysis, when compared with all groups. In the M group significant differences were attained between the sham and Ovx animals, as the sham animals show higher values in the densitometric analysis. The Ovx animals in the MB group attained lower values when compared with the sham animals, but no significant differences were noticed. In both groups M and MB in the sham and Ovx animals, a markedly increase of the densitometry values was observed between the two evaluation points.
RESULTS

Table 23 – Densitometry of mandibular bone defects of Sham and Ovx animals, at 3 month.

<table>
<thead>
<tr>
<th>Densitometry</th>
<th>Mean (AU)</th>
<th>sd (AU)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>3955</td>
<td>976</td>
<td>0,281</td>
</tr>
<tr>
<td>Ovx</td>
<td>3558</td>
<td>511</td>
<td></td>
</tr>
<tr>
<td>Membrane and biomaterial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>4773</td>
<td>470</td>
<td>0,003</td>
</tr>
<tr>
<td>Ovx</td>
<td>3883*</td>
<td>253*</td>
<td></td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test between the Sham and Ovx group.

*p<0.05 Vs. Sham group; sd: standard deviation; Au: Arbitrary unit

Figure 203 – High magnification of the mandibular defect. A – Sham animal M group; B – Ovx animal M group; C - Sham animal MB group; D – Ovx animal MB group, at month 3.
In Figure 204 is presented the graphical distribution of the densitometry values of mandibular bone defects in the Membrane group and the Membrane and biomaterial group at month 3, in Ovx and Sham groups.

**Figure 204** – Box plot of the densitometry values of the mandibular bone defect with the Membrane (A) and Membrane and Biomaterial (B), at month 3 of Sham and Ovx groups. *p<0.05 Vs. Sham group.
3. MICRO-CT

Micro-tomographic analysis was conducted for samples of Sham and Ovx animals, 1 and 3 months following the mandibular bone defect and with implantation of biomaterial and a membrane of biomaterial implantation, depending on the group; Membrane (M) or Membrane and Biomaterial (MB). Due to the capacity to differentiate between mineralized tissue density, a different color is presented differentiating the highly mineralized tissue (light pink) from the less mineralized tissue (dark pink), within the created CSD. Overall, the defect was not closed in any essayed experimental situation, at the assayed time point, in both experimental groups. At the defect margin, a centripetal growth of the newly formed radiopaque tissue could be identified at the axial view, in both Sham and Ovx samples (Figures 205-208).

In all the images was not possible to distinguish the bone from the biomaterial. The radiographic densities of the bone and biomaterial are similar, precluding the possibility of discriminate the new bone formation from the biomaterial. This also justifies the small granules that are observed around the defect which must correspond to a portion of the biomaterial. The biomaterial is expected to be gradually resorbed, since the defect is no longer completely filled as observed in Figure 207.

In the Membrane group, in Sham animals, we can observe an increased area of newly formed bone tissue (pink and grey), at the margin of the defect, comparing to the Ovx animals also implanted with the membrane alone (Figures 205-206).

In the Membrane and Biomaterial group, Sham animals, increased bone tissue regeneration could be branded, as evidenced by the greater amount of newly formed mineralized tissue in light and dark pink. In Ovx animals, the evidence of newly formed mineralized tissue was far less scarce within the defect (Figures 207-208).
RESULTS

MODULATION OF THE BONE REGENERATION PROCESS IN SYSTEMIC IMPAIRED CONDITIONS

Figure 205 – Mandible from Sham animal treated with a Membrane at month 1.

Figure 206 – Mandible from Ovx animal treated with a Membrane at month 1.

Figure 207 – Mandible from Sham animal treated with a Membrane and biomaterial at month 1.

Figure 208 – Mandible from Ovx animal treated with a Membrane and Biomaterial at month 1.
The mineralized tissue volume was attained in the samples of all the groups in the two timepoints of evaluation. In the month 1, Sham animals of the MB groups present the highest values for bone volume, followed by the Sham animals of the M group and by the Ovx animals of the MB group and finally the Ovx animals of the M group.

At month 3 as observed previously, Sham animals in the MB group present the highest values of bone volume. Between the Sham and Ovx animals in the M and MB groups, the sham animals continually show higher values than the Ovx animals. Although no significant differences were attained between the group at all timepoints of evaluation.

Table 24 – Microstructural analyses of mandibular defect in the Membrane (M) and Membrane and Biomaterial (MB) groups of Sham and Ovx animals, at month 1 and 3.

<table>
<thead>
<tr>
<th>Month</th>
<th>Membrane</th>
<th>MTV</th>
<th>sd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>1.785</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>Ovx</td>
<td>0.965</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>Membrane and Biomaterial</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>1.990</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>Ovx</td>
<td>1.280</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>3.061</td>
<td>0.067</td>
</tr>
<tr>
<td></td>
<td>Ovx</td>
<td>1.675</td>
<td>0.152</td>
</tr>
<tr>
<td></td>
<td>Membrane and Biomaterial</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>3.661</td>
<td>0.140</td>
</tr>
<tr>
<td></td>
<td>Ovx</td>
<td>2.775</td>
<td>0.141</td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test between the Sham and Ovx group.
*p<0.05 Vs Sham group.; sd: standard deviation; MTV: mineralized tissue volume.
4. HISTOLOGY

Histological sections were analysed and scored to address the tissue response at the bone–material interface and the tissue ingrowth within the grafted defects. Throughout the analysis, a special focus was set at the defect margins.

MONTH 1: MEMBRANE

At 1 month following the mandibular surgery, at the defect margin, it was verified the typical formation of a cone with the vertex oriented toward the center of the defect, in which the centripetal tissue growth was attained. Osteogenic activity is observed with a pattern of intramembranous ossification, especially along the margins of the bone defect, for both Sham and Ovx animals. The new bone was interspaced by fibrous tissue in the vast majority of the sample. In the close vicinity to the defect margin, a small part of newly formed bone tissue is observed, both in Ovx and Sham samples (Figures 209-213).

The Sham animals in the Membrane group display in the peripheral zone of the central area of the defect the residual fibers of the collagen membrane, finding that this was not completely resorbed. When comparing the sham and Ovx animals, we can observe a more developed and compact trabecular new bone on the limits of the original defect in the Sham animals, than in the Ovx animals, with a thinner bone trabaculae (Figures 209-213).
Figure 209 – Representative image of the Sham CSD filled with a Membrane Evolution®, at month 1. 40x magnification. Toluidine blue staining. 1 – New bone; 2 – Old bone; 3 – Connective tissue; 4- Bone marrow.

Figure 210 – Representative image of the Sham CSD filled with a Membrane Evolution®, at month 1. 100x magnification. Toluidine blue staining. 1 – New bone; 2- Connective tissue; 3- Bone marrow.
Figure 211 – Representative image of the Ovx CSD filled with a Membrane Evolution®, at month 1. 40x magnification. Toluidine blue staining. 1 – New bone; 2 – Old bone; 3 – Connective tissue.

Figure 212 – Representative image of the Ovx CSD filled with a Membrane Evolution®, at month 1. 100x magnification. Toluidine blue staining. 1 – New bone (mature); 2 – Connective tissue; 3 – New bone (immature).
MONTH 1: MEMBRANE AND BIOMATERIAL

At month 1, osteogenic activity is visualized at the margins of the defect, with the characteristic cone formation in both Ovx and Sham samples (Figures 214-220). In the representative images of the Sham animals in the Membrane and Biomaterial group, there is a significant amount of newly formed bone tissue when compared with Ovx animals. The Membrane and Biomaterial group display a larger area of new bone formation when compared with animals of the Membrane group, at the same timepoint.

The Sham and Ovx animals display residual fibers of the collagen membrane, finding that this was not completely resorbed. Although no biomaterial is perceptible, we assume that it has been reabsorbed in a cell-mediated process. When comparing the Sham and Ovx animals, we can observe a more developed and compact trabecular new bone on the limitis of the original defect in the Sham animals, than in the Ovx animals, with a thinner bone trabaculae (Figures 214-220).
RESULTS

**Figure 214** – Representative image of the Sham CSD filled with Putty® and a Membrane Evolution®, at month 1. 40x magnification. Toluidine blue staining. 1 – New bone; 2 – Old bone; 3 - Connective tissue; 4 - Masseter muscle layer; 5 – Bone marrow.

**Figure 215** – Representative image of the Sham CSD filled with Putty® a Membrane Evolution®, at month 1. 100x magnification. Toluidine blue staining. 1 – New bone; 2 – Old bone; 3 - Connective tissue; 4 - Bone marrow.
Figure 216 – Representative image of the Sham CSD filled with Putty® a Membrane Evolution®, at month 1. 100x magnification. Toluidine blue staining. 1 – New bone; 2-Connective tissue; 3- Remnants of the residual membrane.

Figure 217 – Representative image of the Sham CSD filled with Putty® a Membrane Evolution®, at month 1. 400x magnification. Toluidine blue staining. 1 – New bone; 2-Osteocytes; 3- BMU; 4 –Bone marrow.
**RESULTS**

**Figure 218** – Representative image of the Ovx CSD filled with Putty® and a Membrane Evolution®, at month 1. 40x magnification. Toluidine blue staining. 1 – New bone; 2 – Old bone; 3– Connective tissue; 4– Masseter muscle layer; 5– Remnants of the residual membrane.

**Figure 219** – Representative image of the Ovx CSD filled with Putty® a Membrane Evolution®, at month 1. 100x magnification. Toluidine blue staining. 1 – New bone; 2 – Old bone; 3– Connective tissue.
MONTH 3 : MEMBRANE

At month 3 of evaluation, a significant amount of bone formation was visualized at the margins of the defect, either in Sham and Ovx animals in the Membrane group (Figures 221-226). In Sham animals, it is evident the residual fibers of the collagen membrane on the peripheral zone of the central area of the defect. The area of the new bone is superior when compared with month 1 of evaluation, with new bone mature in the center of the defect and new trabeculae being formed (Figure 221). In Ovx samples, significant newly formed bone tissue is visualized when compared with month 1, but with a smaller area when matched with the Sham animals, presenting a thinner bone trabaculae and a wider bone marrow (Figures 221-226).
RESULTS

Figure 221 – Representative image of the Sham CSD filled with a Membrane Evolution®, at month 3. 40x magnification. Toluidine blue staining. 1 – New bone; 2 – Old bone; 3 – Masseter muscle layer; 4 – Remnants of the residual Evolution® membrane.

Figure 222 – Representative image of the Sham CSD filled with a Membrane Evolution®, at month 3. 100x magnification. Toluidine blue staining. 1 – New bone (immature); 2 – New bone (mature); 3 – Remnants of the Evolution® membrane; 4 – Connective tissue.
RESULTS

Figure 223 – Representative image of the Sham CSD filled with a Membrane Evolution®, at month 3. 100x magnification. Toluidine blue staining. 1 – New bone immature; 2 – New bone mature; 3 – Old bone.

Figure 224 – Representative image of the Ovx CSD filled with a Membrane Evolution®, at month 3. 40x magnification. Toluidine blue staining. 1 – New bone; 2 – Old bone.
RESULTS

MODULATION OF THE BONE REGENERATION PROCESS IN SYSTEMIC IMPAIRED CONDITIONS

Figure 225 – Representative image of the Sham CSD filled with a Membrane Evolution®, at month 3. 40x magnification. Toluidine blue staining. 1 – New bone immature; 2 – New bone mature; 3- Old bone.

Figure 226 – Representative image of the Ovx CSD filled with a Membrane Evolution®, at month 3. 400x magnification. Toluidine blue staining. 1 – New bone immature; 2 – New bone mature; 3- Osteocytes.
MONTH 3: MEMBRANE AND BIOMATERIAL

At month 3 of evaluation, a significant amount of new bone formation was perceived at the margins of the defect, either in Sham and Ovx animals in the Membrane and Biomaterial group (Figures 227 e 231). In Sham and Ovx animals, it is clear the residual fibers of the collagen membrane, and with a higher magnification (Figure 233). In the Sham animals the area of the new bone is superior when compared with month 1 of evaluation, with new bone mature in the center of the defect and new trabeculae being formed. In Ovx samples, significant newly formed bone tissue is visualized when compared with month 1, but with a smaller area when matched with the sham animals, presenting a thinner bone trabaculæ and a wider bone marrow (Figures 231-235).

Figure 227 – Representative image of the Sham CSD filled with Putty® and a Membrane Evolution®, at month 3. 40x magnification. Toluidine blue staining. 1 – New bone; 2 – Old bone; 3- Connective tissue; 4- Masseter muscle layer.
**RESULTS**

**Figure 228** – Representative image of the Sham CSD filled with Putty* and a Membrane Evolution*, at month 3. 100x magnification. Toluidine blue staining. 1 – New bone immature; 2 – New bone mature; 3 – Masseter muscle layer; 4 – Blood vessels.

**Figure 229** – Representative image of the Sham CSD filled with Putty* and a Membrane Evolution*, at month 3. 100x magnification. Toluidine blue staining. 1 – New bone immature; 2 – New bone mature; 3 – Old bone; 4 – Blood vessels.
RESULTS

Figure 230 – Representative image of the Sham CSD filled with Putty® and a Membrane Evolution®, at month 3. 400x magnification. Toluidine blue staining. 1 – New bone immature; 2 – New bone mature; 3- Old bone; 4- Bone marrow; 5 – Osteoblasts; 6 – Osteocytes; 7- Osteoclast.

Figure 231 – Representative image of the Ovx CSD filled with Putty® and a Membrane Evolution®, at month 3. 40x magnification. Toluidine blue staining. 1 – New bone; 2 – Old bone; 3- Connective tissue; 4- Masseter muscle layer.
RESULTS

Figure 232 – Representative image of the Ovx CSD filled with Putty® and a Membrane Evolution®, at month 3. 100x magnification. Toluidine blue staining. 1 – New bone immature; 2 – New bone mature.

Figure 233 – Representative image of the Ovx CSD filled with Putty® and a Membrane Evolution®, at month 3. 100x magnification. Toluidine blue staining. 1 – Remnants of the residual membrane; 2 – Connective tissue.
**Figure 234** – Representative image of the Ovx CSD filled with Putty® and a Membrane Evolution®, at month 3. 1000x magnification. Toluidine blue staining. 1 – Residual membrane; 2 – Connective tissue; 3 – Fibroblasts.
5. HISTOMORPHOMETRIC ANALYSIS

In order to validate the qualitative histologic findings by a quantitative approach, the relative amount of the newly formed bone was determined. At month 1 and 3 of evaluation the bone volume (BV) was lower in Ovx animals in the M and MB group, comparing to Sham animals, throughout the experimental period. Significant differences were attained at the month 1 and 3 following the mandibular bone defect procedure. The bone volume was higher in the MB group in sham animals at month and 3, comparing to all groups in the timepoint of evaluation (Table 25).

Table 25 – Morphometric analysis of histological samples. Histomorphometric analysis of the bone regeneration process in Sham and Ovx animals, throughout 3 months of healing following mandibular bone defect.

<table>
<thead>
<tr>
<th>Time following surgery</th>
<th>Regenerated area</th>
<th>BV (%)</th>
<th>sd</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Membrane</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Month 1</td>
<td>Sham</td>
<td>38.0</td>
<td>5.8</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Ovx</td>
<td>28.0*</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Membrane and biomaterial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>39.9</td>
<td>4.8</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Ovx</td>
<td>29.25*</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Month 3</td>
<td>Membrane</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>59.0</td>
<td>2.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Ovx</td>
<td>43.8*</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Membrane and biomaterial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>63.4</td>
<td>9.0</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Ovx</td>
<td>51.6*</td>
<td>4.7</td>
<td></td>
</tr>
</tbody>
</table>

Comparisons performed by the Mann-Whitney test:

*p<0.05 Vs. Sham group, sd: standard deviation, BV: bone volume.
RESULTS

**Figure 235** – Box plot of the Regenerated area (%) values of the mandibular bone defect with the Membrane (A) and Membrane and Biomaterial (B), at month 1 of Sham and Ovx groups. *p<0.05 Vs. Sham group.

**Figure 236** – Box plot of the Regenerated area (%) values of the mandibular bone defect with the Membrane (A) and Membrane and Biomaterial (B), at month 3 of Sham and Ovx groups. *p<0.05 Vs. Sham group.
IV. DISCUSSION
Osteoporosis is a major health problem worldwide; approximately 200 million women have osteoporosis. Although the likelihood of developing osteoporosis currently is greatest in North America and Europe, it will increase in developing countries as population longevity in these countries continues to increase.[108] This disease reports a gradual deterioration of the bone mineral content over time, a process that can significantly enhance the risk of bone fracture.[343] Despite the acknowledged alterations in the osteoporotic bone, the regeneration process is far less understood in this systemically affected circumstance, especially in a process mediated by biomaterials implantation. This is of the highest importance since fractures related to osteoporosis often cannot be readily repaired by a self-healing approach, and thus the use of degradable or nondegradable biomaterials is required.[344-346]

A proper experimental animal model for the study of osteoporosis reduces bioethics problematic and minimizes the limitations associated with studying the disease in humans, namely time and behavioral variability among test subjects.[194] There is a range of animals that allows us to study the osteoporotic model, although the rat, especially the ovariectomized model, provides a wide assortment of benefits when compared with other models. Numerous studies showed that this model offers a similar characteristics as the postmenopausal bone loss; i.e., Increased rate of bone turnover with resorption exceeding formation; initial rapid phase of bone loss following by a much slower phase; greater loss of cancellous than cortical bone; decreased intestinal absorption of calcium; some protection against bone loss by obesity; similar skeletal response to therapy with estrogen, tamoxifen, bisphosphonates, parathyroid hormone, calcitonin, and exercise.[206, 213, 214, 347]

Accordingly, this work aimed to establish and characterize the representative model of the human osteoporotic condition – the ovariectomized rad model. Moreover we aimed to address the bone regeneration process, either mediated or not by the presence of biomaterials, in a model without biomechanical load – the critical sized calvarial model; and the guided bone regeneration process by the presence of biomaterials in a model with biomechanical load – the mandibular critical sized defect.
There is extensive literature studying the Ovx rat in which regards to bone histomorphometric changes, biochemical markers and biomechanical changes. In accordance, following ovariectomy a rapid loss of cancellous bone mass and strength occurs, which then proceeds, in a less rapid rate and in a site-specific fashion, to reach steady state phase of bone mass with an increase in the rate of bone turnover. [213-215, 348] Wronski et al. revealed osteopenia in ovariectomized female rats, as early as 14 days after the ovariectomy.[349]

In the present study, the expected biochemical and structural responses of the Ovx rat model were demonstrated. The ovariectomy procedure was confirmed by failure to detect ovarian tissue and observation of an atrophic uterus in the Ovx group, at the necropsy. Moreover, significant differences were attained regarding the weight of the uterus in Sham and Ovx animals, at all time points. Substantiating the efficacy of the ovarian ablation, plasmatic levels of estradiol were shown to be below the detection limit of the test used for the animals in the Ovx group, while in Sham animals, estradiol levels were kept broadly constant, around 20pg/mL, throughout the study period, which is in accordance with the published data on ovariectomy-induced experimental osteoporosis.[350, 351]

Results showed that Ovx animals presented an increased body weight, comparing to the group of Sham animals. This is in accordance with literature reports substantiating an increased hyperphagia and augmented body weight associated with the ovariectomy-mediated disruption of the normal hypothalamic-pituitary-gonadal (HPG) axis cycling, in adult female rats.[352, 353] It has been shown that the ovarian inhibition of feeding involves two specific components: one, related with the tonic inhibition that is revealed by the increased basal level of food intake; and other, associated with the phasic or cyclic inhibition that is revealed by the decrease in food intake during estrus in intact female rats, which is naturally absent in ovariectomized animals.[354, 355] Additionally, it has been demonstrated that a cyclic, near-physiological pattern of plasma estradiol treatment is sufficient to restore the HPG axis cycling and maintain normal levels of body weight, spontaneous feeding patterns, and total food intake, in Ovx rats.[356]
Serum levels of the ALP activity were slightly higher in our study, but with no statistically significantly differences between the two experimental groups, throughout the experimental period. Similar changes were observed by other authors were the serum ALP levels were increased in osteoporosis and other bone metabolic disorders.[357] Serum levels of Ca and P, were shown not to differ significantly between the two experimental groups, throughout the experimental period. The unchanged levels of serum calcium and phosphorus in Sham and OVX group indicated that homeostatic mechanisms were able to maintain serum levels of these minerals despite ovariectomy.[358, 359]

Plasma values of Albumin (ALB), Creatine kinase (CK), Total Protein (TP) and Triglycerides (TG), in Sham and Ovx animals at months 3 and 5, were evaluated. No significant differences were verified in ALB and CK in both time points. There are statistically significant differences in plasma values of TP and TG plasma concentrations at month 3 of evaluation. The levels of TP and TG were lower on the Ovx groups. The lower values of TG complies to other studies that revealed that Ovx-induced estrogen deficiency caused prominent changes in lipid metabolism with a significant elevation in the polyunsaturated fatty acids levels and depletion of saturated fatty acids and TG. They also observed concurrent elevation of lipid b-oxidation products (3-HB and acetoacetate) in the plasma of Ovx rats.[360] The marked decrease in plasma triglycerides observed here also agreed well with previous findings of reduced hepatic TG secretion in Ovx rats.[361]

Skeletal alterations were also observed in animals submitted to the ovariectomy procedure. Image analysis of left tibia X-rays revealed a decreased trabecular structure in Ovx animals, specially focused in the proximal metaphysis, both at month 5 (animals with 7 months of age) and month 8 (animals with 10 months of age) of the study. Moreover, conventional radiographic bone densitometry was conducted at a ROI involving the proximal epiphysis, metaphysis and part of the diaphysis, and revealed an increased intensity for Sham animals, which attained significance at month 8 (animals with 10 months of age). Conventional radiographic bone density has been shown to be a valid technique to address the bone mineral density (BMD) loss, reflecting the status of the crystalline component of bone.[362] This technique has been shown to correlate
strongly with quantitative computed tomography (qCT), in the assessment of BMD of human cadaveric femurs.[362] It has also been used to detect osteopenia in the tibia of young ovariectomized rats.[363]

Attained tibial differences between Ovx and Sham animals are in accordance with data reported from the literature. As so, ovariectomized rats have been shown to be characterized by osteopenia and histomorphometric indices of increased bone turnover, at the proximal tibia, 2.5 months following ovariectomy.[347] Increasing the period of assessment regarding the effect of ovariectomy over the tibial bone, a biphasic pattern of cancellous bone loss was observed in the proximal metaphysis: an initial rapid phase of bone loss was verified until around 3 months following ovariectomy, and was followed by a period of relative stabilization of cancellous bone volume, at the markedly osteopenic level of 5-7%, until approximately 12 months.[214] Both phases of ovariectomized-dependent bone loss were associated with increased bone turnover. In Sham animals, cancellous bone volume of the tibial metaphysis remained relatively constant at 25-30%, out to 12 months of age.[214] Female rat ovariectomy has also been shown to cause deterioration of the three-dimensional trabecular microstructure, notably structure model index and connectivity density, as assessed by microtomography of the proximal tibia, 9 months following the surgical ablation of the ovaries.[364] These data are in agreement with our work that revealed a marked reduction in the BV/TV, CD, Tb.Th, and Tb.N and an increased Tb.Sp, in Ovx animals in month 5 and 8 after the ovariectomy procedure when compared with the Sham animals. In fact, microtomography and DXA have been shown to detect bone loss earlier than bone histomorphometry, in the tibia assessment of a rat model of osteoporosis.[365] Accordingly, a decrease in the total BMD and trabecular BMD of the tibia of the Ovx rat has been reported by several authors.[366, 367] Whit the decrease of these parameters, the biomechanical strength ends up to be inferior in the Ovx animals, as showed in our work by the values of the biomechanical tests at the tibia. Some studies assert that the biomechanical tests at long bones provide maximum information as they are composed of pure cortical bone and they are covered by periosteal tissue and can thus react by periosteal apposition, as seen in human bone.[209]
The biomechanical parameters of the femur in our study were evaluated at month 3 of the osteoporotic condition and we attained a significant difference in the ultimate stress between the Ovx and sham animals, enabling the susceptibility of fractures the femurs in an osteoporotic condition. Available data in the literature substantiate these findings. Ovariectomy has been shown to promote periosteal modeling and induce endocortical and cancellous femoral bone remodeling, with a net loss of bone mass due to excess bone resorption.[368] Bagi et al. observed in the Ovx-induced an increase resorption resulted in reduced trabecular number, thickness, and endocortico-trabecular connectivity at the proximal femur, 3 months following Ovx procedure in female rats.[368] Moreover, significant reduction in femoral trabecular BMD was observed in vivo, by qCT, at 6 and 14 weeks following ovariectomy, in a study of Breu et al.[369] Cortical femoral thickness in Ovx rats has also been shown to be reduced because of the high endocortical resorption, which, in addition to cancellous bone resorption, resulted in fewer endocortico-trabecular connections.[370] Giavaresi et al. also observed a decrease in the mechanical results of Ovx animals when compared to sham animals, with a significantly decrease in the maximum load assessed on the femur of Ovx animals, affirming that important changes in the femoral shaft mechanical competence arise in adult rat 4 months after ovariectomy. [371]

Alternatively, the ovariectomy of young skeletally immature animals may also lead to site-specific bone loss, particularly focused on trabecular component, while cortical bone seems to respond differently to estrogen deficiency attained during the skeletal growth. For instance, increased cortical density, bone mass, and bone size were verified in the femur. [372] In the study a significant trabecular bone loss was observed in the vertebra measured by the densitometry technique on the x-rays, in accordance with some studies.[373] These differences were significant and attained in the month 3 and 5 of our study. Wronski et al. observed the osteopenic changes of vertebral ovariectomized rats and following 180 days after the surgery the cancellous bone volume was decreased to 30-35%. [373] Toolan et al. observed a significant reduction in the stiffness and ultimate load of L6 and the area fraction of trabecular bone of L5, in ovariectomized rats.[374]
Bone loss and a reduced mineral density were verified in the cranio-maxillofacial complex, namely at the mandible and calvarial bone of young ovariectomized female rats.[375, 376] In our work we found a significant decreased in the density of calvarial bones at month 3 and 5 in the Ovx animals when compared with the Sham animals, by the densitometry evaluation of the x-rays.

In the present study, we found the density and trabecular bone decrease of the molar region of the mandible and maxilla in Ovx rats, manly in the month 5 in the maxilla. Although the mechanism of the low susceptibility of the molar region of the mandible to estrogen deficient condition is not clear, it is likely that mechanical stress derived from functional occlusion is preventing the bone loss in this pathological condition. Elovic et al. have clearly demonstrated that maxillary molar extraction together with ovariectomy causes more bone loss in the mandible than maxillary molar extraction alone, [377, 378] which funds this theory. Yang et al. investigated the oestrogen deficiency on the rat mandible and tibial microarchitecture after ovariectomy, and reported alterations of the trabecular bone both in the mandible and the tibia within 16 weeks. They also realized that the size of marrow spaces and the shape of the trabeculae in the mandible correlate with the osteoporotic changes in the long bones.[379]

Tanaka et al. studied the effects of ovariectomy on trabecular structures of rat alveolar bone and observed a significant decrease of bone mass in the alveolar interradicular septum of the rat first molar. The microtrabecular stiffness was lower when compared with the sham animals and revealed a thinner distal alveolar bone.[380] These same authors found in another study a consistent bone loss in manly in the posterior region of the mandibular condyle of ovariectomized rats.[381]

The rat mandibular cortical bone as been studied by Fu et al. using spectroscopy and a multivariate analysis, they observed the mandibles at 2, 4 and 8 months following the ovariectomy and noticed a significant reduction in the mineral-to-matrix ratio in the mandibular cortical bone after Ovx.[382]

The biomechanical test in our study of the mandible shows that the Ovx-group presents a lower threshold of the ultimate stress when compared with the sham group (27,5Mpa Vs. 38,9Mpa). The biomechanical property of the mandible in osteoporotic
conditions is an understudied subject. However the rat mandibular cortical bone as been studied by Fu et al. using spectroscopy and a multivariate analysis, they observed the mandibles at 2, 4 and 8 months following the ovariectomy and noticed a significant reduction in the mineral-to-matrix ratio in the mandibular cortical bone after Ovx.[382]

Elovic et al. studied the effect of the ovariectomized rat in the bone fraction of the rat mandible; they performed a test in three-point bending to evaluate the load failure, stiffness and bone area fraction between Ovx and Sham rats within the day 14, 114 and 200 following the post-Ovx. The ovariectomy led to an increase in failure load (15%) and stiffness (28%) of the mandible of Ovx rats in the 114 day of evaluation. In the 200 day of evaluation a decreased of 8% in bone area fraction of the mandible was assessed. The stiffness (20%) and bone area fraction (7%) of the mandible decreased in the Ovx rats at day 200 when compared with the Ovx rats at day 114. The increase in structural properties of the mandible may be a consequence of an increase in masticatory function as it has been reported that Ovx rats eat approximately 10% more than sham controls.[383] These theory is in accordance with other authors that affirmed that the normal (hard) diet may limited the negative effects of oestrogen deficiency on mandibular architecture four months after ovariectomy.[384]

These findings are in accordance with several studies like Comelekoglu et al. that investigate the effect of ovariectomy on rat femur biomechanical parameters. They observed an Ultimate stress decreased 21% in Ovx rats and the cortical thickness was significantly decreased in Ovx rats when compared with the controls. They affirmed that the femur biomechanical parameters are decreased in osteoporosis.[385] Peng et al. evaluated the maximal load of the femoral neck and the tibia in three different rat models of experimental osteoporosis and found a significant decrease in the Ovx rats in both bones in evaluation. [386] He affirmed that mechanical strength of the femoral neck was a sensitive indicator of bone loss associated with Ovx. [386]

In this study, in order to evaluate the bone regeneration process in osteoporotic conditions and to address the biomaterial-mediated bone regeneration and the bone-biomaterial interaction, a CSD (5 mm in diameter) filled with a commercial xenograft (Bio-oss®) with proven clinical success was accomplished, on the calvaria of both Ovx and Sham animals.
Regarding the analysis of the regeneration process, radiographic, micro-
tomographic, histological methodologies and quantitative PCR analysis expression
were used. Radiological observation revealed the validity of the 5 mm CSD, in which
negative controls – defects left unfilled – were unable to regenerate throughout the
duration of the study. In fact only residual new bone formation was verified on the
margins of the defects, as stated for the definition of critical size defect.[331] Defects
filled with Bio-oss® revealed the integrity of the biomaterials’ granules within the
defect margin. Regions of interest (ROIs) for both microtomographic and histological
analysis were set manually. Nonetheless, giving the high feasibility and resolution of
the used analytical tools, manual selection allowed the fine tuning of ROIs, since no
fixed anatomical landmarks could be of use to delimit standardized ROIs in relation to
the healing bone defect.

Micro-tomographic analysis of the implanted CSD defects, in a longitudinal
view, showed an increased bone formation at the margin of the defect, in Sham
animals, compared to Ovx animals, at month 6 – animals with 10 months of age. The
axial view analysis of the defect area also revealed an increased new bone formation
zone, at the margin of the defect, in Sham animals. There were no evident areas of
biomaterial resorption, neither zones of new bone formation apart from the continuity
with the defect margin. Histological analysis revealed an increased amount of bone
formation throughout the assayed time points of the study – 1 month, 3 months and 6
months, both for Sham and Ovx animals. When the two groups were compared, at
each time point, the process of new bone formation was more evident within animals
of Sham group, either in which regards the bone formation at the defects margin, and
the new bone formation at the biomaterial granules’ border. Even so, the vast majority
of the space between Bio-oss® granules was occupied by fibrous tissue, at all time
points.

In this study, Bio-oss®, a bovine-derived xenograft material which has found
clinical success in several bone regenerative applications, namely in those associated
with the cranio-maxilofacial area (e.g., dental implant placement and sinus lift
procedures), was used.[235, 236, 315, 321] In this particular experimental setting, Bio-
oss® allowed only a modest process of bone regeneration, at the border of individual
granules, both in Sham and Ovx animals, throughout the assayed time points. Literature reports of the use of Bio-oss® in the assessment of the bone regeneration process, in rats, present data which is in accordance with the attained results. Slotte et al. showed that following the implantation of non-permeable silicone domes filled with Bio-oss® in rat calvaria, significantly less augmented bone was found, comparing to control – in which the domes were left empty – 8 weeks post-surgically.[387] A similar experimental design, in which Teflon® capsules were filled with Bio-oss® or left unfilled (control), analysis was used to address bone augmentation in the surface of the mandibular ramus of rats. Following 2 and 4 months post-operatively, the mean volume of the newly formed bone was significantly increased in the control versus the Bio-oss® group.[388] The same authors used this methodology to evaluate the mandibular bone formation, one year following the implantation of the Teflon® capsules, filled or not with Bio-oss®. The mean volume of newly formed bone occupied only 23% of the total space in the animals grafted with Bio-oss®, while in control animals, the mean volume increased to around 88%.[389] This research group also addressed the mandibular bone regeneration, with the same experimental methodology, with a re-entry surgery, one year following capsule implantation. In this second surgical intervention, capsules were removed and animals were immediately sacrificed (baseline), or euthanized at 3 or 6 months following re-entry surgery. Newly formed bone within the capsule reached between 92 to 94% at the control, and 20 to 23% within the Bio-oss® group, throughout the assayed time points.[390] Bio-oss® was also evaluated in the bone formation process in the rat mandibular ramus, in association with enamel matrix proteins (EMP), in filled polytetrafluoroethylene (PTFE) capsules.[391] It was shown that neither the application of EMP, or the use of Bio-oss®, or even the combination of both, resulted in the enhancement of the bone formation, comparing to control – unfilled capsules – either at 2 months or 4 months, following implantation.[391]

On another report, the effect of Bio-oss®, associated with enamel matrix proteins (EMP), or a resorbable collagen membrane was evaluated in a 5mm CSD established on the rat calvaria.[392] It was shown that the predictability of the bone formation was essentially dependent on the presence or absence of the barrier
membrane alone, and that the combined use of Bio-oss® and/or EMP did not significantly increase the potential for complete healing of the defect.[392]

In the appraised works, the newly formed bone was always in continuity with the host bone and extended only a limited distance from the pre-existing bone surface. In the reports addressing the capsule model, in the remaining volume of the capsule, granules were embedded in fibrovascular connective tissue. This pattern of regeneration/repair, also verified in this study, has been observed following the grafting procedure of Bio-oss® in various types of bone defects, either regarding in vivo research or clinical application.[387-391, 393-395] New bone formation mediated by Bio-oss® seems to be confined to the neighborhood of the host bone, even in long periods of healing. Nonetheless, the bone tissue formed in association with Bio-oss® seems to be biologically and biomechanically stable on a long-term basis, which may substantiate the reported long-term clinical success of implant placement associated with Bio-oss® grafting.[318, 396, 397] This is in line with the published in vivo experimental reports that failed to detect a reduction of the amount of grafted Bio-oss® over time[398, 399] and to the finding of large amounts of unresorbed graft granules 44 months[400] and 6 years[401] following clinical implantation.

In our study, the combined analyze of the bone regeneration in osteoporotic conditions, either in which regards CSD implanted with Bio-oss®, revealed an impaired biological process of new bone formation in Ovx animals, compared to Sham animals. Literature reports have shown that osteoporosis modified bone cell proliferation and functional activity, cell reactivity to local factors, and the availability and number of progenitor cells.[1, 402, 403] These biological differences, in association with differences in biomechanical and micro-architectural bone properties, may be involved in the etiology of the impairment of the bone regeneration process. Although some biological highlights have been acknowledged for the bone regeneration process in osteoporotic conditions, limited data is available regarding the study of orthopedic biomaterials behavior following implantation in osteoporotic bone. Kin et al. showed that ovariectomy may act as a negative factor for new bone formation, as assessed in rat calvarial CSD implanted with a mixture of tooth ash and plaster of Paris. Data is presented for 1, 2 and 4 months following ovariectomy procedure.[404] Fini et al.
studied the effect of osteopenia on the osteointegration of biomaterials in rats and displayed the detrimental effects of the osteopenic condition, due to estrogen deficiency, on osteointegration of titanium, zirconia and a bioglass (AP40).[367] The biological behavior of synthetic hydroxyapatite was also addressed in the femoral condyle of Ovx and Sham rats and a significant decrease in the osteointegration of the material was attained in Ovx animals.[405] Interestingly, Hayashi et al. found no significant differences between the affinity index (the length of bone directly apposed to the implant/the total length of the bone-implant interface ×100%) of tibia implanted hydroxyapatite cylinders in Sham, Ovx or Ovx plus neurectomized rats, at 2 or 6 months following implantation.[406]

Most recently, an experimental report aimed to address the effect of ovariectomy in the consolidation of implanted methyl methacrylate capsules filled with Bio-oss®, in rat calvaria. The rate of new bone formation was similar in Ovx and control animals – not submitted to Sham surgery – both at 4 and 8 weeks following implantation. In both groups, the amount of new bone formation was low and insufficient for quantification by microtomography.[407]

In both Sham and Ovx animals, with the used experimental design – a grafted critical size defect – the bone regeneration process was found to be slow-paced, even following 6 months of healing, and broadly distributed within the close vicinity of the margin of the defect. Grafted critical size defects must rely on the osteoconductive, osteoinductive or osteogenic capabilities of the implanted constructs to achieve tissue regeneration and, in the absence of potent osteoinductive or osteogenic stimuli, broadly fail to output a significant tissue regeneration.[329] Thus, in this model, the use of a bone biocompatible xenograft, which is endowed with only osteoconductive properties, might determine a slow bone healing process. This seems to be particularly relevant in bones with high cortical content (such as the calvaria), in which the healing seems to be slower than in bones with high trabecular content (e.g. tibia).[408]

Apart from the observed slow-paced bone regenerative process, qualitative data from microtomographic and histological analysis, as well as acquired histomorphometric data revealed reduced bone regeneration in osteoporotic conditions, being significant differences attained at 3 and 6 months healing time.
Some previous literature reports have addressed the biomaterial-mediated bone regeneration in osteoporotic conditions in sub-critical size models, outputting distinct results. On one hand, some experimental studies failed to identify significant differences between the biomaterial-mediated bone formation process in osteoporotic conditions, as compared to sham controls [409-411]. Some other authors substantiate the impaired biomaterial-mediated bone regeneration process in osteoporotic conditions [412-414]. Attained variances in the available literature might be, at least in part, justified by differences in the experimental design. Distinct animal models, methodologies for the induction of the osteoporotic condition, biomaterials, bone defects and evaluation periods were used and thus, the establishment of direct correlations between studies is difficult.

Despite these substantiations, biological evidence seems to sustain an impaired regenerative potential of the osteoporotic bone. In our model, we found an altered pattern of osteoblast-related gene expression in the regenerating calvarial bone tissue of Ovx animals, i.e., Col I, OC and RUNX2 mRNA expression were significantly decreased, as comparing to control. While the gene expression pattern has not been previously evaluated in the biomaterial-mediated regenerating osteoporotic bone, previously reports addressing the osteogenic differentiation of mesenchymal stem cells (MSCs) derived from osteoporotic environments revealed significant alterations [415-417]. Indicated differences set ground on intrinsic deficiencies in the self-renewal capability and differentiation potential of the osteoporotic stem cells, particularly nourishing a down-regulation of specific osteogenic-related markers, thus sustaining an impaired function of MSCs in the osteogenic differentiation pathway [415-417].

The expression of estrogen receptors was also found to differ in the regenerating bone of Ovx and Sham animals. ER-alpha expression was reduced in the ovariectomized rat regenerating tissue, while the expression of ER-beta was increased. This pattern of expression was found to occur in the non-regenerating bone of the Ovx rat [418], as well as on the healing of drilled femoral defects on the Ovx mice [419]. ER-alpha activation is known to induce the intramembranous and endochondral ossification processes in rodent’s femur, and to promote the bone formation at the periosteal surface of the cortex and the prevention of the resorption at the
endocortical surface [420]. Likewise, signaling through the ER-beta receptor seems to inhibit both intramembranous and endochondral ossification processes in a gene knock-out model [421], and to retard periosteal bone formation and suppress gain in bone size and bone strength [422]. This altered expression of estrogen receptors may though contribute to the altered bone regenerative potential of the osteoporotic bone.

We also verified an increased expression of PPAR-gamma, a master regulator of the adipogenic differentiation, in the regenerating bone of Ovx animals. It has been proposed that the bone marrow fat content is increased during the osteoporotic development [342]. Furthermore, mesenchymal stem cells derived from osteoporotic conditions revealed an increased adipogenic potential, as shown by the higher expression of PPAR-gamma and inactivation of osteogenic transcription factors [423]. This fact seems to disrupt the normal osteogenic equilibrium and favor the adipogenic differentiation of recruited mesenchymal stem cells, within the bone milieu [424]. The increased adipogenesis, either in terms of cell number and/or cellular function, seems to further increase osteoblasts apoptosis, as well as to promote osteoclast recruitment, activation and function, resulting in a net increase of the bone resorption process [425, 426].

In this study, in order to evaluate the bone regeneration process in the presence of functional biomechanical loading in osteoporotic conditions, and to address the guided bone regeneration process, a CSD (4 mm in diameter) filled with a commercial xenograft (Putty®) and surrounded by a with saleable membrane (Evolution®) proven clinical success was accomplished, on the mandible of both Ovx and Sham animals.

Regarding the analysis of the regeneration process, radiographic, microtomographic and histological methodologies were used. Radiological observation revealed the validity of the 4 mm CSD, in which negative controls – defects left unfilled – were unable to regenerate throughout the duration of the study. This methodology is consistent with that described by Schmitz and Hollinger, a circular through and through defect of a diameter varying from 4 to 7 mm drilled into the mandibular ramus.[326] In fact only residual new bone formation was verified on the margins of the defects, as stated for the definition of critical size defect. [326, 333]
The rat mandibular defect model has been used to evaluate bone regeneration of biomaterials and osteoconductive properties of membranes with or without growth-stimulatory factors and permits the evaluation of the response to related to clinical applications of the implanted material. [427-431]

Kaban et al. define the mandible as a distinctive bone that is subjected to continuous motion and significant compressive and shearing forces; therefore bone defects created in other craniofacial bones or in long bones may not apply to the mandible, given the functional biomechanical loading submitted to this bone. Considering the increase in the life expectancy of humans, this experimental model is of clinical interest for the relevance of bone repair in the craniofacial region in cases of osteoporosis.[332]

Defects covered with the membrane Evolution® (M) and the defects filled with Putty® and covered with the membrane Evolution® (MB) in a macroscopic evaluation revealed the integrity of coating of the defects in both time points of evaluation. The original defect of the animals in the control group in both sham and Ovx animals were invaginated by the masseter muscle, impairing the diameter of the original defect in all time points of evaluation.

The x-ray evaluation at month 1 – animals with 5 months of age, allows to observe an increased area in the margins of the original defect in Sham animals when compared with the Ovx animals, both in the M group and the MB group. The densitometry analysis of the mandibular bone defects demonstrated that the defects were more closed in the Sham animals, in the group MB, when compared with the other groups. The Ovx animals in both groups presented lower values of regeneration. Although no significant differences were achieved. At month 3 – animals with 7 months of age, the results are in accordance with the ones observed at month 1, but with a significant difference in the M group between the Sham animals with a higher densitometric values, comparing with the Ovx animals.

Micro-tomographic analysis of the implanted CSD defects shows the apposition of the bone in the defect margin, a centripetal growth of the newly formed radiopaque tissue could be identified, in both Sham and Ovx samples in M and MB groups. An increased bone formation was observed at the margin of the defect, in Sham animals,
compared to Ovx animals, at month 1, in the M group. In the MB group, at month 1, in the center and proximity of the defect we can observe some spots distributed in and around the defect that correspond to the biomaterial. The membrane was not observed in the micro-Ct images. The Sham animals in the MB group reveal increased bone regeneration in the margins of the defect when compared with the Ovx group. Regions of interest (ROIs) for both microtomographic and histological analysis were set manually, which may be a source of errors/bias throughout data analysis. Nonetheless, giving the high feasibility and resolution of the used analytical tools, manual selection allowed the fine tuning of ROIs, since no fixed anatomical landmarks could be of use to delimit standardized ROIs in relation to the healing bone defect.

Quantitative analysis of the amount of bone formed in rat mandibular defects covered by the M and the MB showed that significant new bone was formed, without statistically significant differences between the animals and groups. At month 1 the M group in the sham animals presented a Bone Volume (BV) of 1.785, that was higher than the 0.965 BV of the Ovx animals. The MB group in the sham animals presented the highest values of BV with 1.990, compared to the 1.280 of the Ovx animals. At month 3 the same tendency was followed as observed at month 1, with an increased BV in all groups evaluated, showing the sham animals with a superior volume of bone, in M and MB group.

Histological analysis revealed an increased amount of bone formation throughout the assayed time points of the study – 1 month and 3 months, both for Sham and Ovx animals. The graft biomaterial wasn’t noticeable in the histological specs, the Putty®, a mix of 80% of granulated cancellous and cortical porcine bone with a granulometry ≤300µm and 20% of collagen gel, could be imperceptible due to his low granulometry. The Evolution® membrane, is a collagen membrane derived 100% pericardium of equine origin with a thickness of 0,5-0,7mm. This one is observed by residual debris that leaves in the margins of the defect. The tissue response to the collagen membrane was characterized by a slighter reaction, possibly due to its resemblance to native collagen. The degradation profile is characterized by a macrophage and polymorphonuclear leukocyte associated degradation as well as an enzymatic degradation profile.
At month 1 and month 3 of observation, the Sham group when compared with the Ovx group manifested a higher bone regeneration process in both experimental M and MB groups. The animals of month 1 of evaluation are in accordance with the results obtained previously in the microtomographic analysis. The MB group in the sham animals at month 1 and 3 attained the higher values in the morphometric analysis of the histological samples, followed by the M group in the sham animals and the MB group of the Ovx animals and at last the M group in the Ovx animals.

When the two groups (M and MB) within the sham and Ovx animals were compared, at each time point, the process of new bone formation was more evident within animals of Sham group in regards of the bone formation at the defects margin. Even so, the vast majority of the space between the immature bone was occupied by connective fibrous tissue, at all time points.

The bone regeneration process applied in this experimental study was based on the guided bone regeneration (GBR) that has proven to be a predictable procedure for many techniques namely in the augmentation of alveolar ridges prior to implant dentistry.[220] Several studies have proven the expected results in these techniques using a resorbable collagen membrane like the one used in our study. The collagen is a material with several advantages such as hemostatic function, allowance of early wound stabilization, chemotactic properties and enabling nutrient transfer, therefore being a membrane of choice for most GBR procedures.[432] Leeuwen et al. evaluated the difference between the regeneration process using three types of membranes to cover a circular CSD of 5mm in the left mandibular angle in Sprague-Dawley rats. The defects were evaluated by micro-radiography, micro-computed tomography and histological analysis at all time points (2, 4 and 12 weeks). They evaluated a ply(trimethylene carbonate), collagen and an expanded polytetrafluoroethylene membrane. They observed that the control group that was left uncovered didn’t show new bone formation, at all time points. The groups treated with a membrane presented substantial bone healing that increased progressively until the 12 weeks, although no statically significant differences were attained between the groups, verifying that all were well suited for use in guided bone regeneration.[433] The use of bioresorbable membranes in the mandibular bone defect was assessed by
Kostopoulos et al. to perceive the difference between the guided bone regeneration in contrast with a control group without a barrier. They observed an increasing bone filling in the group with an occlusive bioresorbable membrane whereas in the control site only 35% of the defect was filled with bone and observed a ingrowth of muscular, glandular and connective tissue in the defect, showing the importance of an occlusive bioresorbable membrane excluding the surrounding soft tissues from the wound.[434]

The Evolution® membrane used in our experimental study has found clinical success in several bone regenerative applications, namely in those associated with the cranio-maxilofacial area (e.g., dental implant placement and sinus lift procedures). Pagliani et al. in a multicenter case series studies used the Evolution® membrane with a porcine bone substitute for augmentation of the alveolar crest or the maxillary sinus floor augmentation prior to implant placement, as the histological results of the samples retrieved 5 or 7 months after the surgical intervention reveals a good clinical results with bone condensation properties and the material proved to be resorbed with time.[435] Scarano et al. also used the Evolution® membrane with a porcine bone graft in sinus augmentation procedures, with a 5-year retrospective clinical evaluation presenting a 92% survival rate on the implants placed on the grafted bone.[436] Additional studies similarly used the Evolution® membrane for maxillary sinus procedures.[281] This membrane has been use also for crestal ridge preservation for posterior placement of implants. Covani et al. used these membranes with porcine bone xenograft on post extraction sites and filled them. After 4 months of the surgery clinical and histological observations showed new bone formation with satisfactory healing without any signs of complications, providing the opportunity to place implants for future prosthetic rehabilitations. [437]

The membrane is useful to allow the space maintenance, although the use of bone grafts materials prevents in situ membranes from collapsing into the defect, thus making it more suitable to bridge defects. Some authors refer that some of these biodegradable membranes are primarily designed for periodontal regeneration, so to aid the membrane adaptation to the defect, a soft configuration has been applied in the fabrication of the membrane. Accordingly, these characteristic are not ideally for bone regenerative purposes, in the specific application of the developed experimental
model, the membranes may not be able to resist the forces from the elevating muscles in the angulus region of the rat mandible, loosing their mechanical strength and, therefore, their space maintaining properties.[430] Therefore the collagen membrane is best used in combination with grafting materials.[438-440]

The lower values attained in the Membrane group in both sham and Ovx animals when compared with the Membrane and Biomaterial group, could be explained by this disadvantage of the collagen membranes used alone in locations subjected to forces, like the mandible.

The graft biomaterial used in this study was the Putty® a mix of 80% of granulated cancellous and cortical porcine bone with a granulometry ≤300µm and 20% of collagen gel. This is a biomaterial that proved its effect and has been clinically used. Nannmark et al., evaluated the different collagen ratios on the bone tissue response to collagenated porcine bone grafts. They produced a bone defect in the maxilla of rabbits and filled with Putty® or Gel40® and, after 8 weeks, observed by x-ray and histological evaluation. They concluded that cortical porcine bone with different ratios of collagen exhibits good biocompatibility and osteoconductive properties. In this model, the two materials were equally, with respect to both bone formation and resorption, which started at the endpoint at 8 weeks.[441]

This biomaterial has been used as a filler for post extraction sockets to preserve ridge volume. One example is the clinical evaluation of Arcuri et al. that inserted, in the residual socket, the Putty® graft and three months later a bone biopsy was taken to evaluate the bone regeneration process. The histological investigation of the graft site in all the samples examined showed complete resorption of the heterologous material and its substitution with bone tissue for the whole extension of the sample collected. No traces of the particulate previously inserted were found. They conclude that the biomaterial shows exceptional malleability and plasticity, thanks to its structural characteristics, which makes it easy to apply, favoring its osteoconductive properties.[324] Although, the biomaterial has yet few studies in animal model. In our study we also observed, in histological examination, the absence of the biomaterial particles allowing us to foresee the total resorption of the biomaterial.
The literature in guided bone regeneration in osteoporotic conditions in the rat mandibular model is scarce. Arisawa et al. investigated the bone promotion in 4 mm in diameter in surgical defects in the mandible angle in normal and ovariectomized female rats treated with calcitonin and a polytetrafluoroethylene barrier. The female rats were divided in four groups: control (C), control treated with calcitonin (CM), ovariectomized control (OV) and ovariectomized treated with calcitonin (OVM); assessed in day 3, 7, 14, 21, and 28 days after surgery. Groups CM and C showed higher levels of bone formation after 7 days compared with the OV and OVM groups. A significant difference was observed between group C and Ov at 3-14 days. The OV group presented slower bone regeneration of the surgical bone defect when compared with the C group. Synthetic salmon calcitonin accelerated bone regeneration in the defect in osteoporotic animals treated with calcitonin when compared with the control group.[442] Jardini et al. evaluated the influence of osteopenia induced by the bone repairing of the receptor site/autogenous bone graft block interface either associated with or without PTFE-e membrane through the analysis of the trabecular bone volume. The rats were submitted to parietal bone graft which was fixed to the lateral wall of the left mandibular ramus, with or without covering by the membrane. They assessed the process of the graft intervention with the receptor site and state that it was negatively affected by the induced osteopenia presence and that the presence or absence of the membrane did not interfere in the integration process.[443]

Overall, and taking into account, the stated biological hindrances associated with the osteoporotic state and the data trend substantiating the impaired biomaterial-mediated bone regeneration process in osteoporotic conditions, new therapeutic approaches ought to be developed in order to find optimized clinical interventions for the treatment of osteoporotic-mediated problems. The controlled release of bisphosphonates from bioceramic materials has been considered[444, 445], as well as the delivery of osteogenic factors[446] or even the development of smart biomaterials.[447] Nonetheless, effective and predictable therapies are far from the
application in the clinical scenario and urge to mitigate the personal, social and economic cost of osteoporosis-mediated complications.
V. CONCLUSION
Osteoporosis is a prevalent disease and a major worldwide health problem reporting a high rate of mortality and morbidity. It is a pathological condition characterized by changes in the microarchitecture of the bone tissue and a low bone mass, which converge to compromise bone strength and increase the risk of bone fracture. Bone regeneration process, in osteoporotic conditions, may be somewhat limited mainly due to a decrease of new bone formation, nonetheless specific mechanisms have not been stated.

In this work, the bone regeneration process was evaluated in an animal model representative of the human osteoporotic condition. Bone regeneration was addressed in the presence of Bio-oss®, in calvarial critical size defects (CSD), and guided bone regeneration process was evaluated, in the mandibular CSD in the presence of a membrane and membrane or biomaterial.

The rat ovariectomized model proved to be a valid system for osteoporosis-related research, reporting significant metabolic (i.e., increased body weight, absence of estradiol production and evidence of uterus atrophy) and bone alterations (i.e., altered tibial and femoral microarchitectural organization and cortical thickness), highly relatable to the clinical post-menopausal osteoporotic state. The biomaterial-mediated bone regeneration of CSD also seemed to be enhanced in Sham animals. Bio-oss® allowed only modest new bone formation, either in Sham and Ovx animals, nonetheless this process was significantly higher in animals of the Sham group. The guided bone regeneration process assessed in the mandibular defect was also enhanced in the Sham animals in the Membrane (M) and Membrane and Biomaterial (MB) group, with enhance outcomes in the MB group. The Ovx animals reported a minor regeneration process in both groups comparing to the Sham animals.

The stated biological hindrances, verified in bone regeneration process in osteoporotic conditions, justify that care should be taken on pre-operative preparation of osteoporotic patients undergoing bone-related interventions, and that a selective choice of biomaterials should be undertaken when facing bone regeneration in systemically-compromised conditions.
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