UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

Characterization and Optimization of Sheep as an Animal Model of Osteoporosis Follow-up of Bone Turnover Markers, Bone Mineral Density and Mechanical Properties and Bone Remodelling After Osteoporosis Induction

- Final Version -

PhD Thesis in Veterinary Sciences - Clinic

José Arthur de Abreu Camassa

Supervisors

Professor Maria Isabel Ribeiro Dias

Professor Nuno Miguel Magalhães Dourado

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I, José Arthur de Abreu Camassa, declare that the contents of this Thesis are my own work and that they have not been presented to any University other than the University of Trás-os-Montes and Alto Douro.

To my Family and Friends

“Que o medo do desconhecido jamais impeça a caminhada.”

(J. A. A. Camassa)

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Abstract

Osteoporosis is a skeleton alteration characterized by a decrease of bone mass and strength, resulting in an increased of bone fragility and consequently fractures. This patology is the most common metabolic disorder, with a prevalence the 200 million of women worldwide affected. Therefore, osteoporosis is a global public health problem associated with a high cost to national health services. The main diagnostic tool is dual X-ray absorptiometry (DXA) or namely bone densitometry. This method allows to monitor bone mineral density (BMD) status, which can be normal, osteopenic, osteoporotic or severe osteoporotic, but not characterizing the early stages of bone loss. Thus, bone turnover markers (BTMs), which can be classified into formation, resorption and osteoclast regulatory protein, are aim of research for decades to assess the early bone remodelling. However, the high biological variability among individuals associated to analytical variability introduced by the different assay techniques is the main limitation to their clinical use due to the difficulty to establish reference ranges for serum and urinary BTM levels.

Thereby, the main purpose of this work (Chapter VI) was the characterization and optimization of sheep (Portuguese Serra da Estrela breed) as an animal model of osteoporosis through the follow-up of BTMs during osteoporosis induction, and assess of BMD and mechanical properties and also bone remodelling after osteoporosis induction. In conclusion, biomarkers showed to be reliable in determining bone remodelling. However, the combination method ovariectomy (OVX) and glucocorticoid to induce osteoporosis resulted in limited trabecular alteration in vertebrae. And finally, it was verified that fouth (L4), sixth (L6) and seventh lumbar vertebrae (L7) are the most useful for further studies of vertebral augmentation or spinal fusion.

Additionally, a review of the literature (Chapter III) was undertaken to summarize research on the use of sheep and goats as large animal models of human osteoporosis for preclinical and translational studies. The available data established that, the majority of studies is perfomed by isolated ovariectomized (OVX) or combined treatment with glucocorticoids (GCs) in sheep, while goat model present a limited use in these type of studies. With the knowledge acquired about the pathophysiological mechanisms, studies with sheep model should be carried out with greater confidence in obtaining transposable results for humans.

A second literature review (Chapter IV) aimed to compile the available information on the use of BTMs in studies on small ruminants, specially highlighting the clinical effectiveness of BTMs in pre-clinical or translational experimental orthopaedic research and reporte a variability influence on BTMs. With a systematic literature search was demonstrated, BTMs are effective to determine the bone remodelling in small ruminants, however, the variability may be a limitation.

Posteriorly, another study (Chapter V) aimed to generate a reference range for a bone resorption biomarker, tartrate-resistant acid phosphatase in sheep and validate the already published data. The data represents a useful tool in clinical orthopedic research studies and any variation verified in this marker can be explained by the well-known physiology.

In summary, the work presented under the scope of this Thesis consolidates investigation in the field of BTMs in small ruminants and contributed to the characterization of the GC-treated OVX osteoporotic sheep model for pre-clinical and translational studies in orthopaedic research.

Keywords: osteoporosis, sheep model, bone turnover markers, micro-computed tomography, biomechanics, bone histomorphometry

Resumo

A Osteoporose é uma alteração do esqueleto caracterizado por uma diminuição da massa e força óssea, resultando num aumento da fragilidade óssea e consequentemente das fraturas. A patologia é reconhecidamente a mais frequente doença metabólica óssea, com uma estimativa de prevalência de 200 milhões de mulheres em todo o mundo. Resultando assim numa significativa questão de saúde pública agregado ao alto custo aos Serviços Nacionais de Saúde. A principal ferramenta de diagnóstico é absorciometria por duplo feixe de raio-X (DEXA) ou densitometria óssea. Este método permite mensurar a densidade mineral óssea (BMD), classificando a BMD em normal, osteopénica, osteoporótica ou osteoporose severa, não caracterizando o estágio inicial de diminuição da massa óssea. Assim, há mais de uma década os biomarcadores de remodelaçao ósseos (BTMs) têm sido estudados como meio de avaliar precocemente a remodelação óssea, sendo geralmente subdivididos em marcadores de formação, marcadores de reabsorção e proteínas reguladoras da função osteoclástica. Contudo a aplicação clínica dos BTMs é limitada devido à elevada variabilidade inter-individual associada a variabilidade analítica causada por diferentes métodos analíticos, dificultando a elaboração de um intervalo de referência para os níveis séricos e urinários.

Desta forma, objetivo principal deste estudo (Capítulo VI) foi caracterizar e otimizar o modelo ovelha (raça portuguesa da Serra da Estrela), monitorizando diferentes BTMs durante a indução da osteoporose e avaliar BMD, propriedades mecânicas e remodelação óssea após a indução da osteoporose. Concluindo que os biomarcadores monstraram-se sensíveis para determinar a remodelação óssea. Contudo o método de indução de osteoporose através de glucocorticóides associado a ovariectomia (OVX) resultou em limitada alteração trabecular em vértebras. E finalmente, verificou-se que a quarta (L4), sexta (L6) e sétima vértebras lombares (L7) são as mais adequadas para futuros estudos associados a fusão espinhal e verterbroplastia.

Este estudo teve adicionalmente uma revisão de literatura (Capítulo III) com o propósito de sintetizar as informações disponíveis sobre o uso de pequenos ruminantes nomeadamente ovinos e caprinos como modelo animal de osteoporose para estudos pré-clínicos e translacionais. Os dados disponíveis estabeleceram que a maioria dos estudos são realizados por meio de ovariectomia (OVX) ou por tratamento combinado com glucocorticóides (GCs) em ovelhas, enquanto que o número de estudos utilizados o modelo caprino é limitado. E com o conhecimento dos mecanismos patofisiológicos adquiridos, estudos com o modelo ovino deveriam ser desenvolvidos com maior confiança com o propósito da obtenção de resultados transponíveis para humanos.

Uma segunda revisão de literatura (Capítulo IV) objetivou compilar informações disponíveis sobre uso de BTMs em estudos com pequenos ruminantes, com atenção à eficácia clínica de BTMs em estudos ortopédicos pré-clínicos e translacionais e sobre a influência da varialibilidade sobre os BTMs. Após uma pesquisa sistemática da literatura científica, ficou demonstrado que os BTMs são adequados para determinar a remodelação óssea em pequenos ruminantes, entretanto a variabilidade pode ser um factor limitante.

Posteriormente foi conduzido outro estudo (Capítulo V) com o objetivo de gerar um intervalo de referência a um biomarcador de reabsorção óssea, a fosfatase ácida resistente ao tartarato em ovelhas, e deste modo validar com dados publicados. Os dados obtidos representam uma ferramenta útil em estudos clínicos de pesquisa ortopédica, e toda a variação verificada no marcador pode ser explicada pela fisiologia conhecida.

Assim, o trabalho apresentado no âmbito desta Tese consolida a investigação no campo dos BTMs em pequenos ruminantes e contribui para a caracterização do modelo

ovino osteoporótico após OVX e administração de GCs para estudos pré-clínicos e translacionais em pesquisas ortopédicas.

Palavras-chave: osteoporose, modelo ovelha, biomarcadores de remodelação óssea, micro-tomografia computadorizada, histomorfometria óssea

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Abbreviations and Acronyms List

ALP: Alkaline phosphatase
ANOVA: Analysis of variance
BALP: Bone specific alkaline phosphatase
BKP: Balloon kyphoplasty
BMD: Bone mineral density
BMP: Bone morphogenetic protein
BPs: Biphosphonates
BTM: Bone turnover marker
BV/TV: Bone volume/Trabecular volume
Ca: Calcium
CLA: Chemiluminescence immunoassay;
CLIA: Chemiluminescent Immunoassay
CNPQ: National Council for Scientific and Technological Development
CT: Computed tomography
CS: Calcium phosphate
Ct.Po: Cortical porosity
Ct.Th: Decrease of thickness
CTX: Cross-linked C-terminal telopeptides of type I collagen
CTX-MMP: CTX – matrix metalloproteinase
DIMA: Dietary-induced metabolic acidosis
DPD: Deoxypyridinoline
ECM: Extracellular matrix
ELISA: Enzyme-Linked Immunosorbent assay
GC: Glucocorticoid
GFs: Growth factors
GLA: γ - carboxyglutamic acid
H&E: Hematoxylin and Eosin
HPD: Hypothalamic-pituitary disconnection
HPLC: High-performance liquid chromatography
HYP: Hydroxyproline
iP: Inorganic phosphate
ICTP: Carboxy-terminal telopeptide of type I collagen
IGF-2: Insulin-like growth factor 2
L1-L7: First lumbar vertebrae to seventh lumbar vertabrae
MDL: Modulator
Mg: Magnesium
M-CSF: Macrophage colony-stimulating factor
MMP: Matrix metalloproteinase
MSCs: Mesenchymal stem cells
NTx: cross- linked N-terminal telopeptides of type I collagen
OC: Osteocalcin
OPG: Osteoprotegerin
OVX: Ovariectomized
P: Phosphorous
PINP: Amino-terminal procollagen propeptides of collagen type I
PICP: Carboxy-terminal procollagen propeptides of collagen type I
PIIINP: Amino-terminal procollagen propeptides of collagen type III
PYD: Pyridinoline

PLGA: Poly-L-glycolic acid
PMMA: Polymethylmetacrylate
p-NPP: Para-nitrophenylphosphate
P_U: Ultimate load
PVP: Percutaneous vertebroplasty
Px: Pinealectomy
RANKL: receptor activator of nuclear factor NF-κB ligand
RANK: receptor activator of nuclear factor NF-Kb
R_0: Initial stiffness
RIA: Radioimmunoassay
ROI: Region of interest
Tb.N: Trabecular number
Tb.Sp: Trabecular separation
Tb.Th: Trabecular thickness
TCP: Tricalcium phosphate
TGF-β1: Transforming growth factor beta 1
TNF: Tumor-necrosis family
TNSALP: Tissue-non-specific alkaline phosphatase
TRAP5b: Tartrate-resistant acid phosphatase isoenzyme 5b
VEGF: Vascular endothelial growth factor
VOI: Volume of interest
σ_u: Ultimate stress

List of Publications / Submitted Manuscripts

Parts of this Thesis have been published or submitted for publication as:

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José A. Camassa, Vera A. Barros, Pedro S. Babo, Fábio A. M. Pereira, José J. L. Morais, Aureliano Fertuzinhos, Camila C. Diogo, João A. Bordelo, Cristina P. Sousa, Jorge T. Azevedo, Rui L. Reis, Manuela E. Gomes, Carlos A. Viegas, Nuno Dourado, Isabel R. Dias. Contribution to the characterization of the glucocorticoid treated ovariectomized osteoporotic sheep model for pre-clinical and translational studies in orthopaedic research. Submitted for publication.

Chapter I - General Introduction

General Introduction

1. Bone Tissue

Bone is a mineralized connective tissue composed by cells and nonmineral matrix of collagen and noncollagenous proteins associated with inorganic mineral salts deposited within the matrix, namely calcified extracellular matrix (ECM) (Feng, 2009). Skeleton stiffness balanced with a certain degree of elasticity allows the body to perform different roles essential to life such as support and protection of the organs such as bone marrow, heart, and the lungs, as well as a place of insertion for ligaments, tendons, and muscles, which are essential for locomotion (Buchwalter et al., 2010). Additionally, the bone tissue supports maintenance of acid-base balance due to storage of calcium, magnesium, and phosphate ions (Downey and Siegel, 2006).

2. Composition and organization of bone tissue

2.1. Woven and lamellar bone

The bone tissue exists in two forms considering the arrangement of collagen fibrils: woven (primary) or lamellar (secondary) bone (Currey, 2002). Woven bone is a rapidly created tissue, synthesized by osteoblasts, formed during skeletal histogenesis and growth, and fracture healing, or in tumors and some metabolic bone diseases (Currey, 2002). Woven tissue, unlike lamellar bone, is characterized by irregular bundles of collagen fibers, loosely organized extracellular matrix, numerous osteocyte population, and delayed, disorderly calcification which occurs in irregularly distributed patches. Although woven bone is highly mineralized, it is often quite porous at the micron level (Currey, 2002).

Lamellar bone is formed by a progressive replacement of woven bone during the bone remodeling that normally follows development or healing (Moreira, 2019). Secondary bone is arranged in sheets (lamellae), which can be parallel to each other or arranged in a precise concentric form, with a final mineralization degree lower than that of primary bone (Moreira, 2019). The parallel lamellar structure can be found in the trabecular bone and periosteum, and the circumferential form in the cortical bone Haversian system (Moreira, 2019). Haversian

system or secondary osteon is formed during bone remodeling (Currey, 2002). In short, secondary osteons differ from primary osteons because the secondary results from a replacement of existing bone, and primary are likely formed by mineralization of cartilage, thus being formed where bone was not present (Petrtýl, 1996). In the remodeling process, first the osteoclasts resorb a portion of bone in a tunnel called a cutting cone (Kenkrel and Bassett, 2018). Next, the osteoblasts partially fill up the cutting cone with lamellae and leave a center portion open (Kenkrel and Bassett, 2018). The central cavity is known as Haversian canals, which contain blood and lymph vessels, and nerves. Another kind of canal in lamellar bone is the Volkmann canal, which present transverse vessels that run perpendicular to the long axis of the cortex of the Haversian systems (Figure I.1) (Kenkrel and Bassett, 2018). Volkmann canals link adjacent osteons and also link the blood vessels of the Haversian canals to the periosteum (Kenkrel and Bassett, 2018).

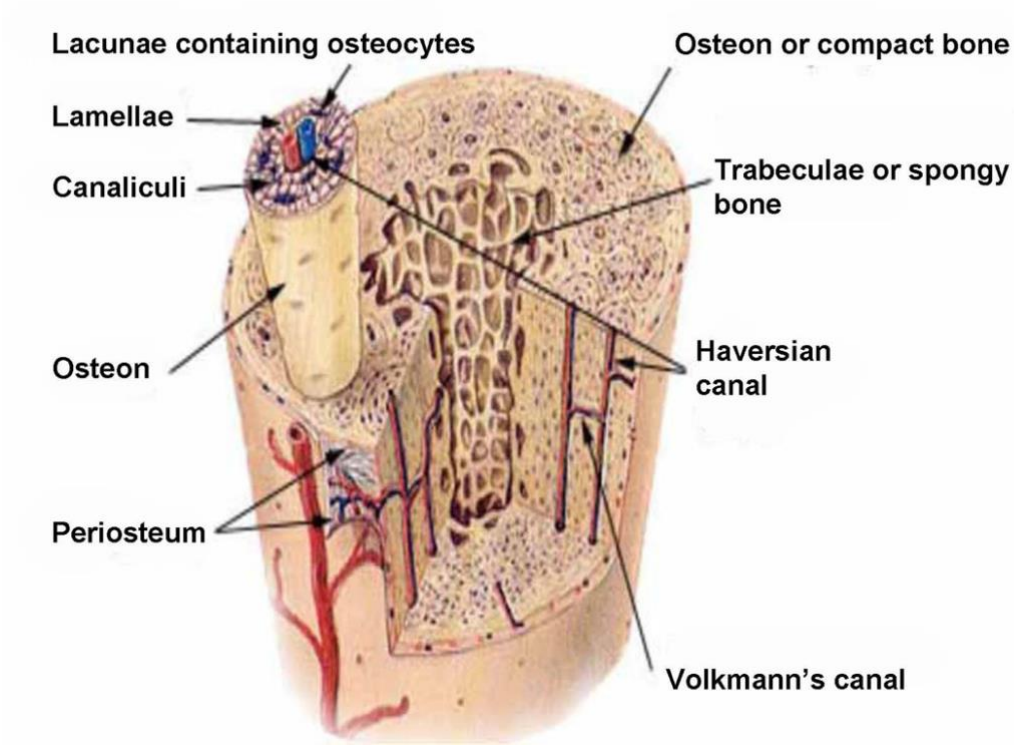

Figure I.1 - Haversian system of mature bone. Adapted from Kenkrel and Bassett (2018).

2.2. Cortical and trabecular bone

The bone matrix in the adult skeleton is generally classified into two types: trabecular (cancellous) and cortical (compact) (Figure I.2). Cortical and trabecular bone are made up of the same cells and the same matrix elements, but there are structural and functional differences (Clarke, 2008). Cortical bone has a porosity ranging between 5% to 10%, while trabecular bone is much more porous, with porosity ranging between 50% to 90% (Morgan et al., 2013). The remainder, which is not occupied by calcified bone, is filled out by bone marrow, blood vessels, and connective tissue (Cashman and Ginty, 2003). Cortical bone is located in the shaft of long bones (Figure I.2) and forms the outer shell around cancellous bone at the end of joints and the vertebrae, while trabecular bone is located at the end of long bones, in vertebrae and in flat bones like the pelvis (Downey and Siegel, 2006; Morrison and Scadden, 2014).

Regarding mechanical properties, cortical or trabecular type definitively has a role in the biomechanical function of bones (Downey and Siegel, 2006). Cortical bone, due to having 70% to 85% of the interface with soft tissues, has great resistance to torsional and bending forces, and has a mechanical and protective function (Downey and Siegel, 2006). Trabecular bone fulfills mainly a metabolic function, although it also participates in the biomechanical function in specific sites such as the vertebrae, with great resistance to compressive loads (Currey, 2002; Morgan et al., 2013). However, trabecular bone has a strong influence on stiffness and strength because of its porosity, which can reach 95% in elderly spines (Currey, 2002).

Bone nutrition in the cortical bone, which presents a low proportion of vascularization to the amount of mass, is poorly metabolically active. On the other hand, the trabecular bone has a quick response to metabolic alterations due to high vascularization (Clarke, 2008). The same occurs in bone remodeling; trabecular tissue has remodeling rates that could be 10 times higher than cortical bone (Morgan et al., 2013).

2.3. Bone marrow

Bone and bone marrow, although often considered individual systems, function as a single tissue (Compston, 2002). Bone marrow is a complex spongy tissue that resides inside of some bones and its cells are precursors of bone remodeling cells (Compston, 2002; Florencio-

Silva et al., 2015). These cells exert a critical influence on the regulatory role both in their own development and in the remodeling process, acting as mediators for the effects of systemic and local factors (Florencio-Silva et al., 2015).

Bone marrow contains hematopoietic and mesenchymal stem cells, which are responsible for renewing elements in the blood and regeneration of tissues, such as muscle, tendon and bone, respectively (Morrison and Scadden, 2014; Gurkan and Akkus, 2008).

Bone marrow recognizes mechanical signals and physiological loads from their environment (Ozcivici et al 2010). Drugs, steroids, aging, osteoporosis, or disuse can affect different properties of bone marrow such as intramedullary pressure and composition (Gurkan and Akkus, 2008; Ozcivici et al 2010). As such, bone turnover is directly affected by activity or inactivity of the bones, and a drastic reduction of mechanical loads leads a reduction of bone mineral density. In contrast, large loads result in an increase of bone mass (Gurkan and Akkus, 2008; Ozcivici et al 2010).

The combination of several factors such as Transforming Growth Factor β (TGF-β) and IGF-2 (Insulin-like growth factor 2) in bone marrow allow the creation of an osteogenic microenvironment (Gurkan and Akkus, 2008; Crane, 2014). Thus, mesenchymal stem cells undergo a differentiation into osteoblasts resulting in bone formation (Knight and Hankenson, 2013). On the other hand, hematopoietic stem cells originate osteoclasts, which play a role in bone resorption (Downey and Siegel, 2006). In brief, osteoblasts and osteoclasts are essential cells in bone remodeling and when the bone marrow environment is affected and disturbed, this results in an imbalance of bone homeostasis (Gurkan and Akkus, 2008; Crane, 2014). Thus, the destination of mesenchymal stem cells is modulated by the bone marrow microenvironment.

The composition of marrow is supportive stromal cells, hematopoietic tissue, and marrow adipose tissue. The last two have the same proportion in a physiological state, however, in osteoporosis, mesenchymal stem cells undergo a higher differentiation into adipocytes (Crane, 2014). Therefore, an inefficiency by mesenchymal stem cells causes a decrease in bone formation and consequently several skeletal disorders (Zaidi, 2007).

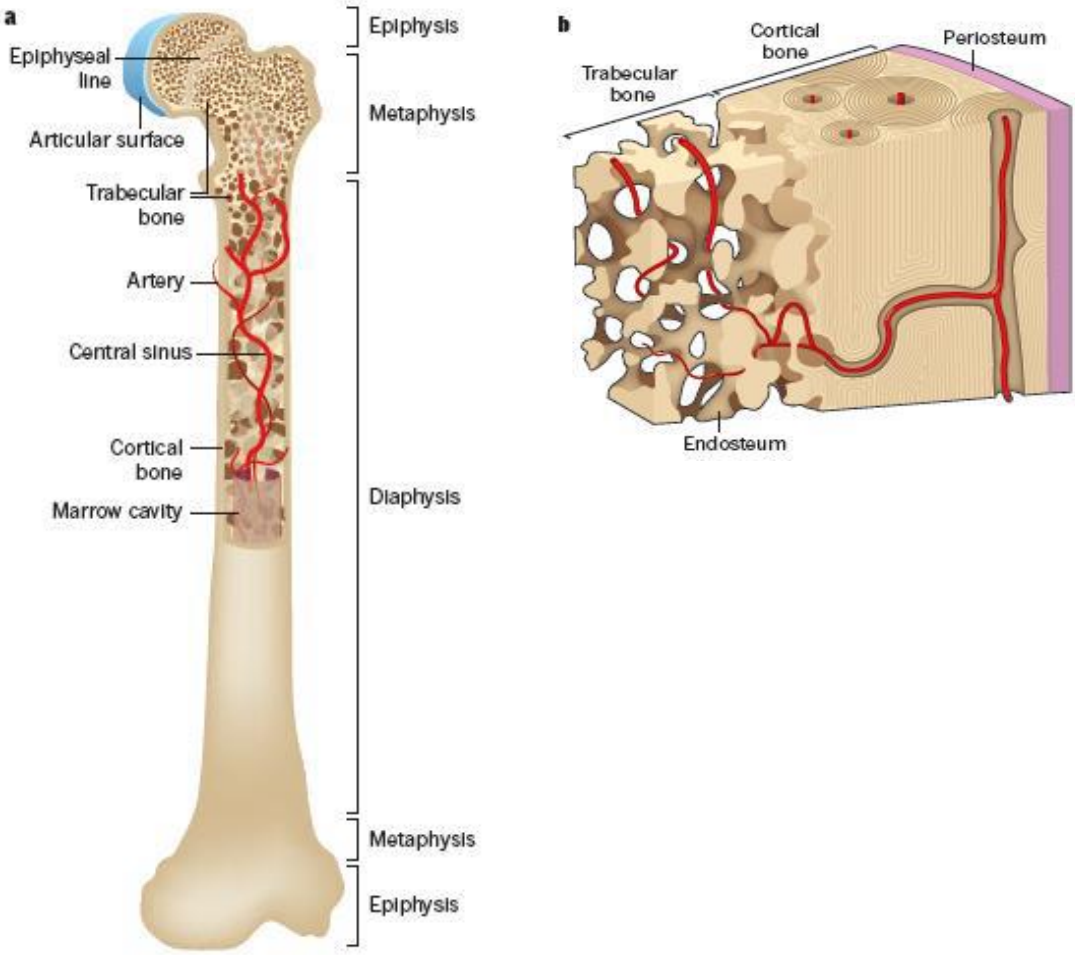

Figure I.2 - Bone organization. a) Epiphyses is the wider section at each end of the bone, which which is filled with spongy bone. The diaphysis is the tubular shaft that runs between the proximal and distal ends of the bone. The hollow region in the diaphysis is called the medullary cavity, which is filled with yellow marrow. The walls of the diaphysis are composed by compact bone. Epiphysis meets the diaphysis at the metaphysis. Cortical bone surrounds the marrow space and is most prominent in the diaphysis. b) Endosteum is the structure between the bone and bone marrow and surrounds the medullary cavity. Periosteum is a membrane that covers the outer surface. Adapted from Morrison and Scadden (2014).

2.4. Extracellular bone matrix

The extracellular matrix is divided into 30 to 35% of organic matter, and 65% to 70% of inorganic matter (Cremers and Seibel, 2008). The organic matrix is constituted mainly by collagenous proteins, as well as bone tissue cells, predominantly represented by type I collagen, noncollagenous proteins, and growth factors (Aszódi et al., 2000). The inorganic material compounds are predominantly phosphate and calcium ions, which form the hydroxyapatite crystals (Datta et al., 2008).

However, also present are components such as bicarbonate, sodium, potassium, citrate, magnesium, carbonate, fluorite, zinc, barium, and strontium (Buckwalter et al., 1996). Association between collagen and noncollagenous matrix proteins form a support for hydroxyapatite deposition resulting in stiffness and resistance of the bone tissue. This way, the mineral fraction contributes to the mechanical and physiological homeostasis of bone tissue (Datta et al., 2008) in which a variation of crystal size and crystallinity of bone mineral leads to some metabolic diseases, such as osteoporosis (Hadjidakis and Androulakis, 2006).

3. Cells of the bone tissue

Bone is composed by four types of cells: osteoblasts, bone lining cells, osteocytes, and osteoclasts (Table I.1) (Cremers and Seibel, 2008). The first three are derived from a mesenchymal cell lineage and osteoclasts are derived from the hematopoietic lineage (Morgan et al., 2013). Location of these cells vary: osteocytes are situated deep in the bone matrix, and osteoblasts, osteoclasts and bone lining cells are situated along the surface of the bone, and these cells are responsible for bone production, maintenance and modeling (Cremers and Seibel, 2008).

Table I.1 - Bone cells: origin, location and functions. Adapted from Barros (2019).

	Origin	Location	Functions
Osteoblasts	Mesenchymal stem cell.	Periosteal and endosteal bone surfaces.	Responsible for bone formation through secretion of the organic components of bone matrix.
Bone lining cells	Mesenchymal stem cell (Terminally differentiated osteoblasts).	Periosteal and endosteal bone surfaces.	Responsible for forming the periosteum and the endosteum.
Osteocytes	Mesenchymal stem cell (Terminally differentiated osteoblasts).	Lacunae surrounded by mineralized bone matrix.	Support bone structure and metabolic function.
Osteoclasts	Hematopoietic stem cell (Monocytes).	Periosteal and endosteal bone surfaces.	Responsible for bone resorption through digest the organic matrix.

3.1. Osteoblasts

Osteoblasts are highly specialized cells composed by mononucleate cuboid cells, abundant rough endoplasmic reticulum, mitochondria, and prominent Golgi apparatus. They are located along the endosteal and periosteal bone surface. Its main role is bone formation through the production and secretion of the organic components of the bone ECM (Capulli et al., 2014). This synthesis of ECM occurs in two steps: depositions of organic matrix and its mineralization (Florencio-Silva et al., 2015). In summary, alkaline phosphatase, which is secreted by osteoblasts, is an enzyme that is involved in the regulation of bone mineralization by pyrophosphate, an inhibitor of mineral deposition (Orimo, 2010). This way, phosphate-containing compounds are degraded by pyrophosphate, dropping phosphate ions inside the matrix vesicles (Glimcher, 1998). Thus, the accumulation of phosphate and calcium ions inside the vesicles result in hydroxyapatite crystals. Next occurs the supersaturation of calcium and phosphate ions, and after the matrix vesicle reaches its limit, it ruptures, spreading hydroxyapatite crystals to the surrounding matrix (Boivin and Meunier, 2002). Furthermore, osteoblasts are one of several producers of other factors, such as cytokine receptor activator of nuclear factor-kB ligand (RANKL) and macrophage colony stimulating factor (M-CSF), which have important functions in bone tissue formation and resorption (Florencio-Silva et al., 2015).

3.2. Bone lining cells

Bone lining cells are inactive flat-shaped osteoblasts located on bone surfaces. These cells display few cytoplasmic organelles, such as rough endoplasmic reticulum and Golgi apparatus. Although knowledge of the functions of bone lining cells is incomplete, it is known that these cells preserve the bone matrix by avoiding its direct contact with osteoclasts when bone resorption should not occur (Miller et al., 1989). Another known function is it contributes to osteoclast differentiation, producing osteoprotegerin (OPG) and the receptor activator of nuclear factor kappa-B ligand (RANKL) (Andersen et al., 2009).

3.3. Osteocytes

Osteocytes are the most numerous bone cells, reaching 95% of all bone cells, and with a life expectancy of up to 25 years (Franz-Odendaal et al., 2006). In summary, during bone tissue formation, a subpopulation of osteoblasts becomes osteocytes, located in newly formed bone matrix (Schaffler et al., 2014). The new cells play important roles in the bone, for example, regulating bone homeostasis through interpretation of external mechanical and chemical inputs via interconnected osteocytes, contributing to the adaptation of the bone. As such, this interconnected network acts as mechanosensors, helping to detect mechanical pressures and loads, leading to adaptation of the bone to daily mechanical disturbances (Currey, 2002). Thus, osteocytes behave as orchestrators of bone remodeling, conducting osteoblast and osteoclast activities (Schaffler et al., 2014). Furthermore, osteocyte apoptosis is a chemotactic signal to osteoclastic bone resorption, in which apoptotic osteocytes are engulfed by osteoclasts during bone resorption (Boabaid et al., 2001).

3.4. Osteoclasts

Osteoclasts are multinucleated and voluminous cells, which originate from mononuclear cells of the hematopoietic stem cell lineage, found in the Howship lacunae, resorption channels, and commonly on bone surfaces (Clarke, 2008; Crockett et al., 2011). The role of these cells is bone resorption, caused by enzyme degradation of the protein fraction and the acid dissolution of minerals in the calcified bone ECM. Stimulus to initiate bone resorption is mediated by osteoblasts, where the most receptors to osteolytic factors are located. This way, parathormone (PTH), interleukin 11 (IL 11), prostaglandin E2 or vitamin D3 may induce osteoblasts to activate quiescence osteoclasts. On the other hand, osteoclasts have receptors for calcitonin, a hormone capable of inhibiting the function of these cells. It is not fully known where bone resorption is selected, however, the first signal on the surface of the endosteum is performed by certain systemic factors, such as PTH, causing a retraction of osteoprogenitor cells from the bone tissue. Afterwards, there is lysis of the usually non-mineralized superficial layer of the ECM. After the osteoclasts are exposed to a mineralized surface, a bonding occurs, where osteoclasts secrete protons to dissolve the mineral fraction of the ECM, and proteolytic enzymes, namely, lysosomal proteases and metalloproteinases, leading to a digestion of the bone matrix proteins (Katagiri and Takahashi 2002). The resorption process results in an output of factors that attract and activate osteoblasts. This way, skeletal remodeling tends to be in

constant balance, however, an abnormal increase or decrease in osteoclast formation and activity result in bone disease, such as osteoporosis (Feng and McDonald, 2011).

Factors, such as macrophage colony-stimulating factor (M-CSF) and RANK ligand (RANKL) promote the activation of transcription factors and gene expression in osteoclasts. The first factor is secreted by osteoprogenitor mesenchymal cells and osteoblasts and the second by osteoblasts, osteocytes, and stromal cells (Yoshida et al., 1990; Yavropoulou and Yovos, 2008). Osteoclastogenesis is induced when RANKL binds to its receptor RANK in osteoclast precursors (Sodek et al., 2000). Another essential factor called osteoprotegerin (OPG) prevents the RANK/RANKL interaction, binds to RANKL, and limits osteoclastogenesis (Boyce and Xing, 2008). During bone pathologies, cytokine tumor necrosis- α (TNF-α) induces RANKL production and consequently promotes osteoclastogenesis, resulting in an abnormal state of bone resorption (Luo et al., 2018).

4. Osteoporosis

Osteoporosis is a multifactorial disorder associated with loss of bone mass and structure, in which a deterioration of bone strength leads to an increase in bone fragility and a higher fracture risk (Zajickova and Zofkova, 2003; Nuti et al., 2018). For decades, osteoporosis was classified as a secondary cause of low mass, however, it is now classified as a primary bone disorder related to metabolic changes, not only in bone, but in whole body homeostasis (Rosen, 2017).

Bone remodeling tends to maintain a balance, where total bone formation and resorption are equal, functioning as a preventive maintenance program, continually removing older bone and replacing it with new bone, maintaining a healthy skeleton. On the other hand, certain factors can increase resorption, such as menopause and advancing age, resulting in an imbalance of bone remodeling leading to bone loss and osteoporosis (Sözen et al., 2016).

Thus, there is a growing consensus on correlation between low BMD and fracture risk, and qualitative aspects of the bone as additional factors contributing to skeletal fragility (Rosen, 2017). In this process, individual trabecular plates of bone are destroyed, resulting in a fragile structure and reduced bone mass with a high likelihood of collapse (Sözen et al., 2016). Regarding changes in trabecular bone, there is decrease of the number of trabeculae, trabecular thickness and the degree of connectivity, where extremities are generally more affected than

central regions. Regarding changes in the cortical bone, with increasing age, the degree of mineralization increases, which is reflected in an increase in mineral content, in porosity and in diameter of the cortex. Afterwards, there is a decrease of cortical bone thickness and deterioration of mechanical properties (Rosen, 2017).

Osteoporosis could be divided into two forms of the disease: primary osteoporosis, which includes juvenile, postmenopausal, and male and senile osteoporosis; primary osteoporosis occurs in the absence of an underlying disease. Secondary osteoporosis occurs in the presence of an underlying disease or medication, such as diabetes mellitus and glucocorticoids, respectively (Mirza and Canalis, 2015; Nuti et al., 2018). Primary osteoporosis can also be divided into two subgroups. Type I is known as postmenopausal osteoporosis, caused by a deficiency of estrogen, mainly affecting the trabecular bone. Type II, senile osteoporosis, is related to bone mass loss due to the aging of cortical and trabecular bones (Mirza and Canalis, 2015).

Osteoporotic fractures present a predisposition for certain anatomic sites, such as femoral neck, and lumbar and thoracic vertebrae; however, they may occur in almost all the skeleton (Warriner et al., 2011). Trauma due to fall is the major cause of fractures affecting long bones (femur, humerus, and radius), while in the vertebral body, the cause and exact time are frequently undetermined due to often going undiagnosed (Nuti et al., 2018).

Osteoporosis is a global epidemic; it is estimated that more than 200 million people are affected, which places a big financial burden on public health systems. However, with an early diagnosis before fracture and an early fast-acting treatment, osteoporosis can be prevented. It is important that the medical sector focuses on informing society of the prevention measures for osteoporosis since this will be the most logical and shortest pathway to control this epidemic (Sözen et al., 2016).

4.1. Estrogen and osteoporosis

Estrogen plays an important role in balancing activities in the bone cells. Suppressing bone resorption and modulating bone formation are important estrogen tools to maintain stability in bone remodeling (Khosla et al., 2011). Suppression of bone resorption is performed by blocking osteoclastogenesis, and estrogen also modulates RANK signaling in osteoclasts, which induces apoptosis of osteoclasts (Shevde et al., 2000; Martin-Millan et al., 2010; Khosla

et al., 2011). Estrogen enhances bone formation by stimulating osteoblast differentiation and function (Eastell et al., 2016). Therefore, the lack of estrogen increases expression of M-CSF, RANKL and TNF-α and reduces OPG expression, resulting in a decrease of osteoclasts apoptosis and subsequent increase of bone resorption (Lerner, 2006). Bone homeostasis is still more affected by withdrawal of estrogen due to increased apoptosis of osteocytes, which is the cell responsible for the remodeling balance described earlier, and also increased production of sclerostin (Figure I.3), which reduces new bone formation; however, the number of osteoblasts is higher than in a physiological state (Tomkinson et al., 1997; Boabaid et al., 2001; Currey, 2002; Lerner, 2006; Khosla et al., 2011).

Estrogen replacement therapy is used for the prevention of postmenopausal osteoporosis mainly for whom non-estrogen medications are not indicated. However, this therapy increases the risk of developing breast and uterine cancer, and it has never been approved for the treatment of osteoporosis (Hutchinson-Williams and Gutmann, 1991; Sözen et al., 2016).

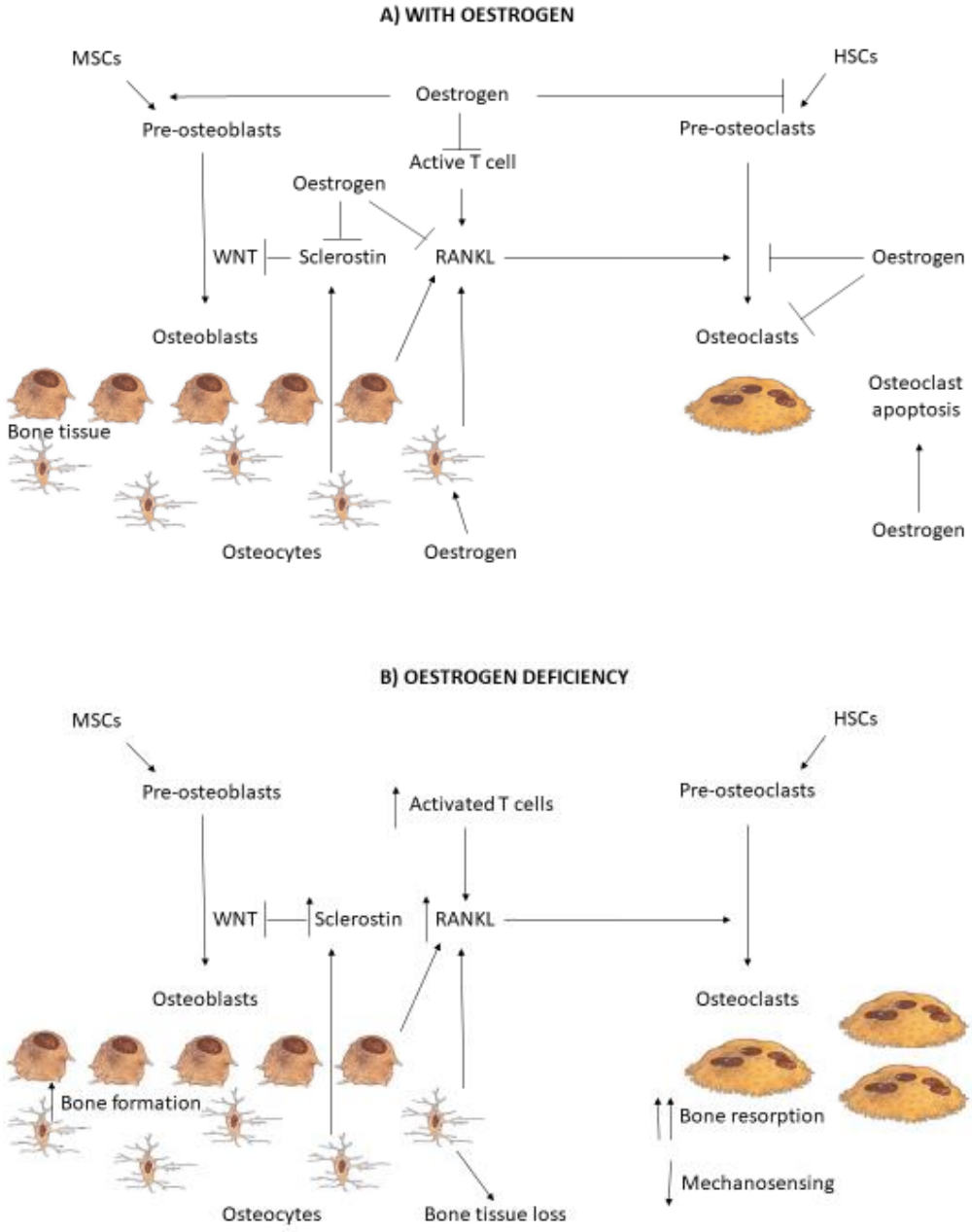

Figure I.3 - Role of oestrogen in bone remodeling. A) Physiological bone remodeling. Oestrogen stimulated a bone formation through osteoblasts and suppress osteoclastogenesis and osteoclast activity and reduce receptor activator of nuclear factor-κB ligand (RANKL) by different vias. B) Oestrogen deficiency. Osteoblasts are suppressed by increased sclerostin, whereas leads higher osteoblast number. RANKL production increase resulting higher osteoclast number and activity.

4.2. Diagnosis of osteoporosis

Diagnosis of osteoporosis and evaluation of fracture risk are based on multiples factors such as history, physical examination, laboratory, and diagnostic tests. History requires a detailed investigation of the patient's medical history, lifestyle, and appropriate assessment of

risk factors (Nuti et al., 2018). Osteoporosis in the family history is an important evidence of a high risk for osteoporotic fracture. Another essential point is to check for any comorbidities or medication that may interfere with bone metabolism, and in women, verify gynaecological history, and date of possible menopause as detailed previously (Fox et al., 1998).

Diagnostic imaging of osteoporosis is performed using several techniques where the purpose is to measure BMD, which is expressed as grams of mineral per area or volume. The most utilized technique is dual energy X-ray absorptiometry (DXA) due to high sensitivity to calcium; however, there are also quantitative ultrasound (QUS) and quantitative computed tomography (QCT) (Lane, 2006; Kanis et al., 2013). Basically, there are two norms used to compare different densitometry methods, Z-score and T-score, the first compared to the BMD of an age-, sex- and ethnicity-matched reference population and the second compared to a young-adult reference population of the same sex. Both norms present result in units of standard deviation (SD), posteriorly classified from normal bone to severe osteoporosis (Table I.2) (Cosman et al., 2014).

Table I.2 - Bone classification by BMD. Adapted from Cosman et al. (2014).

Classification	BMD	T-score
Normal	Within 1 SD of the mean level for a young-adult reference population.	T-score at -1.0 and above.
Low bone mass (Osteopenia)	Between 1.0 and 2.5 SD below that of the mean 1 level for a young-adult reference population.	T-score between -1.0 and -2.5.
Osteoporosis	2.5 SD or more below that of the mean level for a young-adult reference population.	T-score at or below -2.5.
Severe or established osteoporosis	2.5 SD or more below that of the mean level for a young-adult reference population with fractures.	T-score at or below -2.5 with one or more fractures.

4.3. Prevention and treatment of osteoporosis

Osteoporosis is a preventable and treatable disease, however, people are not diagnosed and treated during an early phase of osteoporosis because of absence of signs prior to fracture. Therefore, prevention must be the first step to prevent or slow the onset of disease, starting with recommendations for all patients. Non-pharmacological intervention consists in correction of

risk factors such as adequate intake of calcium and vitamin D, lifelong regular weight-bearing and muscle-strengthening exercises, smoking cessation, limit alcohol and caffeine intake, and assessment of the home environment to prevent falls (NIH, 2000; Cosman et al., 2014; Sözen et al., 2016.

Drug intervention is indicated when the patient is included in one of the categories. First, is development of hip or vertebral fractures. Second, the presence of T-score ≤−2.5 at the femoral neck, total hip, or lumbar spine by DXA. The last category is seen in postmenopausal women and men age 50 and older with low bone mass (T-score between −1.0 and −2.5 at the femoral neck or lumbar spine) at the femoral neck, total hip, or lumbar spine by DXA and a 10-year hip fracture probability ≥3 % or a 10-year major osteoporosis-related fracture probability ≥20 % based on the USA-adapted WHO absolute fracture risk model (Fracture Risk Algorithm (FRAX®) (Cosman et al., 2014).

Anti-osteoporotic drugs can be divided into two categories: anti-resorptive or anti-catabolic such as bisphosphonates, estrogens, calcitonin; and selective estrogen-receptor modulators and stimulators of bone formation or anabolics, such as parathyroid hormone (PTH) (Table I.3). Combining anti-resorptive therapies is not recommended due to a possible oversuppression of bone turnover, accompanied by a higher risk of major osteoporotic fractures. Otherwise, anti-resorptive and stimulators of bone formation therapy, specifically PTH and bisphosphonate combined, present a better BMD result than any other treatment, but PTH is expensive and must be administered daily via subcutaneous injection. However, it is necessary to have a personalized therapy for each patient because of high interindividual variability, and monitor therapy efficacy with bone markers, BMD, DXA and vertebral imaging (Lane, 2006; Papapoulos and Makras, 2008; Cosman et al., 2014).

Adherence to medical therapy is poor mainly because osteoporosis is a silent disease; patients are not aware how severe the condition is due to the absence of signs. To improve adherence to therapy, it is necessary to raise awareness in patients about the risks and benefits expected with and without therapy, such as fracture risk and quality of life Kanis et al., 2013; Jaleel et al., 2018).

Table I.3 - Drugs for osteoporosis treatment. Adapted from Barros (2019).

Drug	Objective	Activity	Side effect	Results
Bisphosphonates (Alendronate, risedronate, zoledronic acid and etidronate)	Prevention and treatment.	Reduces bone resorption: inhibits osteoclasts activity and shortening their lifespan.	Esophageal and gastric irritation.	Reduces the risk of vertebral fractures (40% to 70%); Reduces the incidence nonvertebral fractures (50%).
SERMs (Raloxifene)	Prevention and treatment.	Estrogen agonist/ antagonist.	Increases the risk of deep vein thrombosis; Increases hot flashes; Leg cramps.	Reduces the risk of vertebral fractures in patients with a prior vertebral fracture (30%) and in patients without a prior vertebral fracture (55%).
PTH (Teriparatide)	Treatment.	Stimulates osteoblastic bone formation.	Muscle cramps and infrequent; Hypercalcemia, nausea and dizziness; Bone tumors (high-dose treatment).	Increases in trabecular bone density and connectivity; Reduces incidence of new vertebral fractures (65%); Reduces incidence of new nonvertebral (53%).
Estrogens (17 β-estradiol)	Prevention.	Stimulates osteoblastic bone formation.	Increases risks of myocardial infarction, stroke, breast and uterine cancer, pulmonary emboli, and deep vein thrombosis.	Reduces the risk of new vertebral fractures and hip fractures (34%); Reduces the risk of new others osteoporotic fractures (23%).
Calcitonin	Treatment.	Inhibits osteoclastic bone resorption.	Injection calcitonin: nausea, local inflammation and flushing of the face or hands; Spay calcitonin: local nasal irritation.	Increases BMD at lumbar spine (3%) and forearm; Reduces the risk of vertebral fracture (33%).

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Chapter II - Objectives

1. Objectives

The overall aim of this Thesis was to give insight into the role of BTMs in small ruminants and contributed to the characterization of the GC-treated OVX osteoporotic sheep model for pre-clinical and translational studies in orthopaedic research. For this purpose, the present thesis was organized in two literature review and two original articles:

- To systematically summarize what is known about the use of sheep and goats as large animal models of human osteoporosis for preclinical and translational studies, providing a comprehensive overview of how important the ovine model is to osteoporosis research (Chapter III).

- To identify and validate in the literature the use of bone turnover markers in studies on small ruminants, specially determining its usefulness and limitations as a tool for researchers (Chapter IV).

- To generate a reference range for a specific bone marker, tartrate-resistant acid phosphatase, comparing different ages and metabolic stages and to validate the already published data (Chapter V).

- To characterize and optimize the glucocorticoid treatd ovarietomized sheep as an animal model of osteoporosis: Follow-up of bone turnover biomarkers monthly during osteoporosis induction, and to evaluate the bone mineral density, mechanical properties, and bone remodeling after 6 months postoperative period (Chapter VI).

Chapter III - Preclinical and Translational Studies in Small Ruminants (Sheep and Goat) as Models for Osteoporosis Research

The content this chapter has been based on the following article:

Isabel R. Dias*, **José A. Camassa***, João A. Bordelo, Pedro S. Babo, Carlos A. Viegas, Nuno Dourado, Rui L. Reis, Manuela E. Gomes. Preclinical and Translational Studies in Small Ruminants (Sheep and Goat) as Models for Osteoporosis Research. Current Osteoporosis Reports 2018; **16**(2): 182-197. [Invited review]. DOI: 10.1007/s11914-018-0431-2.
*Isabel R. Dias and José A. Camassa contributed equally and were considered co-first authors of this review article

Large animal models for osteoporosis research: The small ruminants (sheep and goat) (por: Isabel R. Dias, Vera Barros, **José A. Camassa**, João A. Bordelo, Pedro S. Babo, Jorge T. de Azevedo, Carlos A. Viegas, Nuno Dourado, Rui L. Reis, Manuela E. Gomes), Atlas of Science, published in 2nd April 2019. https://atlasofscience.org/large-animal-models-for-osteoporosis-research-the-small-ruminants-sheep-and-goat/

Preclinical and Translational Studies in Small Ruminants (Sheep and Goat) as Models for Osteoporosis Research

1. Introduction

In 2000, the National Institutes of Health Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy, from the US Department of Health and Human Services, defined osteoporosis as "a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture" (NIH, 2001).

Osteoporosis is the most frequent metabolic disease worldwide, resulting in a significant public health issue due to high prevalence of fragility fractures in aged populations and a significant economic cost on society (Burge et al., 2007). In the USA, the medical cost of osteoporosis and related fractures was estimated at 22 billion dollars (Blume and Curtis, 2011). For instance, along with the increase in life expectancy in the USA, namely women are the fastest growing group within the veteran population, expecting to increase from 10.3% in 2013 to 15% in 2030 (Cauley, 2017), with a 24% higher risk of hip fracture due to osteoporosis relative to non-veteran women, which may represent an important future high-risk group (LaFleur et al., 2016) and one more proof of the importance of research related to postmenopausal osteoporosis. Therefore, there is a wide need for orthopedic research where the use of laboratory (rodents (mice and rat), lagomorphs (rabbits)) and large animal models (non-human primates, dogs, small ruminants (goat and sheep), pigs) of osteoporosis are strictly necessary and useful to test novel therapies. Although, a perfect osteoporotic animal model that absolutely reproduces all pathophysiologic characteristics developed in humans has not yet been established (Turner, 2011; Oheim et al., 2016). The non-human primates are the most similar animal model to man, especially with regard to bone loss pathophysiology and immune, reproductive, and cardiovascular systems (Smith et al., 2009; Smith et al., 2011). Nevertheless, the use of non-human primates in scientific research carries major ethical concerns (Quiley, 2007). Therefore, the rodents, mainly mice and rats, still are the most common osteoporotic models (Sophocleous and Idris, 2014). Although, when the research purpose consists in prevention, pharmacological therapy of low bone mass and/or surgical treatment of osteoporotic fractures, a large animal model should be used in view of the experimental design (Bonjour et al., 1998). Hence, the US Food and Drug Administration has indicated the necessity of evaluating new osteoporosis drug therapies using at least two species (Thompson et al., 1995): (1) a well characterized ovariectomized (OVX) rodent model (Lelovas et al., 2008;

Leitner et al., 2009) and (2) a non-rodent large animal model (e.g., adult non-human primates, dogs, goats and sheep, and pigs) (Reinwald and Burr, 2008; Smith et al., 2009; Checa et al., 2011; Turner, 2011). These large animal models should exhibit bone tissue macro- and microstructure, composition, biochemical properties, and bone mineral density (BMD), as well as secondary Haversian bone tissue remodeling in the skeletally mature stages of their lifespan more similar to humans. Nevertheless, species-dependent variations in bone and cartilage tissues composition and mechanical properties can be found Aerssens et al., 1998; Pearce, 2007). Nevertheless, the most relevant differences between the large animal model species currently available for orthopedic research and humans are the rapid growth of the first, with a predominance of plexiform, or woven-fibered and lamellar bone in the areas adjacent to the periosteum and endosteum of long bone cortices during the first years of their lifespan (namely, in the dog and small ruminants) (Pearce, 2007), and the quadruped locomotion in apposition with the biped locomotion of the human species that use exclusively the hind limbs for this purpose. In scientific literature, there are a considerable number of publications on the development of large animal models for osteoporosis research, namely goats and sheep (Turner, 2002; Egermann et al., 2005; Reinwald and Burr, 2011, Beil et al., 2012; Oheim et al., 2012a; Zhang et al., 2016). However, references to the subsequent use of these animal models in practical preclinical and translational situations, such as for the treatment of fragility metaphyseal and spinal fractures (vertebral augmentation, spinal fusion), to evaluate the efficacy of implant fixation and improvement of the fracture-healing process in the osteoporotic bone tissue or in the repair of bone defects in osteoporotic skeleton are very scarce or non-existent. Hence, this review article was intended to compile small ruminant osteoporotic models that report to the fracture healing processes and tissue engineering defect repair of osteoporotic bone, and it summarizes concluding remarks of these studies in order to provide a comprehensive overview of the use of these large animal models for preclinical assessment.

2. Small ruminant models for osteoporosis research

Opposite to the relatively high incidence of osteoporosis in human populations, osteoporosis is not endemic and is hardly physiologically developed by other animals. The physical stature and weight of small ruminant models, namely sheep and goats, has been an important criteria for the choice of this animal model to conduct orthopedic research (Reinwald and Burr, 2011). Large animals present obviously a wide skeletal structure that allows for

realistic clinical situations, namely in osteoporosis research for drug therapy evaluation, examination of orthopedic implants and prostheses in an osteoporotic environment, and biomaterials, like bioactive ceramics specially for vertebral augmentation and spinal fusion (Turner, 2002; Yu et al., 2014). Other points such as cooperation and compliance (Newman et al., 1995), availability, facility of handling and housing, as well as low cost compared with other large animals, and acceptance as an experimental animal model by the public opinion of the majority of society (Turner, 2007) need to be validated by these animal models. However, environmental conditions could directly influence the bone metabolism of sheep and goats, such as the season and photoperiod cycle, geographical location (altitude), and diet. Sheep and goats are seasonal breeders, with higher estrogen levels between the autumn and winter and lower BMD during the winter (Johnson et al., 2002; Arens et al., 2007; Healy et al., 2010). A diet composed by legumes or leguminous silage or pasture very rich in phytoestrogen could promote alterations similar to estrous and consequently affect bone metabolism (Lans et al., 2007; Reinwald and Burr, 2011).

3. The sheep model

3.1. The combined treatment – ovariectomy, calcium/vitamin D diet, and glucocorticoids application for 6 months

Lill et al. (2000; 2002a) conducted the first studies on different osteoporosis induction regimens in sheep to establish a large animal model to evaluate treatment for fragile fractures. They concluded that after 6 months, the combined treatment of OVX, calcium (Ca)/vitamin D-restricted diet, and glucocorticoids was the most effective in view of the induction of severe osteoporosis in sheep. Subsequently, Lill et al. (2002b) concluded that this osteoporotic sheep model met the criteria for an osteoporosis model for fracture treatment with respect to mechanical and morphometric bone properties. Goldhahn et al. (2005) performed a study with the combined treatment of OVX, Ca/vitamin D-restricted diet, and steroids, to evaluate the osteoporosis sheep model with regard to normalization of hormonal status and possible BMD rebound after 28 weeks. Herein, they observed a slow BMD rebound in cancellous bone subsequently to the termination of steroid application, applied during the initial 12 weeks post-operatively. Egermann et al. (2008) evaluated the treatment of OVX sheep, with the

combination of restricted diets and methylprednisolone. Therefore, OVX sheep were subjected to a daily Ca/vitamin D-restricted diet (1.5 versus 5 g Ca; 100 versus 1000 IU vitamin D3 day−1) and 1800– 2000 mg/IM methylprednisolone in different regimens 6 months post-operatively. BMD reduction was observed over 30% concomitant with a reduction of bone biomechanical properties after 12 weeks of the combined treatment. It was also noted that the rebound of cancellous bone loss following steroid application stopped, as previously reported (Goldhahn et al., 2005). Nevertheless, given the severity of the side effects of the glucocorticoid medication in this combined treatment, such as alopecia, abscess, immunosuppression, and compromised animal welfare, Egermann et al. (2008) considered that the model had to be refined. Klopfenstein et al. (2007) suggested that the administration of equal amounts of steroids could reduce the adverse side effects, as it allows for decreasing the number of administrations, without reducing the effect regarding corticosteroid-induced osteoporosis. Dvorak et al. (2011) evaluated in the combined treatment osteoporotic sheep model the catabolic jawbone turnover to explain the increase on cortical bone porosity and thickness, which may affect the mechanical stability of dental implants. Also, Veigel et al. (2011) evaluated the lumbar and maxillofacial BMD changes in the combined treatment osteoporotic sheep model at 1, 3, 6, 9, and 12 months, observing generalized microstructural changes, reinforcing this sheep model for further investigation in the maxillofacial area. Zarrinkalam et al. (2012a; 2012b) in the combined treatment osteoporotic sheep model observed changes in osteocyte density corresponding with changes in the osteoblast and osteoclast activities with the regional histological differences suggesting different pathophysiological mechanisms operating at the different anatomical sites. Additionally, it was verified that the osteoporotic characteristics persistent in the spine of OVX sheep after corticoid administration stopped.

3.2. Ovariectomy in aged animals with a post-operative period longer than 12 months

Newman et al. (2004) evaluated OVX sheep after 12 months as a model for human bone loss, suggesting the validity of this model based on the observed changes in the trabecular bone architecture of the iliac crest (bone volume and thickness reduction and increased in trabecular separation), associated with estrogen deficiency. Kennedy et al. (2008a) assessed in an OVX sheep model the biomechanical properties of osteoporotic L3 vertebrae by dual-energy X-ray absorptiometry and compressive tests. The authors performed the quantification of bone

turnover by epifluorescence microscopy and micro-architecture by micro-computed tomography scanning. The results showed that BMD and micro-architecture remained unchanged after 12 months; however, bone turnover increased in cortical and trabecular compartments. In the same model, Kennedy et al. (2008b) also evaluated the behavior of fatigue-induced micro-damage in compact bone samples, verifying that the number of cycles to failure was lower in the OVX group relative to controls by approximately 7% while crack density was higher in the OVX group compared with controls. Wu et al. (2008) evaluated that in the OVX sheep, bone biomechanical and architectural properties change 6 and 12 months post-operatively. A decrease superior to 30% on trabecular BMD was observed at the femoral neck and condyle, as well as micro-architectural deteriorations in the lumbar spine and femoral neck with reduced mechanical properties. The authors concluded that this model seemed to satisfy the criteria for an osteoporosis model, though the duration post-OVX might be longer than 12 months to ensure the osteoporotic condition of the animal model to develop. Kennedy et al. (2009) observed that in the period of 12 months post-OXV, the bone turnover and porosity were significantly increased, while stiffness and yield strength decreased in ovine mid-diaphyseal metatarsal cortical bone. Zhang et al. (2014) compared the variation of cancellous bones at different skeletal localizations in OVX sheep, verifying that the sensibility of cancellous bone to estrogen deficiency was site specific, showing a specific pattern: lumbar vertebra, femoral neck, mandibular angle, and rib. Kreipke et al. (2014) assessed the longterm OVX sheep model (12 and 24 months) observing changes in micro-architectural and mechanical properties of trabecular bone tissue stabilized at the ovine spine and distal femur. In 2016, Kreipke et al. (2016) studied in the osteoporotic OVX sheep model the effects of microarchitecture and estrogen depletion on micro-damage susceptibility in trabecular bone, supporting the concept that bone micro-damage is dependent on trabecular microarchitecture and pre-existing micro-damage. Zarrinkalam et al. (2009) suggested and contributed to the evaluation of the OVX sheep subjected to glucocorticoid treatment for biomaterial research and to the study of vertebral osteoporosis.

3.3. Pinealectomy and hypothalamic-pituitary disconnection

In 2011, Egermann et al. (Egermann et al., 2011) proposed pinealectomy (Px) to induce osteoporosis in sheep, as the melatonin released from pineal gland may influence bone metabolism. It was observed that the bone resorption activity after Px causes non-transient bone loss as evidenced by a continuous decrease in BMD until 30 months. And, in 2012, Beil et al. (2012) proposed hypothalamic-pituitary disconnection (HPD) associated to OVX in sheep, which developed severe osteoporosis due to low bone turnover, characteristic of senile osteoporosis in humans (Oheim et al., 2013). The rationale for this technique was the fact that the hypothalamus and intact hypothalamo-pituitary axis are of critical importance in regulating bone remodeling, as demonstrated by the osteopenia-induced intracerebroventricular application of leptin in ewe (Pogoda et al., 2006; Oheim et al., 2012b). Oheim et al. (Oheim et al., 2017) demonstrated in the OVX + HPD sheep model the importance of peripheral hormones – 17β-estradiol and thyroxin/L-T4, for a balanced bone remodeling and a physiological bone turnover.

3.4. Dietary-induced osteoporosis

As alternative options to induce osteoporosis in sheep, MacLeay et al. (2004; 2010) suggested a compensated dietary-induced metabolic acidosis (DIMA)—a dietary model for human high-turnover osteoporosis, which significantly decreased the lumbar BMD and arterial pH, and increased the fractional excretions of Ca and P in mature OVX ewes. The susceptibility of sheep to DIMA suggests this species as a possible animal model to study the influence of diet on the development of osteoporosis. Ding et al. (2010) proposed an osteoporotic sheep model induced just by the administration of glucocorticoids, associated to restricted diet with low Ca and P. In this study, a biomaterial was assessed for spinal fusion and the osteoporotic inductive conditions maintained for 7 months after surgery. At the term of the experiment, a significant decrease of cancellous bone volume fraction, trabecular thickness, and bone strength in lumbar vertebra was observed comparable with those observed in humans after long-term glucocorticoid treatment.

4. Other important aspects to consider in small ruminant models of osteoporosis

Although the vast majority of studies have concluded that the ovine model is feasible, the isolated OVX in sheep generally do not promote skeletal alterations on the magnitude of the ones observed in women. The reestablishment of bone parameters within 6 months post-OVX indicates that the isolated OVX sheep model is inappropriate to mimic human osteoporosis, being in fact partly possibly justified by the sheep metabolism capacity to synthesize estrogen extragonadally in the adipose tissue and via conversion of adrenal-derived C19 steroids (Sigrist et al., 2007). It should be noted that small ruminants (sheep and goat) also exhibit significant differences in their metabolism, namely concerning the gastrointestinal system with a completely different diet, reproductive and endocrine systems relatively to the human species. This marked physiological specificity could also influence hard tissue composition and micro-structural properties. Sheep and goats are small ruminants (polygastric stomach), herbivores, and seasonally polyestrous, beginning at a 10-month period of estrous cycling when daylight hours diminish in the fall (Jainudeen et al., 2000). So, another explanation that reinforces the observations of Sigrist et al. (2007) is the hypothesis that sheep are more sensitive to the effect of extragonadal estrogen, since the average ovine exposure to estradiol is very low in sheep in comparison with humans (Fabre-Nys et al., 2007; Reinwald and Burr, 2011). As a polygastric animal, the microbial fermentation that occurs in the sheep rumen could potentially convert ingested food compounds, therapeutic drugs, or food containing large percentages of bioactive principles, such as fodder rich in phytoestrogens (Wilkinson, 1999) before the absorption by the digestive system altering their potency (Reinwald and Burr, 2011). The conclusion reported by Sigrist et al. (2007) may also explain the necessity that when using just the OVX sheep model, the post-operative period after OVX should be longer than 12 months to trigger osteoporosis (Giavaresi et al., 2001; Wu et al., 2008; Brenann et al., 2011a; Brenann et al., 2012).

5. The goat model

In the goat model, studies concerning the induction of osteoporosis published in English are very limited. In goats, Reinwald and Burr (2011) refer to a decrease of 42.3% from the baseline serum estradiol after 1 month and of 52.39% after 6 months. Leung et al. (Leung et al.,

2001) developed an osteopenic model in this small ruminant by the association of OVX with a restricted Ca diet of 0.5%, during 6 months, which induced a significant deterioration of mechanical properties and BMD decrease of the cancellous bone tissue. These results were confirmed by Siu et al. (2004) and Tam et al. (2009). Yu et al. (2015) studied the long-term effects of OVX on the properties of bone in goats concluding that after 24 months, OVX goats are a reproducible animal model for osteoporosis.

6. Preclinical and translational studies using the osteoporotic small ruminant models

6.1. Studies performed in the sheep model

In clinical orthopedics, total joint replacements, vertebral augmentation, and spinal fusions are routinely performed with their instrumentation success greatly affected by the presence of osteoporosis (Aldini et al., 2002). Alternative methods have been widely developed to enhance fixation strength in osteoporotic bones although there is no one gold standard method (Goldhahn et al., 2006; Li et al., 2013). For those reasons, the in vivo investigations on biomaterial and surgical techniques in the sheep model of osteoporosis are mostly focused on the study of efficacy of vertebral augmentation in fragility compression fractures by percutaneous vertebroplasty (PVP) or balloon kyphoplasty (BKP), and implant fixation for spinal fusion (spondylodesis or spondylosyndesis), or for long bone fragility fracture treatment, which generally require joint replacements for their surgical resolution, namely the hip fractures.

6.1.1. Vertebral augmentation

Presently, the treatment of fragile vertebral fractures by PVP or BKP normally is performed by injecting bone cement into the body vertebrae (Galovich et al., 2011; Li et al., 2013). Many of the preclinical and translational studies in the osteoporotic sheep model for vertebral augmentation intend to test alternatives to the bioinert, supraphysiologically stiff polymethylmetacrylate (PMMA) cement, which leads to possible complications (difficulty in removing, risk of thermal injury promoted by the exothermic reactions, leakage, and compression), requiring caution in its use (Table III.1). Alternative biomaterials to the use of

PMMA cement have been tested such as biocements, in combination with osteogenic mesenchymal stem cells (MSCs) and/or osteoinductive growth factors (GFs). Galovich et al. (2011) reported that Ca phosphate (CaP) biocement intravertebral bodies inserted via percutaneous injection was not effective in osteoporotic sheep. However, recently, Maenz et al. (2017) proved that a poly-L-glycolic acid (PLGA)-reinforced CaP cement was highly biocompatible and its PLGA fiber component enhanced bone formation, improved the mechanical properties of brittle CaP cement, with potential applicability in load-bearing areas, representing a suitable alternative to the PMMA cement. Moreover, PLGA-reinforced CaP cement with bone morphogenetic protein (BMP)-2 [104], the GDF5 (also called BMP-14) (Bungartz et al., 2017) or the biologically optimized GDF5 mutant BB-1 form (Gunnella et al., 2017), significantly enhanced bone formation induced by PLGA fiber-reinforced CaP cement in the lumbar osteopenic bone tissue in sheep. Another biocement, the calcium sulfate (CS) cement, when injected through a lumbar midline approach significantly improve the amount, density, and biochemical performance of the bone trabeculae in osteoporotic vertebrae, decreasing the potential risk of fracture (Liu et al., 2016). Association between CaP cement with bisphosphonates (BPs) had a beneficial impact on the microarchitecture of the adjacent trabecular bone with good promise as a local approach for the prevention of fragile vertebral fractures (Verron et al., 2014). Eschler et al. (2015) studied the application of expansive mesh cages and its treatment by intrabody application of eptotermin α, verifying that increased mineralization occurred, without correlation to biomechanical properties. In another study, a cementless method like titanium mesh implants fixation was tested to avoid complications associated with cements (Eschler et al., 2016), with concern over the use of GFs in vertebral augmentation procedures, and in view to improve bone tissue quality of vertebral bodies, Philips et al. (2006) used BMP- 7, associated to PLGA biospheres or carboxymethylcellulose. This solution promoted the increase of mechanical strength and histomorphometric parameters which led to inconsistent change in BMD. Wu et al. (2011) tested the recombinant human BMP-2, associated to a fibrin sealant, with increased strength and reducing the fracture risk, as alternatives to current treatment modalities. Though BMP-2 has been the best substitute for autogenous bone grafts, supraphysiological dosing of BMP-2 presents adverse effects in humans not seen in animal models. Those effects are inflammation, soft tissue swelling, and ectopic bone formation (Zara et al., 2011). As a consequence, James et al. (2016) tested a new osteoinductive factor that possesses both pro-osteogenic and anti-osteoclastic properties, preventing bone resorption by decreasing osteoclast number and increasing osteoblast

number—for example, using the Nel-like molecule-1, onto hydroxyapatite (HA)-coated β-tricalcium phosphate (TCP), observing an absence of adverse effects.

Table III.1 – Overview of published studies performed in the sheep as surgical model for osteoporosis research listed by chronological order.

Reference	Animal model (duration)	Type of study	Aims	Study lenght	Results / conclusion
Aldini et al. (2002) [description of animal model by Fini et al. (2000)]	OVX (24 months)	Efficacy of implant fixation for spinal fusion	To evaluate the osteointegration of HA-coated stainless steel pedicle screws implanted in the osteoporotic bone of L3-L5 vertebral pedicles	4 months post-operatively	Long-term ovariectomy sheep it's an adequate model to study in vivo osteointegration in the osteoporotic spine; HA coating improved bone purchase and bone-screw interface strength
Fini et al. (2002)	OVX (24 months)	Efficacy of implant fixation	To evaluate the osteointegration of titanium alloy Ti6AI4V screw implanted in osteopenic tibial diaphysis	12 weeks post-operatively	Bone formation around Ti6AI4V was not associated with complete bone maturation, even in healthy animals; in the osteoporotic bone tissue, both bone formation and maturation were delayed
Rocca et al. (2002)	OVX (24 months)	Efficacy of implant fixation	To compare osteointegration of HA-coated and uncoated titanium screws in osteoporotic cortical bone of femoral and tibial diaphysis	12 weeks post-operatively	The biochemical and histomorphological results achieved suggest employing HA-coated screws in the presence on osteopenic cortical bone
Fini et al. (2003)	OVX (24 months)	Efficacy of implant fixation for spinal fusion	To evaluate the osteointegration of HA-coated stainless steel screws in the osteopenic lumbar vertebral pedicle	4 months post-operatively	HA coating significantly enhances bone-implant contact in osteopenic bone
Lill et al. (2003)	OVX + diet + glucocorticoid (7 months)	Efficacy of implant fixation for long bone fractures	Assessment of fracture healing in a non-CSD long bone in osteoporotic bone using a standardized 3 mm gap transverse mid-shaft tibial osteotomy fixed with an external fixator	90 days after corticoid medication has stopped; 0, 4, and 8 weeks post-operatively	Results show a delay of fracture healing in osteoporotic bone with respect to callus formation, mineralization and mechanical properties
Sachse et al. (2005)	Aged animals (8 – 12 years, with significant radiologic signs of osteoporosis and adipocytic bone marrow)	Efficacy of implant fixation on long bone	To evaluate the osteointegration of HA-titanium implants coated with nonglycosylated rhBMP-2 (0 to 380 µg) below aged-compromised bone tissue of tibial plateaus	20 weeks post-operatively	The application of nonglycosylated BMP-2 coated on solid implants could foster bone healing and regeneration even in aged-compromised bone tissue

Egermann et al. (2006)	OVX + diet + glucocorticoid (6 months)	Fracture healing process improvement	Assessment of the role ovine osteoblasts and MSCs transduced with a recombinant adenovirus carrying BMP-2 cDNA (Ad.BMP-2) in tibia fracture osteotomies	8 weeks post-operatively	Direct, local adenoviral delivery of an osteogenic gene enhanced healing of the osteotomy site in osteoporotic sheep model
Goldhahn et al. (2006)	OVX + diet + glucocorticoid + movement restriction (6 months)	Efficacy of implant fixation for spinal fusion	Osteointegration assessment of hollow perforated cylinder spinal implant coated with Bonit® (HA/brushite 1.67/1.1) in osteoporotic vertebrae (ventral corpectomy, replacement with iliac strut, and fixation with testing implant)	2 months after corticoid medication has stopped; 16 weeks post-operatively	Adequate fixation, with adequate anchorage to promote vertebral fusion in this animal model, even in the osteoporotic bone tissue
Phillips et al. (2006)	OVX + diet (6 months)	Vertebral augmentation	Assessment of systemic treatment with BMP-7 (osteogenic protein 1 – OP-1) (0 to 370 mg) and different carriers – PLGA biospheres with different release kinetics (allowing or not sustained BMP release) or carboxymethylcellulose inserted in an 8 mm Ø defect in the mid intra-vertebral body implantation at two nonadjacent levels	6 months post-operatively	OP-1 improve mechanical properties but without significant BMD changes in the osteoporotic vertebrae
Borsari et al. (2007a,b)	Aged animals (9±1 years) / OVX (24 months)	Efficacy of implant fixation on long bones	To evaluate osteointegration rate of titanium (TI01) and duplex titanium plus HA (HT01) coating systems with high surface roughness and of sandblasted titanium implants in osteoporotic tibial diaphysis and epiphyses	3 months post-operatively	The performance of TI01 and HT01 surfaces might be useful for aged and osteoporotic patients; bone quality affected the biological response of bone to sandblasted titanium implants in both trabecular and cortical bone
Stadelmann et al. (2008)	OXV (6 months)	Efficacy of implant fixation	To evaluate the positive effect in titanium alloy (TA6V) cylinder periprosthetic bone remodelling of local BP zoledronate (2.1 μg / each implant) delivery in osteoporotic femoral condyle	4 weeks post-operatively	The results support that zoledronate could increase the fixation of an implant in weak bone tissue
Verron et al. (2010)	OVX (6 months)	Bone augmentation	Assessment of the implantation of the BPs (zoledronate) load CaP biocement within proximal osteoporotic femurs (into the femoral head and neck to achieve bone augmentation in the proximal femur)	3 months post-operatively	The implantation of the BPs-loaded biomaterial led to a significant increase I relative bone content and an improvement of proximal femur micro-architecture
Wan et al. (2010)	OVX + diet (12 months)	Efficacy of implant fixation for spinal fusion	To evaluate the implant fixation of an expansive pedicle screw in osteoporotic L1-L5 lumbar spine	6 months post-operatively (with continuity	The expandable pedicle screw improved biochemical and histological properties over the standard screw in the osteoporotic spine

				of low calcium diet)	
Galovich et al. (2011)	OVX + diet + glucocorticoid (7 months)	Vertebral augmentation	Compare percutaneously injected CaP biocement (Calcibon®, Biomet, Varsaw, Indiana, USA) and PMMA cement (Osteopal®V, Biomet, Varsaw, Indiana, USA) in osteoporotic vertebral bone	7 days, 3 and 6 months and 1 year post-operatively	Percutaneous injection of CaP biocement is not predictable and not recommended its use presently as a substitute for PMMA cement in vertebral reinforcement procedures
Giavaresi et al. (2011) [description of the animal model by Giavaresi et al. (2001)]	OVX (18 months)	Efficacy of implant fixation for spinal fusion	To evaluate the anchorage of pedicle screws, for fixation of the Dynesys® System, with different surface treatments – rough blasted (uncoated) and bioactive coated (bioactive), in osteoporotic L2-L5 vertebral bodies	4 months post-operatively	Both surface treatments of pedicular screws provided advantage in terms of bone tissue quality and osteointegration when implanted in osteoporotic vertebrae
Liu et al. (2011)	OVX (12 months)	Efficacy of implant fixation for spinal fusion	To investigate the long-term biochemical performance of augmentation of pedicle screws with CS cement in osteoporotic bone and evaluated the screw-bone interfacial bonding	3 months post-operatively	CS cement significantly improved screw-bone interfacial bonding and strengthen the long-term stability of pedicle screws in osteoporotic bone
Wu et al. (2011)	OVX + diet (12 months)	Vertebral augmentation	Assessment of local (injected into the assigned vertebra transpedicularly) treatment effect of rhBMP-2 with FS in osteoporotic L3-L6 vertebrae	3 months after treatment	Local administration of rhBMP-2/FS improved BMD, microarchitectural parameters and mechanical strength of osteoporotic vertebra, increasing bone strength and decreasing fracture risk
Shi et al. (2012a)	OVX (12 months)	Efficacy of implant fixation for spinal fusion	To evaluate if a low elastic modulus expansive pedicle screws could further improve fixation strength compared to the standard expansive pedicle screws in osteoporotic lumbar L1-L5 vertebrae	6 months post-operatively	The screw with low elastic modulus matching with surrounding bone was helpful to distribute stress uniformly, relieve the stress shielding effect and strengthen the screw-bone interface
Shi et al. (2012b)	OVX + diet (12 months)	Efficacy of implant fixation for spinal fusion	To evaluate the improvement fixation of pedicle screw by microarc oxidation treatment in osteoporotic L2-L5 lumbar spine	3 months post-operatively	The pedicle screws with bioactive surface treated by microarc oxidations improved screw fixation strength in osteoporotic bone tissue in spine
Bindl et al. (2013)	OVX + HPD (6 months)	Fracture healing evaluation	Assessment of metaphyseal fracture healing in distal femoral condyle in HPD sheep including a treatment group receiving thyroxine T4 and 17β-estradiol to elucidate potential pathophysiology mechanism	8 weeks after fracture osteotomy	Fractures healing requires central regulation and thyroxine T4 and 17β-estradiol contribute to complex pathophysiology mechanism of delayed metaphyseal bone healing in HPD sheep

Li et al. (2013)	OVX + glucocorticoid (12 months)	Efficacy of implant fixation for spinal fusion	Assessment of biomechanical stability of pedicle screws augmented by BG and PMMA cement in osteoporotic bone and histologically observe the bone-screw interface	3 and 6 months post-operatively	BG can be useful to improve bone microstructure of the interface bone-implant in osteoporotic bone and increase the hold strength of the pedicle screw
Liu et al. (2013)	OVX + glucocorticoid (10 months)	Efficacy of implant fixation for spinal fusion	To compare expansive pedicle screw and PMMA-augmented pedicle screw in osteoporotic lumbar vertebrae	6 and 12 weeks post-operatively	Expansive pedicle screw markedly enhanced screw stability in initial surgery in osteoporotic bone, with better biological interface between implant-bone and having no risk of thermal injury, leakage and compression
Xiao et al. (2013)	OVX (12 months)	Efficacy of implant fixation for dental implants	To investigate the osteointegration of an expansive implant placed in mandibles	12 weeks post-operatively	The implant stability quotient values, maximal pull-out forces and bone-implant contact and micro-CT bone tissue parameters also improved significantly with the expansive implant
Verron et al. (2014)	OVX (6 months)	Vertebral augmentation	Assessment of an optimized CaP cement to promote new bone formation and locally deliver in situ BP alendronate directly at the implantation site in osteopenic bone defect in vertebral augmentation, through a percutaneous two level lateral corpectomy on L3 and L4 vertebrae	3 months post-operatively	BPs-containing apatitic cement shows good promise as a local approach for the prevention of fragility vertebral fractures
Eschler et al. (2015a) [description of animal model by Eschler et al. (2015b)]	OVX + diet + glucocorticoid (5.5 months)	Efficacy of implant fixation for vertebral augmentation	Development standardized creation of lumbar vertebral compression fractures type AO A3.1 and consecutive fracture reduction and fixation using expansive mesh cages and its treatment by intra-body application of eptotermin α	4 weeks after corticoid medication has stopped; 2 months post-operatively	The model is reproducible and workable; use of eptotermin α increased mineralization with association with cementless augmentation via an expansive cage; higher BMD did not lead to superior biomechanical properties
Eschler et al. (2016)	OVX + diet + glucocorticoid (5.5 months)	Efficacy of implant fixation for vertebral augmentation	Assessment of standardized vertebral compression fracture model type AO A3.1 fixed using intra-vertebral cementless titanium mesh implants	4 weeks after corticoid medication has stopped; 2 months post-operatively	Titanium mesh implant shows to be efficient solution already applied in clinical practice, allowing to avoid cement implants
James et al. (2016)	OVX + diet + glucocorticoid (4 months)	Vertebral augmentation	Use of rhNELL-1 protein lyophilized onto β-TCP mixed with HA treatment (in comparison with HyA with HA-coated β-TCP) to increase	8 weeks after corticoid medication has stopped; 3	The pro-osteogenic and anti-osteoclastic properties of the osteoinductive factor rhNELL-1 improved cortical and cancellous bone regeneration in lumbar vertebrae

			bone formation in 2 mm Ø created osteoporotic lumbar vertebral body defect	months post-operatively	
Liu et al. (2016)	OVX + glucocorticoid (12 months)	Vertebral augmentation	Assessment of CS cement treatment, by injection into the vertebral body transpedicularly in osteoporotic L1-L4 lumbar vertebrae	3 months post-operatively	Lumbar injection of CS significantly improve the amount, density and biochemical performance of the bone trabeculae in osteoporotic vertebrae, decreasing the potential risk of fracture
Andreasen et al. (2017)	OVX + glucocorticoid (7 months)	Efficacy of implant fixation	To evaluate the efficacy of HA/β-TCP granules coated with HyA or PDLLA on titanium alloys implant fixations when applied as graft materials in 2 mm size defects created in the osteoporotic femur condyles	12 weeks post-operatively	Bone substitutes infiltrated with PDLLA and HyA proof to possess osteoconductive properties comparable to allograft, being considered valuable as ne coating materials for composite ceramics even in osteoporotic bone tissue
Bungartz et al. (2017) Gunnella et al. (2017a,b) Maenz et al. (2017) [description of animal model by Bungartz et al. (2016)]	Aged, osteopenic animals (6 – 9 years)	Vertebral augmentation	To test the in vivo biocompatibility and osteogenic potential of a PLGA fiber-reinforced bioabsorbable, brushite-forming CaP cement, alone or associated to BMP-2, GDF5 or GDF5 mutant BB-1 (1, 5, 100 and 500 µg) in L4 and L5 lumbar osteopenic vertebrae	3 and 9 months post-operatively	PLGA-reinforced CaP cement was highly biocompatible and its PLGA fiber component enhanced bone formation, improved the mechanical properties of brittle CaP cement, with potential applicability in load-bearing areas, representing a suitable alternative to the PMMA cement; BMP-2, GDF5 and BB-1 significantly enhanced the bone formation induced by PLGA fiber-reinforced CaP cement in lumbar osteopenic bone tissue

β-TCP – β-tricalcium phosphate; BG – bioactive glass; BMD – bone mineral density; BMP – bone morphogenetic protein; BPs – bisphosphonates; CaP cement – calcium phosphate cement; CS cement – calcium sulfate cement; CSD – critical size defect; CT – computed tomography; diet – calcium/phosphorus and/or vitamin D-restriction; FS – fibrin sealant; HA – hydroxyapatite; HPD – hypothalamo-pituitary disconnection; HyA – hyaluronic acid; MSCs – mesenchymal stem cells; NNEL-1 – Nel-like molecule-1; OVX – ovariectomy; PDLLA – poly-$_{D,L}$ -lactic acid; PLGA – poly-L-glycolic acid; PMMA – polymethylmetacrylate cement; rh – recombinant human

6.1.2. Spinal fusion

Spinal fusion is a neuro-orthopedic surgical technique that promotes the union of two or more vertebrae at the cervical, thoracic, or lumbar levels, preventing the movement between the fused vertebrae and stabilizing the spine length and anatomy. This surgical intervention is frequently performed to relieve the pain and pressure on the spinal cord that results when the disc wears out in degenerative disc disease. This surgery is also performed to treat the clinical cases of spinal stenosis, spondylolisthesis, spondylosis, scoliosis, kyphosis, and spinal fractures. There are several spinal fusion procedures that require bone grafting techniques or the use of bone substitutes, associated to osteosynthesis implants, such as screws, plates, or cages, to support the vertebral segmental structure in place during the fusion healing process between vertebrae. With regard to the spinal fusion techniques by the transpedicular screw system in the osteoporotic vertebrae, patients with osteoporosis can be affected by screw loosening or failure in clinical situations. These situations may be motivated by incorrect pedicle screw fixation and/or mechanical overload of the stabilized spine (Liu et al., 2013). The osteoporotic sheep models are also frequently used in view of the improvement of this technique (Table II.1). Referring to the used methods, one can mention: the application of HA-coated screws (Aldini et al., 2002; Fini et al., 2003), the employment of a hollow cylinder-based implant (Goldhahn et al., 2006), expansive pedicle screw fixation (Wan et al., 2010; Liu et al., 2013), the use of expansive pedicle screws with different surface treatments—rough blasted and bioactive coated (Giavaresi et al., 2011), pedicle screws associated to CS cement (Liu et al., 2011), low elastic modulus expansive pedicle screw (Shi et al., 2012a) or subjected to micro-arc oxidation treatment (Shi et al., 2012b) or augmented by bioactive glass (Li et al., 2013), and also as surgical options to substitute PMMA cements.

6.1.3. Improvement of the fragile fracture healing process and bone defect repair

Another type of research that studies have been focused on is the improvement of the fracture-healing process and repair of bone defect in the osteoporotic skeleton. Fracture healing is a complex biological process of bone regeneration, and once associated with osteoporosis, corresponds to an increase of mechanical failure of the implant fixation due

to the delay in the mineralization process, callus formation, and decrease of local mechanical properties (Lill et al., 2003). Hence, in the last decade, studies have also been developed in the osteoporotic sheep model to improve the efficacy of implant fixation on bone diaphysis and epiphyses for fracture fixation in osteoporotic long bones (Fini et al., 2002; Rocca et al., 2002; Lill et al., 2003; Sachse et al., 2005; Borsari et al., 2007a; Borsari et al., 2007b; Stadelmann et al., 2008), bone augmentation for clinical cases of proximal fragile fractures of the osteoporotic femur (Verron et al., 2010), and dental implant fixation in the osteoporotic mandibles (Xiao et al., 2013) (Table II.1). For studies aimed at improving the bone-healing process in patients with osteoporosis, Egermann et al. (2006) studied the role of ovine osteoblasts and MSCs transduced with a recombinant adenovirus carrying BMP-2 cDNA in tibia osteotomies verifying that the osteogenic gene enhanced healing at the osteotomy site in an osteoporotic sheep model. Bindl et al. (2013) studied the metaphyseal fracture during the healing process in the distal femoral condyles in HPD sheep. The present study allows the elucidation of the pathophysiology of fracture in the mechanism of healing the process, concluding that thyroxine T4 and 17β-estradiol substitution considerably improved bone-healing process. This requires central regulation involved in the complex pathophysiology of delayed metaphyseal bone healing in HPD sheep.

6.1.4. New pharmacological treatment assessment

There are also a few studies published in the scientific literature regarding the evaluation of new pharmacological drugs for the treatment of osteoporosis, particularly post-menopause, which are tested in sheep models of osteoporosis. In this field, we should mention the study of Chavassieux et al. (2001) that evaluates through analytical biochemistry and bone tissue histomorphometric and densitometric methods, the effects of a new selective estrogen receptor modulator (MDL 103,323) on cancellous and cortical bone in OVX ewes. Thomas et al. (1995) investigated the effect of salmon calcitonin on immobilization-induced osteoporosis in the sheep, concluding that it was ineffective in preventing disuse osteoporosis. However, later Jiang et al. (2005) in OVX ewes also used salmon calcitonin, evaluating the trabecular bone microstructure by magnetic resonance imaging and observing a prevention of structural deterioration of bone tissue by this treatment. Brenan et al. (2011b; 2011c; 2014) studied in the OVX ovine model, after a

long post-operative period of 31 months of estrogen deficiency, the effects of the BP zoledronate on bone mechanical and biological properties, concluding that zoledronic acid treatment may contribute to reduce fracture occurrence during osteoporosis. Burket et al. (2013) performed a study in an ovine model of osteoporosis obtained through compensated DIMA in sheep, to compare the BP zoledronate and raloxifene to examine their effects on bulk tissue properties, nanoscale tissue composition, and mechanical properties within bone trabeculae, concluding the efficacy of these anti-resorptive drugs with improvement in trabecular bending stiffness and decreased fracture risk.

6.1.5. Studies performed in the goat model

Regarding the use of a goat as an animal model of osteoporosis for use in later studies on the efficacy of implant fixation on osteoporotic bone, the fracture-healing process improvement and for tissue engineering studies on osteoporotic bone-defect repair, these are very rare with respect to sheep and only recently have some of these studies emerged in the scientific literature (Table III.2). Leung et al. (2006), in view of the efficacy of implant fixation and bone augmentation, evaluated in the osteopenic goat the bone healing and remodeling around thread screws surrounded with an optimized CaP cement. In the osteoporotic goat model, Cao et al. (2012) evaluated the use of autologous bone marrow MSCs associated to β-TCP for the repair of bone critical size defects in the osteoporotic medial femoral condyles, and Alt et al. (2016) tested a new nanocrystalline HA associated to collagen type I injected in osteoporotic metaphyseal bone defects, both therapies proving to be effective in the treatment of bone defects in osteoporotic bone tissue. Yu et al. (2014) studied the effect of long-term estrogen deficiency in the ovariectomized goat, verifying the diffuse micro-damage and linear cracks accumulated up to day 21 and then gradually repaired at 4 and 8 months after surgery in cortical bone of the tibial diaphysis around screw implantation, concluding that the estrogen depletion decreased bone tissue quality with increasing probability of screws to induce mechanical damage that could compromise the initial implant stability. Tam et al. (2009) applied at 9 months an extracorporeal shockwave-stimulation treatment at the level of the calcaneus, distal radius, and femoral condyle in an established osteoporotic goat model, verifying the induction of new bone formation in weight-bearing sites, with trabecular BMD of the treated calcaneus increasing significantly by 2.90%by shockwave. Li et al. (Li et al.,

2009) evaluated the effects of strontium (Sr) (24 and 40 mg kg−1 day−1) and Ca (100 mg kg−1 day−1) in the osteopenic OVX goat, verifying that Sr–Ca administration increased the osteogenic expression of induced bone formation.

7. Conclusions

Most of the preclinical and translational studies in small ruminant models of osteoporosis have been performed by the induction of osteoporosis through isolated OVX sheep 12 months post-operatively or long before its use as a surgical model for biomaterial research, bone augmentation, efficacy of implant fixation, fragile fracture-healing process improvement, or bone-defect repair studies or by the combined treatment of OVX, Ca/vitamin D-deficient diet, and glucocorticoid applications for 6 months in sheep. Other authors selected other effective methods to induce osteoporosis for preclinical and translational studies in sheep, like OVX associated to Ca/ vitamin D-restricted diet, OVX associated to glucocorticoid application, and OVX associated to HPD, and in some studies also, simply the aged osteopenic sheep model was selected. The goat model for osteoporosis research has been used in a very limited number of studies using specially the OVX model after a post-operative period of 24 months. In 2015, Andreasen et al. (2015) concluded that glucocorticoid-treated OVX aged sheep induced a significant bone loss, promoted by an arrest of the reversal phase, resulting in an uncoupling of bone formation and resorption during the reversal phase, as recently demonstrated in postmenopausal women with glucocorticoid-induced osteoporosis (Jensen et al., 2011; Andersen et al., 2013), supporting the importance of this large animal model for the study of the pathophysiology of this disorder and as a preclinical model for orthopedic implant and biomaterial research. Another very recent study elucidates the osteocyte regulation of receptor activator of NF-κB ligand/ osteoprotegerin (RANKL/OPG) in a sheep model of osteoporosis, concluding that in the late progressive phase of the osteoporosis induced by steroids, the RANKL expression is stimulated in osteocytes (El Kahassawna et al., 2017).With the increase in the knowledge of the pathophysiological underlying mechanisms involved in the induction of osteoporosis in the small ruminants, the studies in these animal models should be carried out with greater confidence in obtaining transposable results for humans.

Table III.2 – Overview of published studies performed in the goat as surgical model for osteoporosis research listed by chronological order.

Reference	Animal model (duration)	Type of study	Aims	Study lenght	Results / conclusion
Leung et al. (2005) [description of the animal model by Leung et al. (2001)]	OVX + diet (6 months)	Efficacy of implant fixation and bone augmentation	To evaluate the bone healing and remodelling around threads screws with an optimized CaP cement histologically at the femoral condyles and the mechanical strength of the screw augmented with bone cement by screw-pull-out tests	1 week, 3 and 6 months post-operatively	Histological analyses demonstrated a tightly-coupled bone apposition on the cement surface and the cement increased significantly the initial screw pull-out force and the energy required to failure; the cement improved the prevention of interfacial micromotions and subsequent fibrous tissue formation at the implant-bone interface resulting in a decreased risk of implant failure
Cao et al. (2012)	OVX (24 months)	Bone defect repair	To evaluate combining autologous bone marrow MSCs with porous β-TCP for repair of a bone CSD in the osteoporotic medial femoral condyle	16 weeks post-operatively	Autologous enriched bone marrow MSCs therapy proved to be a good option to treat osteoporotic bone defects since bone formation as occurred
Yu et al. (2014)	OVX (28 months)	Efficacy of implant fixation and bone repair	To assess the effect of a long-term estrogen deficiency on the creation and repair of microdamage in tibial diaphyseal cortical bone adjacent to titanium screw implantation	0 and 21 days, 4 and 8 months post-operatively	Estrogen depletion decreases bone quality and makes it vulnerable to screw-induced mechanical damage, which could compromise the initial stability of an orthopaedic implant
Alt et al. (2016)	OVX (24 months)	Bone defect repair	To evaluate a new treatment with nanocrystalline HA or without collagen-type I injected in osteoporotic metaphyseal bone defects (L3-L5 vertebrae, iliac crest, distal femur)	42 days post-operatively	Nanoparticulate HA with and without collagen type-1 demonstrated good new bone formation and biocompatibility

β-TCP – β-tricalcium phosphate; CaP cement – calcium phosphate cement; CSD – critical size defect; diet – calcium/phosphorus and/or vitamin D-restriction; HA – hydroxyapatite; MSCs – mesenchymal stem cells; OVX – ovariectomy

8. References

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Chapter IV - Bone Turnover Markers in Sheep and Goat: A Review of the Scientific Literature

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Bone turnover markers in sheep and goat: A review of the scientific literature

1. Introduction

In the last decades, small ruminants - sheep and goats - have been widely accepted as animal models in orthopaedic research (O'Loughlin et al., 2008; Reichert et al., 2009) especially due to their low cost, availability, acceptance as an experimental model, facility of handling and housing (Turner 2007a), compliance, and docility (Newman et al., 1995).

The suitability of small ruminants as animal models for orthopaedic research results mainly from having the most similar body weight and long bones with dimensions compatible with application of implants and prostheses developed for humans (Newman et al. 1995; Anderson et al., 1999; van der Donk et al., 2001). In this manner, compared with other species used in orthopaedic research, sheep and goats have an adequate body weight and long bones, with a macrostructure more similar to humans (Newman et al., 1995), despite the bone microstructure of small ruminants being less similar to humans than other animal models such as dogs (Pearce et al., 2007). Sheep have a predominance of plexiform bone until 3 to 4 years of age (Newman et al., 1995) due to fast growth in weight and size (Reinwald and Burr, 2011) and just a predominance of secondary Haversian systems after 7 to 9 years of age with the presence of bone remodelling (Newman et al., 1995). Sheep also presents a trabecular bone density, mineralization and subsequently elevated strength relative to humans, that are variable according to skeletal location (Nafei et al., 2000; Liebschner 2004), nevertheless the bone mineral composition being apparently similar between small ruminants and humans (Ravaglioli et al., 1996).

Despite these macro- and micro-structural differences in bone tissue, studies with small ruminants used as animal models in orthopaedic research have increased considerably (Pearce et al., 2007), and more recently they have also been used for studying bone turnover markers (BTMs) (Sousa et al., 2014a). The BTMs are proteins which indicate bone metabolism (Sousa et al., 2014b), and are generally divided into collagenous bone formation markers, bone resorption markers and osteoclast regulatory protein markers (Leeming et al., 2006). Analysis of BTMs might supply information in a fast, effective, sensitive, specific, and low cost manner (Allen, 2003). Nowadays, it is used in human medicine to help evaluate fracture risk, delayed fracture healing and

consolidation process, and development of metabolic bone diseases (Vasikaran et al., 2011).

These similarities in biochemistry, biomechanics, and bone histology make BTMs a resource in sheep and goats for pre-clinical and/or translational orthopaedic research studies and veterinary and animal science studies (Turner, 2007b). Nevertheless, the reported biological variability of BTMs among age, gender, disease, recent fractures, exercise, time (Seibel 2005), diet (Nicodemo et al. 1999; Liesegang and Risteli, 2005; Liesegang et al., 2013), seasonal changes (Arens et al., 2007) and circadian variation (Liesegang et al. 2003), which can contribute substantially to the variability of these parameters (Smith et al., 2011), are their main limitation (Cremers et al., 2008).

Therefore, the aim of this review was to collect the studies published in scientific literature until the present date concerning the use of BTMs in small ruminant research or to investigate the clinical effectiveness of BTMs in pre-clinical or translational experimental orthopaedic research related to human medicine when sheep and goat are used as experimental animal models for this latter purpose.

2. Bone turnover markers

Bone tissue undergoes turnover along the animal lifespan (Seibel, 2006) and that process is divided into two parts: modelling and remodelling (Clarke, 2008).

Modelling is a longitudinal and circumferential growth process due to mechanical and/or physiological influences (Clarke, 2008), with longitudinal growth located at the epiphyseal plates until their fusion uniting the epiphysis and metaphysis through endochondral ossification (Altman et al., 2015). It also allows the adaptation of bone tissue, removing damage and maintaining its strength (Seeman, 2009), and requires that the process of bone formation and resorption are independent from one another regarding time and location (Raggatt and Partridge, 2010). Remodelling is a process of bone replacement where bone formation outpaces bone resorption (Altman et al., 2015), to maintain bone strength and mineral homeostasis, regulated by osteoclasts and osteoblasts that sequentially carry out resorption of old bone and formation of new bone, keeping the new bone healthy (Clarke, 2008). Bone remodelling predominates when bone is reaching maturity (Iglesias et al., 2011), but it does not influence the size and shape, although the internal architecture may have slight changes caused by external forces (Hadjidakis and

Androulakis, 2006). Bone formation and resorption are present in same site, but not at the same time in order to maintain bone mass (Raggatt and Partridge, 2010).

The proteins produced during bone turnover are detectable mainly in serum in bone formation markers, whereas many of the bone resorption markers are detectable in both serum and urine (Allen, 2003), and there are a significant number of commercial kits developed for use in humans that have cross-reactivity with other species, including sheep and goats (Tables IV.1 to IV.3).

Table IV.1 - Bone formation markers, method of analysis and available commercial assay kits.

Marker	Tissue of origin	Sample	Method of analysis	Available commercial Assay kit	Cross-Reactivity Sheep / Goat
BALP	Bone	Serum	Colorimetric	No commercial kit available	NO / NO
			Electrophoretic	No commercial kit available	NO / NO
			Precipitation	No commercial kit available	NO / NO
			CLA	LIAISON BAP Ostase, Stillwater, MN, USA	? / ?
			ELISA	MicroVue BAP, Quidel Corporation, San Diego, CA, USA	YES / YES
			RIA	Tandem-R-Ostase, Beckman Coulter, Brea, CA, USA	YES / ?
OC	Bone	Serum	CLA	LIAISON Osteocalcin, Stillwater, MN	? / ?
			RIA	BTI Human Osteocalcin RIA, Biomedical Technologies Inc, Stoughton, MA	? / ?
			ELISA	MicroVue Osteocalcin, Quidel Corporation, San Diego, CA, USA;	YES / YES
				BTI Intact Osteocalcin, Biomedical Technologies Inc, Stoughton, MA, USA;	? / ?
				Osteocalcin, SIGMA, Saint Louis, Missouri, USA;	? / ?
				Osteocalcin, GenWay Biotech, San Diego, CA, USA	NO / NO
PINP	Bone and Soft Tissue	Serum	CLA	PINP Roche Diagnostics, Penzberg, Germany	? / ?

			RIA	UniQ Intact PINP, Orion Corporation, Espoo, Finland	? / ?
		Serum or Urine	ELISA	PINP, Neobiolab Inc, Cambridge MA, UK	YES / YES
PICP	Bone and soft tissue	Serum	RIA	PICP, Orion Corporation, Espoo, Finland; PICP DiaSorin., Stillwater, MN, USA	? / ? YES / ?
			ELISA	MicroVue CICP, Quidel Corporation, San Diego, CA, USA;	YES / ?
		Serum or Urine		PICP, Neobiolab Inc, Cambridge MA, UK	YES / YES

BALP: Bone specific alkaline phosphatase; OC: Osteocalcin; PINP: Amino-terminal procollagen propeptides of collagen type I; PICP: Carboxy-terminal procollagen propeptides of collagen type I; RIA: Radioimmunoassay; ELISA: Enzyme-linked immunosorbant assay; CLA: Chemiluminescence immunoassay; YES: presence of cross-reactivity; NO: absence of crossreactivity; ?: no data available.

Table IV.2 - Bone resorption markers, method of analysis and available commercial assay kits.

Marker	**Tissue of origin**	**Sample**	**Method of analysis**	**Available commercial Assay kit**	**Cross-Reactivity Sheep / Goat**
HYP	Bone, soft tissue, cartilage	Serum or Urine	ELISA	HYP, Neobiolab Inc, Cambridge MA, UK	YES / YES
		Urine	Colorimetric HPLC	No commercial kit available	NO / NO
DPD	Bone, dentin	Urine	ELISA	MicroVue DPD, Quidel Corporation, San Diego, CA; USA	YES / ?
			HLPC	No commercial kit available	NO / NO
		Serum or Urine	ELISA	MicroVue tDPD, Quidel Corporation, San Diego; USA	YES / ?
				DPD, Neobiolab Inc, Cambridge MA, UK	YES / YES
PYD	Bone, cartilage, blood vessels	Urine	HPLC	No commercial kit available	NO / NO
			ELISA	MicroVue Serum PYD, Quidel Corporation, San Diego, CA; USA	YES / ?
			RIA	No commercial kit available	NO / NO
		Serum or Urine	ELISA	PYD, Neobiolab Inc, Cambridge MA, UK	YES / YES

ICTP	Bone, skin	Serum	Colorimetric	No commercial kit available	NO / NO
			RIA	ICTP, Incstar Corporation, Stillwatter, MN, USA;	? / ?
				UniQ ICTP, Orion Corporation, Espoo, Finland;	? / ?
				ICTP DiaSorin, Stillwatter, MN, USA	NO / NO
			ELISA	UniQ ICTP EIA, Orion Corporation, Espoo, Finland;	? / ?
		Serum or Urine		ICTP, Neobiolab Inc, Cambridge MA, UK	YES / YES
CTX	Bone	Serum	CLA	β-Crosslaps Roche Diagnostics Penzberg, Germany	? / ?
			RIA	No commercial kit available	NO / NO
			ELISA	Serum CrossLaps, Biointernational, Yvette, France	YES / ?
		Urine	CLA	β-CrossLaps Roche Diagnostics Penzberg, Germany	? / ?
			RIA	CrossLaps RIA, Osteometer Biotech, Herlev, Denmark	? / ?
			ELISA	CrossLaps, Osteometer Biotech, Herlev, Denmark	YES / YES
		Serum or Urine	ELISA	CTX, Neobiolab Inc, Cambridge MA, UK	YES / YES
NTX	Bone	Serum	RIA	No commercial kit available	NO / NO
			ELISA	Osteomark Ostex International Inc., Seattle, WA, USA; NTX MyBioSourse, San Diego, CA, USA	YES / ?
		Urine	RIA	No commercial kit available	NO / NO
		Serum or Urine	ELISA	NTX, Neobiolab Inc, Cambridge MA, UK	? / YES
Cathepsin k	Bone	Serum	ELISA	Cathepsin k ELISA Kit, Antibodies-online, Atlanta, Georgia, USA	? / ?
TRAP	Bone	Serum	RIA	No commercial kit available	NO / NO
			ELISA	MicroVue TRAP 5b, Quidel Corporation, SanDiego, CA, USA;	YES / YES
				Osteolink-TRAP b, Nitto Boseki Corporation, Tokio, Japan;	? / ?

		BoneTRAP SBA, Science, Boldon, UK	? / ?
	Serum or Urine	TRAP, Neobiolab Inc, Cambridge MA, UK	YES / YES

HYP: Hydroxyproline; DPD: Deoxypyridinoline; PYD: Pyridinoline; ICTP: Carboxy-terminal telopeptide of type I collagen; CTx: cross-linked C-terminal telopeptides of type I collagen; NTx: cross- linked N-terminal telopeptides of type I collagen; TRAP 5b: Tartrate-resistant acid phosphatase isoenzyme 5b; RIA: Radioimmunoassay; ELISA: Enzyme-linked immunosorbant assay; CLA: Chemiluminescence immunoassay; HPLC: High-performance liquid chromatography

Table IV.3 - Osteoclast regulatory proteins, method of analysis and available commercial assay kits.

Marker	Tissue of origin	Sample	Method of analysis	Available commercial Assay kit	Cross-Reactivity Sheep / Goat
RANKL	Bone and blood	Serum	ELISA	Human Serum RANKL Free ELISA Kit, Biomedica Medizinprodukte, GmbH & Co. KG, Wien, Austria;	? /?
				RANKL, Immundiagnostik AG, Bensheim, Germany	? /?
		Serum or Urine		RANKL, Neobiolab Inc, Cambridge MA, UK	YES / YES
RANK	Bone	Serum	ELISA	RANK R&D Systems, Minneapolis, MN	? / ?
OPG	Bone	Serum	ELISA	Human Osteoprotegerin ELISA kit, BioVendo Laboratory Medicine, Inc., Labogen, Czech Republic;	NO / NO
				Osteoprotegerin, Immundiagnostik AG, Bensheim, Germany;	? / ?
				Osteoprotegerin R&D Systems, Minneapolis, MN, USA	? / ?
		Serum or Urine		OPG, Neobiolab Inc, Cambridge MA, UK	YES / YES

RANKL: receptor activator of nuclear factor NF-κB ligand; RANK: receptor activator of nuclear factor NF-κB; OPG: osteoprotegerin; ELISA: Enzyme-linked immunosorbant assay.

During the process of bone formation by osteoblasts, formation markers are represented by serum total (ALP) and the bone-specific isoform of alkaline phosphatase (BALP), serum osteocalcin (OC) and two molecules which are released during the type I collagen molecule synthesis - serum procollagen type I carboxy- and amino-terminal propeptides (PICP and PINP, respectively) (Seibel, 2002). In the bone resorption process there is a breakdown of type I collagen, so resorption markers are represented by serum C-terminal telopeptide of type I collagen (serum ICTP), urinary collagen type I cross-linked C- and

N-telopeptide (CTx and NTx), urinary hydroxyproline (HYP), total and free urinary pyridinoline and deoxypyridinoline (PYD and DPD) and also by serum tartrate-resistant acid phosphatase (TRAP) as an enzyme produced by osteoclasts during their bone resorption activity (Seibel 2002) (Figure IV.1).

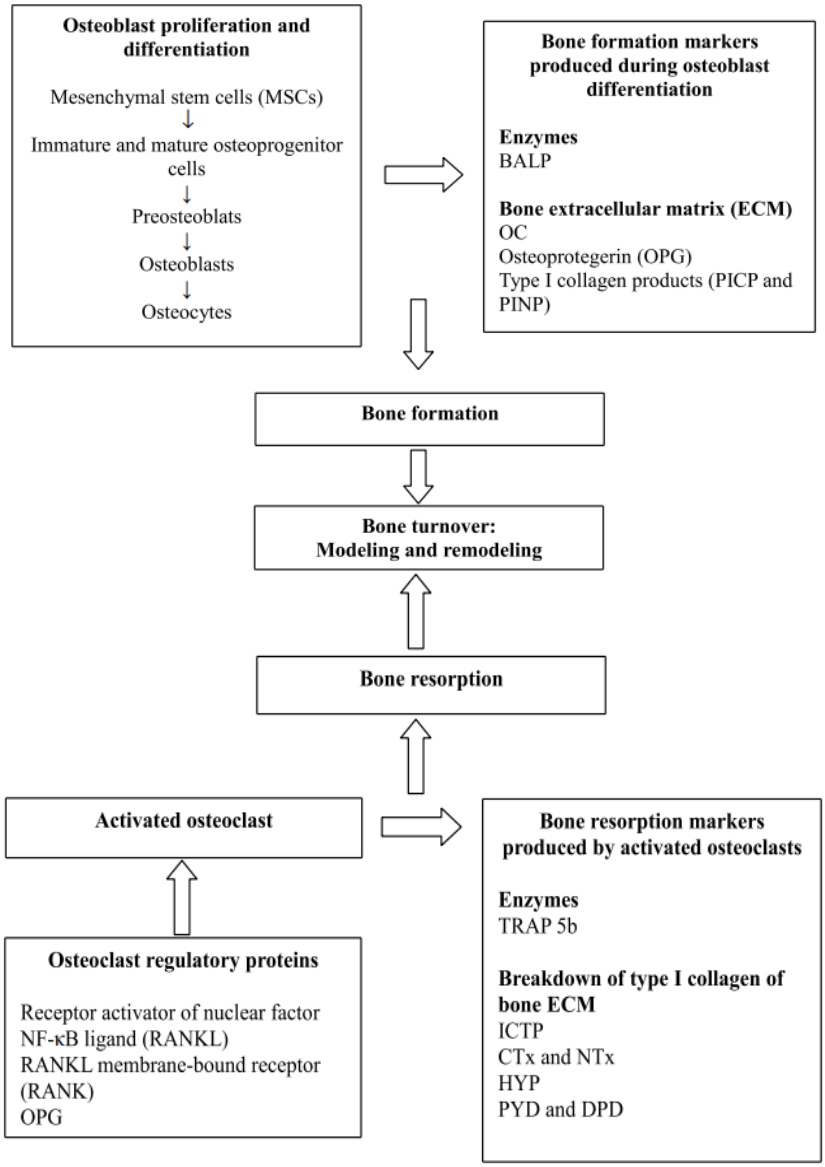

Figure IV.1 - Flow diagram of BTMs produced during the bone turnover process.

2.1. Bone formation markers

2.1.1. Alkaline phosphatase

Alkaline phosphatase (ALP) is a glycoprotein that is connected to the extracellular surface of cells and is synthesized in a variety of tissues, such as intestines, placenta, and germ cells (Millan, 2006). Animals have four isoforms of ALP – bone-specific ALP (BALP), intestinal ALP, liver ALP, and in dogs also the corticosteroid-induced ALP. This variation would render difficult the interpretation of possible variations of the ALP isoenzymes (Allen, 2003). Bone ALP has been used due to its high sensitivity as bone formation marker (Seibel, 2006). It is produced by osteoblasts (Millan, 2006) and is involved in the calcification of bone matrix (Masrour and Mahjoub, 2012) through the hydrolysis of phosphate esters on the osteoblast cell surface, resulting in a high extracellular inorganic phosphate concentration (Whyte, 1994).

2.1.2. Osteocalcin

Osteocalcin (OC) is synthesized by mature osteoblasts, odontoblasts, and hypertrophic chondrocytes and it is vitamin K dependent protein. It has three residues of the calcium-binding amino acid, γ - carboxyglutamic acid (Gla). Its function is poorly understood, although it is primarily deposited in the bone extracellular matrix (ECM), with a small amount present in the blood stream (Cremers et al., 2008). Serum OC is a marker of osteoblastic activity and its serum level thus reflects the rate of bone formation (Seebeck et al. 2005), influences bone mineralization by binding calcium and consequently hydroxyapatite (Neve et al., 2013).

2.1.3. Pro-collagen type I propeptides

Collagen type I is produced by osteoblasts in the last stage of new bone formation (Allen, 2003). The procollagen undergoes enzymatic cleavage producing the C- and N-terminal procollagen type I extension peptides (PICP and PINP, respectively), both extension are cleared by the liver and may be added to the bone ECM (Watts, 1999).

Nevertheless, type I collagen does not depend exclusively on the bone tissue turnover because it is also a component of other soft tissues as fibro-cartilage, tendon, skin, gum, intestine, heart valve, large vessels, and muscle. However, as the metabolism of type I collagen is faster in the bone tissue than in other tissues, changes in type I collagen are considered representative of bone collagen synthesis (Cremers et al., 2008).

It is suggested that PINP is useful in early detection of non union processes with potential for study of the fracture healing process (Coulibaly et al., 2010), although in humans it is unknown whether there exists a correspondence between PINP and the progression of fracture healing (Moghaddam et al., 2011).

2.2. Bone resorption markers

2.2.1. Deoxypyrinoline and pyridinoline

The collagen fibrils recently deposited in bone ECM are stabilized by intra- and intermolecular cross links helping to build the mature collagen molecule (Cepelak and Cvorišcec, 2009).

The pyridinium cross links – deoxypyridinoline (DPD) and pyridinoline (PYD) are formed during extracellular maturation of fibrillar collagens (Gerrits et al., 1995). The PYD is found in bone and cartilage tissues and ligaments (Watts, 1999) while DPD is found in bone and dentin (Delmas et al., 2000), so in the bloodstream PYD is generally more abundant (Cremers et al., 2008), although DPD is more specific as a resorption marker for bone tissue (Seibel et al., 1992). In a study with sheep after ovariectomy, this animal model demonstrated relevance as a model for osteoporosis due to the values of PYD and OC found (Newton et al., 2004).

2.2.2. Carboxy-terminal telopeptide of collagen type I and amino-terminal telopeptide of collagen type I

The N-terminal (NTx) and C-terminal telopeptide of collagen type I (CTx) are fragments of the type I collagen molecule composed by a short peptide sequence from the non-helical domain of this molecule (Chubb, 2012), attached by a pyridinium crosslink (Allen et al. 2000). Both markers are sensitive and reliable indicators of the

bone resorption process (Cremers et al., 2008) and final products of the metabolism of bone ECM, amino acids, and free or peptide-bound PYD or DPD (Allen et al. 2000).

The CTx is not specific as a resorption marker for bone tissue since it is identified not only in bone, but also in skin, dentine, and tendon, and these peptide fragments could also be derived from other types of collagen (Chubb, 2012). However, CTx could be used for monitoring the bone healing process because it was detected that variations in its levels corresponded to bone resorption in an experimental fracture healing study performed in dogs where two different osteosynthesis techniques were used (Paskalev and Krastev, 2010).

2.2.3. Carboxy-terminal telopeptide of type I collagen – matrix metalloproteinase

Cleavage of the type I collagen molecule by the matrix metalloproteinases (MMP) results in the formation of cross-linked C-terminal telopeptide of type I collagen (CTX-MMP or ICTP) (Cremers et al., 2008), suitable to represent osteoclastic activity (Allen et al., 2000).

The ICTP is an indicator for mobilization of bone tissue around parturition and at the beginning of lactation in sheep and goats (Liesegang et al., 2007). In dogs with osteosarcoma (Hintermeister et al., 2008) and horses during physical training, this marker has not revealed itself suitable for determining bone resorption since it did not show correlation with other resorption markers, however it was an indicator of the rate of bone turnover (Price et al., 1995).

2.2.4. Tartrate-resistant acid phosphatase

Tartrate-resistant acid phosphatase (TRAP) is a bone resorption marker, but not originated from the degradation of type I collagen (Hannon et al., 2004). It is a glycoprotein produced by osteoclasts, activated macrophages, and dendritic cells (Leeming et al., 2006). There is an isoenzyme 5, from a total of 6 isoenzymes of the acid phosphate identified by electrophoresis, which through protease cleavage presents two isoforms (a, b) – the TRAP 5a is sialylated and TRAP 5b is produced by osteoclasts, and the latter proposed to reflect osteoclast activity (Delmas et al., 2000, Leeming et al.,

2006). The TRAP could be a suitable resorption marker for detection of normal or delayed fracture healing process in sheep (Seebeck et al., 2005) or dogs (Sousa et al., 2011).

2.2.5. Cathepsin K

Cathepsin K is part of the cysteine protease family and has the ability to cleave both helical and telopeptide regions of collagen type I (Leeming et al., 2006). This enzymatic cleavage is able to degrade, at low pH, several proteins of the bone ECM, namely the telopeptide and helical regions of the collagen type I molecule, the OC and osteopontin (Cremers et al., 2008). This marker could be used as a tool to measure bone resorption, such as in canine osteosarcoma clinical cases (Schmit et al., 2012).

3. Variability of bone turnover markers

The BTMs could suffer constant variation throughout the lifetime of an individual (Sousa et al. 2014b). However, variation between individuals is also a great cause of oscillation in markers, specifically due to biological variability, together with the analytical variability introduced by the different assay techniques (Vasikaran et al., 2011).

Biological variability can be influenced by many uncontrollable factors (Cremers et al., 2008), such as growth (Sousa et al., 2014a), geographical location (Liesegang et al., 2013), pregnancy and lactation (Liesegang et al., 2006; Liesegang et al., 2007), and controllable factors, such as diet (MacLeay et al., 2004a; MacLeay et al., 2004b; Liesegang et al., 2013), and season of the year (Arens et al., 2007), which can be mitigated in clinical studies (Liesegang, 2008). In short, biological variability is affected by any factor that influences the bone remodelling (Watts, 1999).

Analytical variability has been minimized due to automated platform technology, however, there could be variations in results between different methods (Cremers et al., 2008) and the development of new analytical techniques requires previous validation (Seibel et al., 2001).

The high inter-individual variability of BTMs is their main limitation for clinical use due to the difficulty to establish reference ranges for serum and urinary BTM levels (Souberbielle et al., 1999), although bone markers are an effective tool in clinical studies

due to reliable, fast, non-invasive, and cost effective assays with improved sensitivity and specificity (Wheater et al., 2013).

4. Sample and storage

Blood collection for measuring BTMs must be done at a specific time (morning) to avoid the influence of circadian variations (Klein et al., 2004; Seebeck et al., 2005; Dias et al., 2008; Sousa et al., 2014a; Sousa et al., 2014b). Blood samples can be collected from the cephalic vein (Klein et al., 2004) or jugular vein (Dias et al., 2008; Sousa et al., 2014a; Sousa et al., 2014b) into serological tubes containing no anticoagulant (Vernon et al., 2010), and centrifuged (3000 rpm for 10 min) within 30 min of collection (Liesegang et al., 2007). Urine can be obtained using a special external urine collector (Windhagen et al., 2002) or collected by cystocentesis (Allen et al., 2000). Urine and serum samples should be stored at -20°C for mineral analyses (Chanetsa et al., 2000, Taylor et al., 2009, Sousa et al., 2014a) and at -80°C until determination of BTMs (Seebeck et al., 2005; Tatara, 2008; Sousa et al., 2014b), which provides molecular stability for several months (Lomeo and Bolner, 2000).

5. Animal and veterinary science studies

Characteristics of the animal and veterinary science studies regarding population, type of studies, time, and conclusion (Table IV.4).

Table IV.4 - Characteristics of the animal and veterinary science studies that reported the use of BTMs in different types of research.

Authors	Population	Type of Study	Markers	Time	Conclusion
Chavassieux et al. (1991)	Fourteen ewe	Determine the effects of ossein-hydroxyapatite compound on bone remodeling	Serum OC, Serum Phosphate, Serum Ca, Serum ALP	90 days	Possibly the OHC is able to reduce the effect seasonal about the bone turnover
Wan Zahari et al. (1994)	Ten sheeps	Effects of nutrition on bone growth	Serum 1,25 VITD, Serum ALP and Serum TRAP	6 weeks	Diets rich in phosphate not have effect on skeletal mineralization

Turner et al. (1995)	Thirty ewe	Represent changes in bone mass in ovariectomized ewes	Serum BALP	6 months	This model may be useful for estrogen deficiency studies induced bone loss
Chavassieux et al. (1997)	Thirty-two ewe	Use of glucocorticoid for decrease a bone formation	Serum OC and Serum BALP	7 months	Use of glucocorticoid in ewe may represent valid model for bone loss
Scott et al. (1997)	Twenty-four sheep	Effects of nutrition on bone growth	Serum TRAP, Serum OC, Serum BALP, Urinary PYD, Urinary DPD	90 days	Suggest that markers may be useful for diagnosis and treatment of bone disease and early detection of nutrition deficiencies
Nicodemo et al. (1999)	Twenty-four sheep	Influence of diet in growth bone	Serum OC, Serum BALP, Urinary PYD, Urinary DPD, Serum Ca, and Serum P	11 weeks	Markers are unsuitable for assessment the bone growth by different diets
Chanetsa et al. (2000)	Forty sheeps	Effects of an estrogen agonist on growth and Bone mineral accretion	Serum BALP, Serum TRAP, Serum 1,25 VITD, Serum Ca, Serum Mg and Serum P	163 days	This is study have clinical relevance for treating children with delays in growth and bone mineral accretion
Chavassieux et al. (2001)	Forty ewe	The effects of ovx in ewe associate or not a lentaron and effects of a new selective estrogen receptor modulator	Serum OC, Serum Balp and Urinary CTX	6 months	The ovx induced an increase in bone turnover and MDL may useful for prevention of postmenopausal
Lill et al. (2002a)	Eight sheep	Which method is more effective for induce an osteoporosis		6 months	The most effective method for induce osteoporosis is a combination of diet, ovariectomy and glucocorticoid
Lill et al. (2002b)	Thirty-two sheep	Induce an ovine model for severe osteoporosis		7 months	The model may be useful for with osteoporosis model
Windhagen et al. (2002)	Fourteen sheep	What is response of turnover markers during the distraction osteogenesis	Urinary DPD, Urinary PYD, Serum OC	74 days	Showed a pattern of osteoblast cellular activation during distraction osteogenesis

Liesegang et al., (2003)	Twelve goats and sheep	Determine the diurnal variation in bone markers	Serum BALP, Serum ICTP, Serum CL, Serum OC	2 weeks	During the day there was a variantion in concentration of markers, goats presented a higher bone turnover than sheep
Klein et al. (2004)	Fourteen sheep	Use the bone markers for represent course of callus consolidation during bone healing	Serum PICP, Serum BALP and Serum PIIINP	9 weeks	The markers used not were useful for represent bone healing
MacLeay et al. (2004a)	Fifty-two ewe	Develop an animal model for human postmenopausal osteoporosis		90 days	Model is sensitive a loss bone for acidosis diet
MacLeay et al. (2004b)	Twenty-four sheep	Influence of diet and OVX for bone turnover	Urinary DPD, Serum BALP, Serum Ca, and Serum P	180 days	Model is sensitive a loss bone for acidosis diet
Newton et al. (2004)	Twelve ewe	Effects of ovariectomy on the trabeculae of ovine iliac bone	Serum OC, Urinary PYR,	12 months	The ovine model is adequate for changes in trabecular bone architecture studies
Liesegang and Risteli (2005)	Six sheep and six goat	Influence of diet	Serum ICTP, Serum BALP, Serum OC, Serum Ca, Serum CL and Serum 1,25 Vit D	8 weeks	Due to short time is difficult associated the diet with bone turnover
Seebeck et al. (2005)	Sixteen sheep	Use the bone markers for represent callus formation during fracture healing	Serum PICP, Serum ALP, Serum BALP, Serum PIIINP, Serum Ca and Serum P	9 weeks	The markers used not were useful for represent callus formation
Liesegang et al. (2006)	Twelve goat and sheep	Determine the effects of pregnancy and lactation on markers	Milk and Serum Ca, Serum OC, Serum BALP, Serum CTX, Serum ICTP, and SerumVITD	11 months	Markers showed that occured bone turnover during the gestation and lactation
Arens et al. (2007)	Eight sheep	Measure the seasonal variations in quantity and quality of bone turnover	Serum BALP, Urinary DPD, Serum PYD	18 months	The seasonal variation must be considered when used the ovine model in osteoporosis studies

Liesegang et al. (2007)	Twelve goats and sheep	Determine the effects of a second pregnancy and lactation on markers in comparison with first	Milk and Serum Ca, Serum OC, Serum BALP, Serum CTX, Serum ICTP, and Serum 1,25 VITD	11 months	The bone loss in the second pregnancy and lactation is lower that first, possibly due to an adaptation of the organism
Sigrist et al. (2007)	Fourteen sheep	The effect of ovariectomy on bone metabolism in sheep	Serum BALP, Urinary DPD, Serum PYD	18 months	The ovine model is not an appropriate for human postmenopausal osteoporosis
Dias et al. (2008)	Eighteen ewe	Measurement of bone markers in ewes under controlled environmental factors, and the study of their correlations with the serum minerals	Serum ALP, Serum BALP, Serum OC, Serum Ca, Serum P, Serum Mg and Serum Ca2+	6 weeks	Reference to the serum values of bone turnover parameters in sheep could be of great value, possibly to obtaining an early prognosis of fracture healing.
Goebel et al. (2009)	Eighty-five sheep	Verification FGF23 with possible marker of bone healing and regeneration	Serum ALP, Serum Phosphate, Urinary Phosphate and Serum Ca	42 days	FGF23 is promissory marker for indicate the bone healing
Ding et al. (2010)	Eighteen sheep	Use of glucocorticoid for osteopenia induce in cancellous bone		10 months	This method is useful for induced osteoporosis in sheep
Vernon et al. (2010)	Twenty sheep	Influence of exercise about the degradation of the articular cartilage	Serum LOX and Serum C2C	5 months	Markers were unable to evidence the effects of forced exercise
Tralman et al. (2013)	Seven Sheep	Compare two methods of osteosynthesis in sheep	Serum ALP and Serum OC	10 weeks	Use RTP fixator is more effective than plate fixation in osteotomies of long bones in sheep
Kreipke et al. (2014)	Thirteen sheep	The effect of ovariectomy on vertebral bodies and femoral condyles of sheeps after 1 and 2 years		24 months	The vertebral bodies are preferable for trabecular microarchitecture studies

Sousa et al. (2014a)	Ninety sheep	Measure the values of the bone markers and to evaluate the correlation between them and the serum minerals in sheep of various ages and different physiologic stages	Serum ALP, Serum BALP, Serum Ca, Serum Mg and Serum P	1 day	The Measure of lifespan sheep is useful in preclinical orthopaedic research and provide information complementary for others analyses with imaging
Sousa et al. (2014b)	Eighteen sheep	Assessment the variation of serum leves in short-term	Serum ALP, SerumBALP, Serum OC, Serum PIIINP, Serum DPD, Serum TRAP, Serum Ca and Serum P	12 weeks	The variability in short-term not seems to be a limitation for studies with bone markers
Liesegang et al. (2013)	Twenty-four sheep	Influence of diet	Serum ICTP, Serum BALP, Serum Ca, Serum P, Serum 1,25 Vit D and Serum 25 Vit D	4 months	Alteration the bone turnover associated by diet

5.1. Diet

According to Liesegang and Risteli (2005) Liesegang et al. (2013), MacLeay et al. (2004a, b) and Nicodemo et al. (1999) nutritional studies using BTMs were influenced by different diets, though this influence was not statistically significant. MacLeay et al. (2004a) concluded that during the administration of a diet that induced metabolic acidosis in mature ewes, there were no significant changes in serum BALP and DPD levels. In another study by Liesegang et al. (2013) with sheep grazing at different altitudes, it was not possible to confirm the interference of diet in the serum variation of ICTP or BALP, but high bone turnover was confirmed. Also, in a study by Liesegang and Risteli (2005) where a diet with varying calcium content was used, it was not possible to demonstrate the influence of the diet on bone mineral metabolism in growing goats and sheep, possibly due to the short duration of this study, where only the sheep showed a variation in BMD due to an increase in calcium intake. However, Wilkens et al. (2010) demonstrated that

sheep were a suitable model for studies with varying diets, calcium deficiency, and calcitrol.

5.2. Exercise

Liesegang and Risteli (2005) demonstrated that sheep in pasture at high altitudes had an increase in bone turnover and bone mineral content without clear cause, one possible factor being the increase in exercise. In another study in lambs, Vernon et al. (2010) concluded that the markers used were not adequate to indicate the effects of forced exercise.

5.3. Gestation and lactation

Liesegang et al. (2006) noticed that the interval between parturition and early lactation in sheep and goats required a high nutritional value of calcium due to losses to the fetus and lactation, occurring inefficiency of calcium absorption, leading to increases in bone remodelling to help replace maternal bone loss classified as a physiological mechanism. During a second pregnancy, bone loss was less significant compared with the first pregnancy and the lactation greater, possibly due to the adaptation of the organism (Liesegang et al., 2007). Finally, it was concluded that sheep were more adapted to the loss of calcium in comparison to goats, that had a lower bone mineral density and bone mineral content before parturition (Liesegang and Risteli, 2005) increased bone turnover, resulting in a higher activity of bone metabolism and sensitivity to changes in calcium during pregnancy and lactation (Liesegang et al., 2003).

5.4. Circadian and seasonal variation

Chavassieux et al. (1991) reported that bone remodelling was influenced by the photoperiod, with decrease in bone remodelling occurring between spring and summer. Arens et al. (2007) confirmed that bone mass increases in summer and decreases in winter, so taking seasonal variation into account is fundamental in studies using BTMs. Liesegang et al. (2003) reported an increase in the rate of bone formation during the

evening and night, indicating the influence of the circadian rhythm in bone turnover. Sousa et al. (2014b) concluded that the short-term variability should be considered during interpretation of data, such as circadian and seasonal variations, nevertheless the short-term biological variability do not represent a limitation for the use of BTMs.

5.5. Skeletal growing

Pastoureau et al. (1991) mention that sheep are a good model to study the bone growth in growing lamb. It was reported that goats showed a more accelerated bone remodelling that sheep, which was demonstrated by ICTP, CTx (Liesegang et al., 2003), BALP (Liesegang et al., 2003; Sousa et al., 2014a), and OC determinations in various studies (Pastoureau et al., 1991; Liesegang et al., 2003). Collignon et al. (1996) demonstrated that bone growth since the fetal stage produces alterations in serum OC and BALP, confirming the usefulness of these markers in bone formation and growth. Scott et al. (1997) reported that OC, BALP, DPD, and PYD may be useful for detection of changes in bone growth caused by deficient diets, and Wan Zahari et al. (1994) reported that high phosphorus diets resulted in increased bone resorption (increased TRAP) in lambs. However, Chanetsa et al. (2000) exposed castrated lambs to an oestrogen agonist. In this study, bone growth was observed, but no effect on markers of bone remodelling was noticed.

6. Pre-clinical and translational orthopaedic research studies

The characteristics of the pre-clinical and translational orthopaedic research studies, such as population, type of studies, time, and conclusion are listed in Table (IV.4).

6.1. Fracture healing process

Tralman et al. (2013) and Windhagen et al. (2002) reported that the serum markers of bone formation are useful for reflecting the bone healing process, and Goebel et al. (2009) suggested that FGF23 is a good marker to indicate the healing process. Seebeck et al. (2005) stated that degradation of soft callus can be determined by serum PIIINP

during the bone fracture healing process and Schmidt et al. (2008) concluded that it is possible to monitor the maturation of bone callus with the total ALP and NTx. However, without individual reference values, BTMs become a weak tool to determine the prognosis of bone consolidation (Klein et al., 2004).

6.2. Osteoporosis

Newton et al. (2004) reported that ovariectomized (OVX) ewe were a useful model due to alterations in trabecular bone architecture along with the decrease in oestrogen levels, which resemble women in early menopause and Turner (2001) suggested that old OVX ewes could be a valid model for bone loss due to oestrogen deficiency. Johnson et al. (2002) reported that 6 months after OVX in sheep there was a decrease in alveolar bone BMD which became serious during the next 6 months. However, Sigrist et al. (2007) reported that in sheep, 6 months after the OVX, the markers for formation and resorption returned to baseline, indicating that the model was not appropriate for human postmenopausal osteoporosis. Kreipke et al. (2014) reported that OVX induces the necessary changes in bone microarchitecture for studying osteoporosis, but after a year, the changes in architecture stabilize in ovine. Chavassieux et al. (2001) reported that in goats, remodelling occurred only in the cortical bone tissue regions, which was also demonstrated by increased levels of CTx one month after OVX and OC three months after OVX.

Ding et al. (2010) and Andreasen et al. (2015) stated that the induction by glucocorticoids in sheep is similar to the change in the microstructure of human bone also induced by long-term glucocorticoid treatment, therefore being a useful model. MacLeay et al. (2004b) though not knowing what the true mechanism is involved in diets that induce metabolic acidosis in bone loss, concluded that the sheep model is useful for studies of osteoporosis induced by diet.

Therefore, small ruminant models are important for the study of human osteoporosis (Chavassieux et al., 1997; Lill et al., 2002a; Lill et al., 2002b; Andreasen et al., 2015; Kielbowicz et al., 2015, Kielbowicz et al., 2016) induced by OVX and with attention to continuous treatment with glucocorticoids to maintain the osteoporotic bone condition (Ding et al., 2010).

7. Conclusions

The suitability of the determination of BTMs in small ruminants is already confirmed in numerous animal and veterinary sciences studies and also in preclinical and/or translational studies in orthopaedic research, in addition to imaging, mechanical, histological and histomorphometric analyses. Their advantage relies on a fast and non-invasive assessment via biochemical analysis of serum or urine samples, although the referred negative aspect of using BTMs in the clinical setting is related with their high biological variability. Particularly in sheep, BTMs have been used to estimate the extent of the osteogenic response at a local level at the fracture healing site, as precocious indicators of possible bone healing disturbances. BTMs could provide important information concerning bone metabolism at a systemic level, namely about bone remodelling process during induction of osteoporosis and its treatment in experimental orthopaedic studies. Recently it was developed a study by Baharuddin et al. (2014) in sheep with osteoclast regulatory protein – receptor activator of nuclear factor NF-κB ligand (RANKL) produced by osteocytes, osteoblasts and immune system cells, its membrane-bound receptor (RANK) in the osteoclast precursor cells and osteoprotegerin (OPG) as new potential bone markers in future (Sousa et al., 2015), nevertheless more studies would be necessary to assess the usefulness of BTMs in this scientific field.

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Chapter V – Tartrate-Resistant Acid Phosphate as Biomarker of Bone Turnover Over the Lifespan and Different Physiologic Stages in Sheep

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Tartrate-Resistant Acid Phosphate as Biomarker of Bone Turnover Over the Lifespan and Different Physiologic Stages in Sheep

1. Introduction

Currently, skeletal and bone tissue assessment is performed by diagnostic imaging such as radiography, computed tomography, magnetic resonance and densitometry techniques, or invasive methods, such as bone biopsy, although these methods are still limited in many situations. Therefore, determination of the serum levels or urinary bone turnover biomarkers (BTMs) could be a good noninvasive analytical method for evaluation of bone metabolism, as it allows predicting bone cell activity in a quick and real time manner (Allen, 2003).

At present, some studies have been performed on BTMs in animal models for orthopedic research not only to improve the knowledge in animal and veterinary sciences, but also in human orthopedic research of metabolic bone diseases, such as post-menopausal osteoporosis. BTMs could be useful in diagnosing early-stage bone diseases, and helping to monitor evolution and efficiency of treatment.

BTMs are generally divided in two groups: the formation and the bone resorption markers, although there is a third group that is still poorly studied, the osteoclast regulatory protein (Baharuddin et al., 2015). During the metabolic process of bone formation by osteoblasts, formation markers are represented by serum alkaline phosphatase (ALP) and its bone-specific isoform (BALP), serum osteocalcin, and two other molecules that are released during synthesis of the type I collagen molecule – serum procollagen type I carboxy- and amino-terminal propeptides (Dias et al., 2008). The ALP is a glycoprotein that is connected to the surface of cells. In humans ALP are expressed in four gene loci code: nonspecific, intestinal, placental and germ cells (Cremers et al., 2008). Nonspecific gene is synthesized in a variety of tissues (bone, kidney, liver and early placenta) (Cremers et al., 2008). BALP has been used due to its high sensitivity as a bone formation marker (Cremers et al., 2008). It is produced by osteoblasts (Millan, 2006) and is involved in the calcification of bone matrix (Masrour and Mahjoub, 2012).

In the bone resorption process, there is a breakdown of type I collagen, so resorption markers are represented by serum C-terminal telopeptide of type I collagen, urinary collagen type I cross-linked C- and N-telopeptide, urinary hydroxyproline, total and free urinary pyridinoline and deoxypyridinoline, as well as by the serum

tartrateresistant acid phosphatase (TRAP), which is produced by active osteoclasts (Seibel, 2002). TRAP is a glycoprotein produced by mature osteoclasts, activated dendritic cells, and macrophages, therefore TRAP is an indicator of osteoclast and macrophage activity (Leeming et al., 2006; Schleicher et al., 2013). There are two known isoforms (TRAP5a,b). TRAP5b is a specific biomarker of osteoclastic resorption activity, while TRAP5a is a non-osteoclastic form (Hallen et al., 2000). In the future, the latter may be useful in clinical evaluation since it is expressed in bone pathologies, such as fracture healing in different mammals or osteoporosis in women (Seebeck et al., 2005; Leeming et al., 2006; Sousa et al., 2011).

The use of sheep as animal models in orthopedic research, also in studies focusing on BTMs, is based on different aspects such as easy handling (O'Loughlin et al., 2008; Reichert et al., 2009) and possibility of blood sample collection several times per day (Liesegang et al., 2003) due to a high blood volume when compared to other laboratory animal species. Nevertheless, bone markers in small ruminants are influenced by circadian and seasonal variation (Arens et al., 2007), among other possible factors causing serum and urinary variability such as diet, exercise, skeletal growth, as well as intra-individual variability (Cremers et al., 2008). As such, BTMs undergo variations throughout a lifetime, namely with age and different metabolic states. It is therefore important to assess BTMs variation during these phases and establish a reference range for BTMs in this species, to which the greatest limitation is the inter-individual variability (Souberbielle et al., 1999).

Therefore, the aim of this study was to generate a reference range for some of the main BTMs – ALP and TRAP, throughout the lifespan and different physiological states in sheep and to evaluate possible correlations of these parameters with serum minerals.

2. Material and Methods

2.1. Animals

Ninety ewes (Churra-da-Terra-Quente sheep) from the same flock, located in Carrazeda de Ansiães, a municipality in the district of Bragança in northern Portugal were used. Average minimum temperature was 1.9 °C and average maximum temperature 10.2 °C in December at Bragança. Sheep were kept in a natural pasture during the day and

housed overnight. The barn is easily approachable and spacious, dry, well-drained, well-ventilated and bedding composed by hay and straw. These animals were chosen among a flock according to their age or physiologic state and divided into 9 groups of 10 animals each. The groups were as follows: 1 month old (mean weight 9.3 kg), 6 months old (mean weight 22.2 kg), 1 year old (mean weight 40.7 kg), 2 years old (mean weight 50.2 kg), 3 to 5 years old dry (mean weight 52.4 kg), 3 to 5 years old with 2 or 3 months of pregnancy (mean weight 55.3 kg), 3 to 5 years old with 2 or 3 months of lactation (mean weight 51.5 kg), 6 to 8 years old (mean weight 52.1 kg), and the last group with animals over 8 years old (mean weight 48.1 kg). The diet was composed by grass hay, supplemented with 0.250 kg of concentrate feed per animal per day and water provided ad libitum. Dry matter and chemical composition of grass hay is made up of dry matter per kg feed (88.5 g), ash per kg dry matter (5.9 g), neutral detergent fiber per kg (73.3 g) dry matter and crude protein per kg dry matter (6.1 g). Dry matter and chemical composition of feed concentrate is made up of dry matter per kg feed (90.4 g), ash per kg dry matter (8.5 g), neutral detergent fiber per kg (31.6 g) dry matter and crude protein per kg dry matter (20.7 g).

All animal handling practices followed Directive 2010/ 63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes.

2.2. Blood sampling

Blood was drawn in December during the European winter. Blood samples were drawn from the jugular vein and placed into serological tubes (S-Monovette®, SARSTEDT, Nümbrecht, Germany). Samplings were performed between 9:00 a.m. and 10:00 a.m. and the blood carried in a thermal box to laboratory facilities immediately. Blood was centrifuged (3000 rpm for 10 min) and the serum stored in Eppendorf tubes at −20°C until analyses.

2.3. Serum biochemical analysis

Following the manufacturer's instructions, the assays were always performed in duplicate to achieve higher accuracy. Determination of ALP (Alkaline Phosphatase, Beckman Coulter, Ref. OSR6004, CA, USA) and TRAP (ACP, Ref. 17,617; Sentinel Diagnostics, Milan, Italy) were performed via an enzymatic method and molecular absorption spectrophotometry using commercially available kits. Calcium (Ca) (Calcium,

Beckman Coulter, Ref. OSR60117, CA, USA), phosphorus (P) (Phosphorus, Beckman Coulter, Ref. OSR6122, CA, USA) and magnesium (Mg) (Magnesium, Beckman Coulter, Ref. OSR6189, CA, USA) were also determined using commercially available kits, via chemical method and molecular absorption spectrophotometry.

2.4. Statistical analysis

Statistical normality was checked using the Shapiro-Wilk W-Test for all groups. Serum BTMs and mineral values are presented as median ± interquartile range (IQR), minimum (Min) and maximum (Max). Spearman's correlation was obtained between the serum biochemical markers. The Kruskal - Wallis Test was used for testing the nonparametric statistical hypothesis and the Kruskal – Wallis pairwise method for multiple comparisons. Statistical analysis was performed using SPSS software (version 23.0, SPSS, Inc., IBM Company, NY, USA). The level of significance was set at $p<0.05$.

3. Results

All parameters in this study revealed a non-normal distribution for age analysis. The median and interquartile range for each marker in different ages are shown in Table V.1.

Table V.1 - Values of serum biochemical markers and serum minerals by ages.

		Median±IQR	Range (Min–Max)
ALP (U/L)	1 month	849.5±270.75	520-1354
	6 months	269.5±136.25	89-454
	1 year	220±135	138-583
	2 years	200.5±90.25	116-295
	3-5 years gestation	104.5±116	66-350
	3-5 years lactation	330.5±159.25	105-390
	3-5 years dry	181±133.5	108-363
	6-8 years	197±127	63-444
	>8 years	119±177	35-277
TRAP (U/L)	1 month	3.15±0.45	2.9-3.7
	6 months	2.65±0.1	2.5-2.9
	1 year	3.1±0.5	2.4-3.6
	2 years	2.95±0.6	2.2-3.2
	3-5 years gestation	2.8±0.25	2.6-3.0

	3-5 years lactation	2.55±0.37	2.3-2.8
	3-5 years dry	2.8±0.25	2.04-3.3
	6-8 years	2.5±0.07	2.4-2.7
	>8 years	2.65±0.47	1.6-3.3
Calcium (mmol/L)	1 month	2.83±0.12	2.57-2.9
	6 months	2.59±0.23	2.35-2.75
	1 year	2.63±0.1	2.55-2.83
	2 years	2.53±0.2	2.33-2.68
	3-5 years gestation	2.49±0.16	2.18-2.68
	3-5 years lactation	2.49±0.18	2.38-2.65
	3-5 years dry	2.54±0.13	2.35-2.65
	6-8 years	2.61±0.10	2.5-2.68
	>8 years	2.33±0.21	1.85-2.48
Magnesium (mmol/L)	1 month	0.91±0.07	0.78-1.03
	6 months	0.96±0.04	0.96-1.04
	1 year	1.02±0.14	0.92-1.29
	2 years	1.04±0.09	0.95-1.12
	3-5 years gestation	1.08±0.17	0.92-1.29
	3-5 years lactation	1.03±0.03	1.02-1.08
	3-5 years dry	0.97±0.07	0.93-1.13
	6-8 years	1.05±0.13	0.89-1.32
	>8 years	1.12±0.26	0.88-1.56
Phosphorous (mmol/L)	1 month	3.21±0.27	3.1-3.52
	6 months	2.21±0.53	1.06-2.87
	1 year	1.87±0.46	1.58-2.29
	2 years	1.59±0.25	1.23-1.81
	3-5 years gestation	1.42±0.63	1.13-1.98
	3-5 years lactation	1.45±0.30	1.16-1.81
	3-5 years dry	1.36±0.24	0.9-1.9
	6-8 years	1.56±0.36	1.1-2.06
	>8 years	1.64±0.05	1.06-2.42

ALP: alkaline phosphatase; TRAP: tartrate-resistant acid phosphatase; IQR: interquartile range; Min: minimum; Max: maximum.

Figure 1a shows a significant difference between the 1 month old group and all the other groups, and a significant difference between animals of 6 months of age and over 8 years for ALP (Figure V.1a). For TRAP, the 1 month old and 1 year old groups were the ones with a significant statistical difference from the other groups (Figure V.1b).

Calcium and phosphorus both suffer a slight decrease throughout the animal's life, with a statistical difference seen in the 1 month old and over 8 years old groups for Ca (Figure V.1c), and 6 months old and 1 month old groups for P (Figure V.1d), with the latter showing a significant difference when compared to all other groups except the 6 months old group. Magnesium had a slight increase with age (Figure V.1e), with the most

significant difference observed between the 1 month old group and the other groups. The degrees of correlation are shown in Table V.2. The results expressed a fair correlation between markers, with the highest correlation observed between ALP and P ($r<0.60$; $p<0.01$).

In the analyses of the groups with animals between 3 and 5 years of age in different physiologic stages, ALP had a significant difference between the gestation and lactation groups (Figure V.2a) and TRAP (Figure V.2b) had a significantdifference between the dry and lactation groups.

Normality analyses of the groups with 3 to 5 years of age had a normal distribution for Ca and P (Figure V.2c and V.2d), ALP, TRAP and Mg (Figure V.2e) had a non-normal distribution. Correlation between the three groups did not reveal a statistically significant difference.

Table V.2 - Correlation between serum biochemical markers and serum minerals.

	ALP	TRAP	Ca	P	Mg
ALP	-	r = 0.44**	r = 0.514**	r = 0.581**	r = 0.229
TRAP	-	-	r = 0.379**	r = 0.261*	r = -0.066
Ca	-	-	-	r = 0.492**	r = -0.322**
P	-	-	-	-	r = -0.289*
Mg	-	-	-	-	-

*Correlation coefficient is significant at the 0.05 level.
**Correlation coefficient is significant at the 0.01 level.

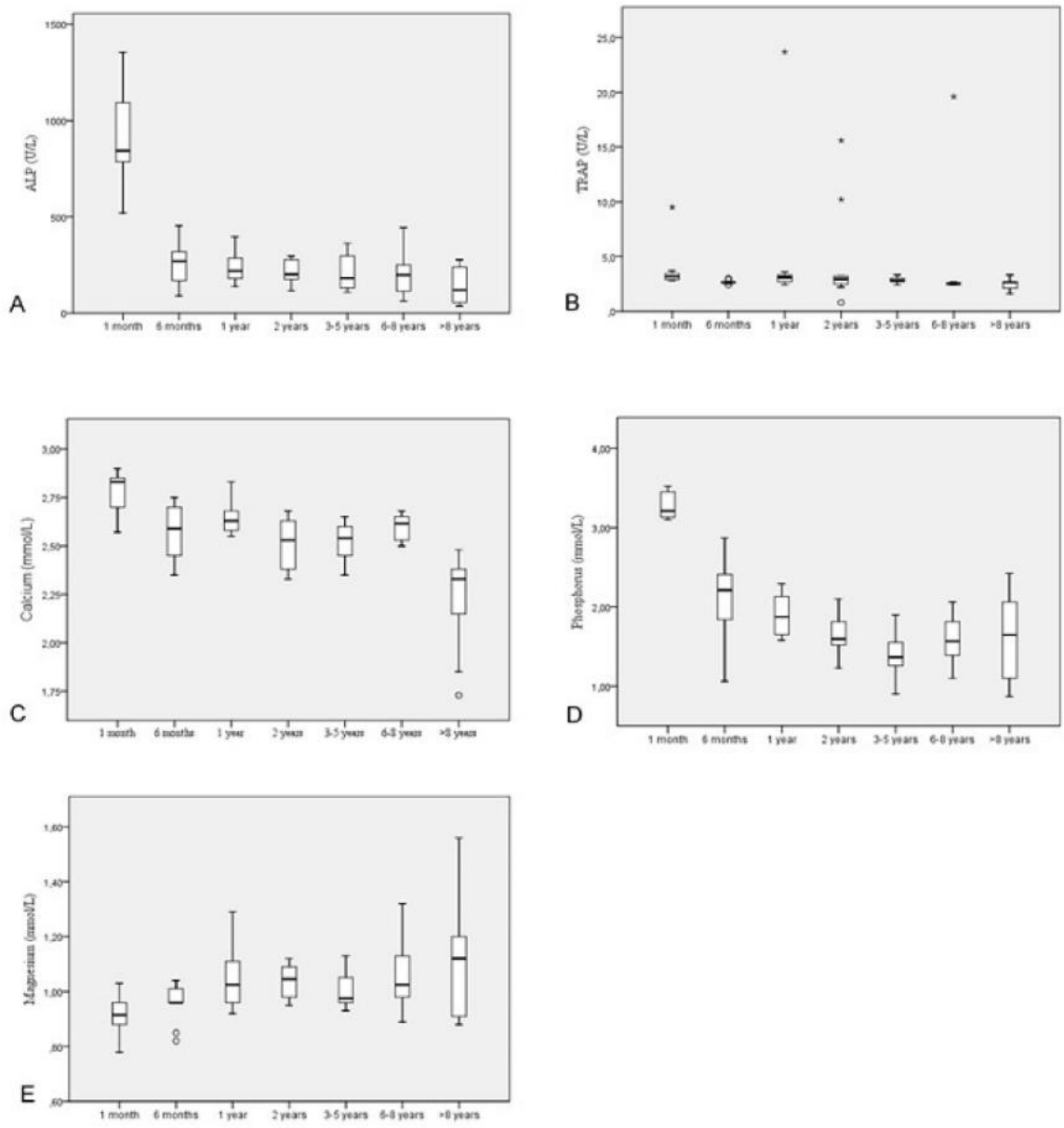

Figure V.1 - Box plot presentations of serum concentrations of biomarkers of bone metabolism by age. (A) Serum ALP activity presented significant difference between 1 month vs. 6 months (P<0.01), between 1month vs. 1 year, 2 years, 3-5 years, 6-8 years and >8 years (p<0.001), between 6 months vs. >8 years (p<0.05). (B) Serum TRAP activity presented significant difference between 1 month vs. 6 months, >8 years (p<0.01), between 1 month vs. 6-8 years (p<0.001), between 1 year vs. 6 months, >8 years (p<0.05), between 1 year vs. 6-8 years (p<0.01), between 2 years vs. 6-8 years (p<0.05). (C) Serum calcium activity presented significant difference between 1 month vs. 6 months, 6-8 years (p<0.05), between 1month vs. 2 years and 3-5 years (p<0.01), between 1 month vs. >8 years (p<0.001), between >8 years vs. 2 years and 3-5 years (p<0.05), between >8 years vs. 6 month and 6-8 years (p<0.01) and between >8 years vs. 1 year (p<0.001). (D) Serum ALP activity presented significant difference between 1 month vs. 6 months (p<0.05), between 1 month vs. 1 year (p<0.01), between 1 month vs. 2 years, 3-5 years, 6-8 years and >8 years (p<0.001), between 6 months vs. 2 years, 3-5 years, 6-8 years and >8 years (p<0.05) and between 1 year vs. 3-5 year (p<0.05). (E) Serum Magnesium activity presented significant difference between 1 month vs. 3-5 years (p<0.05) and between 1month vs. 1 year, 2 years, 6-8 years and >8 years (p<0.01). Outliers are identified with small circle for out values and star for extreme values.

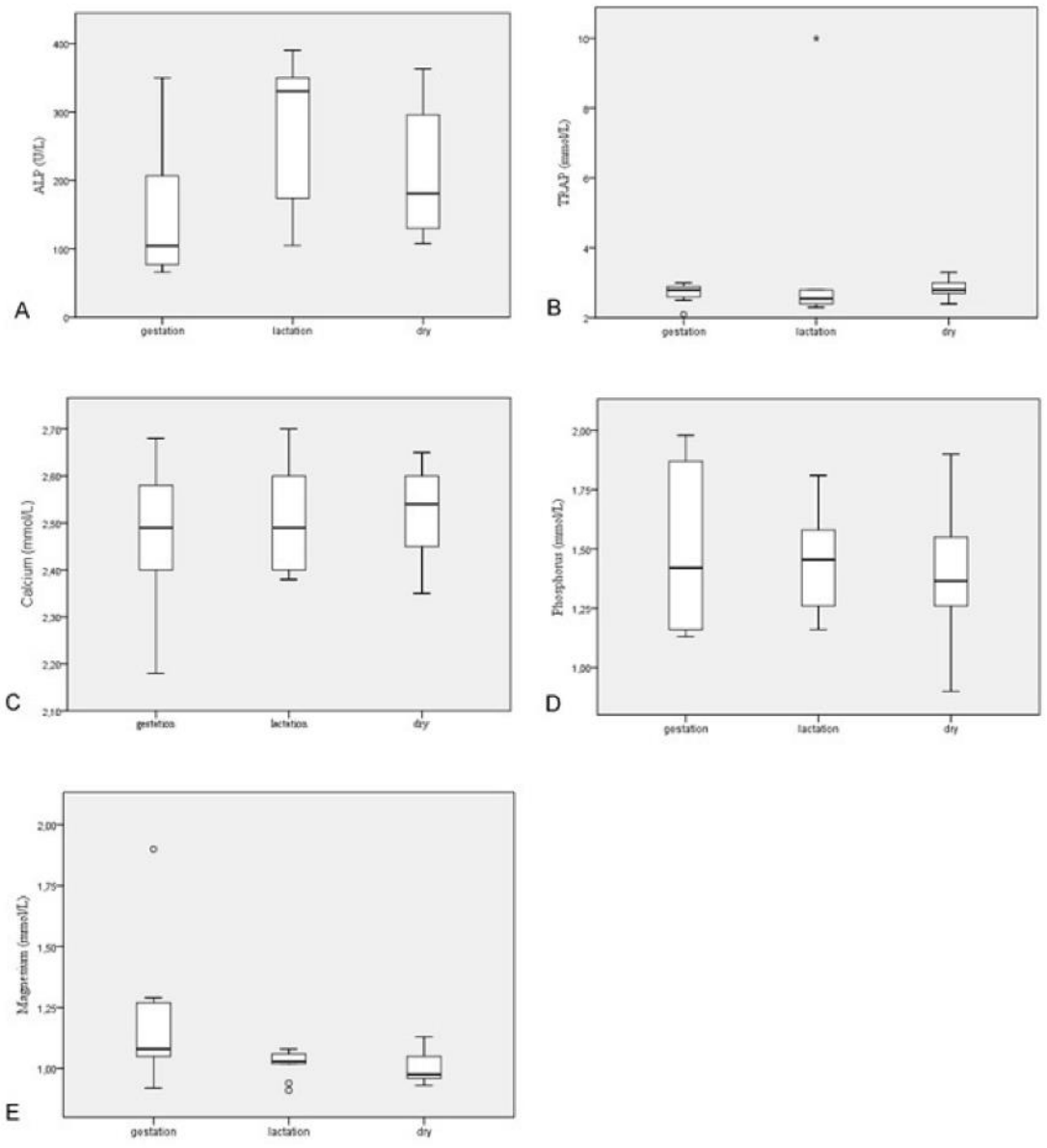

Figure V.2 - Box plot presentations of serum concentrations of biomarkers of bone metabolism by physiologic stages. (A) Serum ALP activity presented significant difference between gestation vs. lactation ($p<0.05$). (B) Serum TRAP activity, there was no significant difference between the groups. (C) Serum Calcium activity, there was no significant difference between the groups. (D) Serum Phosphorus activity, there was no significant difference between the groups. (E) Serum Magnesium activity, there was no significant difference between the groups. Outliers are identified with small circle for out values and star for extreme values.

4. Discussion

The aim of this study was to assess the behavior of serum ALP, TRAP, and minerals in different ages and physiologic stages of the ewe's life, therefore providing a dataset and information to assist in research studies focusing on BTMs in sheep.

In this study, the median ALP value was within the normal range reported for this species (68–387 U/L) (Kahn, 2010) and the variations over the lifespan in sheep can explained by physiological changes. The interval range was wide, however, possibly due to such factors as seasonal influence, with decreased formation marker (BALP) during Autumn (Arens et al., 2007), or circadian influence, in which there is a variation of bone markers throughout the day. Because of the latter, blood samples sampling must be drawn at a standardized time (Liesegang et al., 2003). Age was another influencing factor, with a higher level of ALP found in 1-month old animals, possibly due to synthesis in a variety of tissues, not only due to the bone isoenzyme (Cremers et al., 2008), and high juvenile metabolism (Chai et al., 2015). Lower levels of ALP were found in sheep over 8 years of age, possibly due to the influence of age in decreasing bone formation. In another study, Haversian remodelling in the caudal aspect of the femur, diaphysis of the radius and humerus was observed in sheep between 7 and 9 years of age (Newman et al., 1995), which justifies a link between advanced age and bone loss.

Previously reported values for P (1.62–3.36 mmol/L) and Mg (0.9–1.31 mmol/L) (Kahn, 2010) were within the interval found in the present study, although Ca levels (2.88– 3.2 mmol/L) (Kahn, 2010) were higher in the aforementioned paper. This Ca value could be caused by a decrease in this mineral in pasture, as previously described (Metson and Saunders, 1978). In the referred works the lowest value of Ca was found in different samples from different grazing areas during winter. Blood samples in the present study were drawn in the same season, however, pasture analyses were not performed which renders impossible to investigate a correlation.

In general, for serum minerals, there was a significant difference between the 1 month old group and the other groups, possibly due to the high bone modelling process that is occurring at this age. In fact, as the skeletal structure in these animals is still in growth, there is an increasing demand for Ca and P (Clarke, 2008). A statistical difference in serum minerals between 1-month-old and 6-months-old groups was not expected since both groups have growing animals. There was, however, a statistical difference in Ca and

P between the two groups, possibly due to a change in feed, with the absence of milk and a diet based on grass and concentrate at 6 months old. The decrease in Ca in animals over 8 years of age when compared to the other groups may be associated with bone remodelling in older sheep, as has been reported (Newman et al., 1995), where the decline in Ca would thus be expected due to a decrease in bone mineralization.

During the analysis of the TRAP marker, the 1-month old animals had a higher range interval, probably related to an accelerated resorption during skeletal growth (Leeming et al., 2006). However, 6-months-old animals had a low level of this marker even though the sheep were undergoing skeletal growth, possibly because of a difference in diet, as previously mentioned. This marker showed an inversely proportional relationship with age, thus the animals with 6 to 8 years of age had lower values among all the groups. However, those over 8 years old had an increased TRAP, possibly due to osteoclastic activity (Leeming et al., 2006) in geriatric animals. An explanation for this increase has not been investigated in this study in older sheep. Nevertheless, in older women, it is associated to osteoporosis (Blumer et al., 2012). Therefore, there may be an osteopenia in old animals related to an increase of this marker, but analyses of the bone, such as bone densitometry, would be necessary to prove that.

It should also be mentioned that the ideal TRAP markers to assess bone metabolism should determine the TRAP5b isoform by itself, since it has an osteoclastic origin, resulting from resorption activity, whereas the TRAP5a isoform is nonosteoclastic (Hallen et al., 2002). The occurrence of seven outliers (three in 6 months old, one in the 2 years and three in 8 years old group) in the determination of TRAP could be justified in this study by the marker being expressed in different tissues, such as muscles and heart or pathological conditions. There are published studies in humans that correlated TRAP with leukemia and AIDS (Hayman et al., 2000).

However, the present study used healthy animals and obtained normal values for the ovine species. In the present study, the values found are within the reported range of minimum value in adults and maximum values in juveniles (0,14–5,9 U/L) (Seebeck et al., 2005). It is thus not possible to state which tissue was responsible for the occurrence of outliers and whether there was a pathological cause for these outliers. All TRAP variations can be explained by physiological or pathological changes as previously described.

This study presented a reasonable degree of correlation between P and Ca, previously described in sheep as being involved in bone mineralization (Sousa et al.,

2014). In rats, the increase of these serum minerals were essential for mineralization of bone tissue developed in vitro (Chang et al., 2000). The degree of correlation present between ALP and Ca is possibly caused by the role of Ca in enzymatic reactions involving ALP, a correlation which has been previously described (Dias et al., 2008; Sousa et al., 2011).

With regard to the three groups of 3- to 5-year old sheep in different physiologic stages, only the pregnant group showed values that were considerably lower than the dry and lactation groups for ALP analyses. A similar increase of ALP throughout pregnancy has been previously described (Khatun et al., 2011). However, Liesegang et al. (2006) (Liesegang et al., 2006) used the BALP isoform and obtained a decrease of the marker during gestation. Therefore, when the animals become pregnant, there is a significant reduction of ALP, which then increases between gestation and lactation, and is slightly decreased in dry sheep (Khatun et al., 2011). In this study, the same marker variation was observed among the three described physiologic states, as has been previously described. However, during the analysis of TRAP, the lactating group showed lower values when compared to the dry ones. This could be due to the abrupt drop in bone resorption during the 2 to 3 months of lactation, with values going back to basal values in dry animals. Another greatly important factor would be that the maximum peak of TRAP would occur closer to parturition, as described (Khatun et al., 2011).

5. Conclusions

This study provided information on the variation of two of the most used BTMs – ALP and TRAP, throughout the different stages of life and metabolic stages in sheep. These bone turnover markers variations can be entirely explained by known physiology, confirming that unexplained changes do not occur during the lifespan of sheep. This information may be a useful tool in clinical orthopedic research studies both in animal and veterinary sciences, as well as when using sheep as an animal model for translational studies for humans.

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Chapter VI – Contribution to the Characterization of the Glucocorticoid Treated Ovariectomized Osteoporotic sheep model for Pre-clinical and Translational Studies in Orthopaedic Research

The content this chapter has been based on the following article:

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Contribution to the Characterization of the Glucocorticoid Treated Ovariectomized Osteoporotic Sheep Model for Pre-Clinical and Translational Studies in Orthopaedic Research

1. Introduction

Osteoporosis is a skeletal disorder characterized by a loss of bone mass and structure in which bone strength is compromised, thus increasing the risk of fractures of the hip, spine and other skeletal sites (Riggs and Melton, 1986; Riggs et al., 2003). Osteoporosis can be divided in type I and type II. Therefore, type I post-menopausal osteoporosis represents mainly a loss of trabecular bone, increasing fractures of the vertebrae and typically affects women after menopause due to lack of endogenous oestrogen (Riggs and Melton, 1986; Riggs et al., 2003). On the other hand, type II senile osteoporosis represent a loss of cortical and trabecular bone in both men and women, as the end result of age-related bone loss and it is characterized by hip, proximal humerus, proximal tibia and pelvis fragility fractures (Riggs and Melton, 1986).

Osteoporosis is a disorder that is associated with an abnormality in the bone remodelling process which depends on the coordination of multiple communication pathways between the osteoblast and the osteoclast lineages (Feng and McDonald, 2011). In the healthy skeleton, constant bone remodelling occurs in which mature bone tissue is removed, in a process called resorption, and new tissue is formed in order to maintain bone strength and mineral homeostasis in continuum with a strict coordination between their phases. This is important not only for the amount of bone resorbed and reconstructed but also in what exact location it must occur (Andersen et al., 2009). The bone remodelling cycle has three phases: the initiation, the transition (reversal) and the termination of bone formation and this process is extremely well described in various published studies (Matsuo and Irie, 2008; Andersen et al., 2009; Bonewald, 2011). The process of bone remodelling involves the osteoprotegerin (OPG)/receptor activator of nuclear factor NF-κB ligand (RANKL)/its membrane-bound receptor (RANK) system on osteoblasts and osteoclasts with OPG and RANKL constituting a ligand-receptor system that directly regulates osteoclast differentiation, OPG acting as an inhibitor of osteoclastogenesis by competing with RANKL for the membrane receptor (Meikle, 2006; Teitelbaum, 2007; Liu et al., 2015; Ikeda and Takeshita, 2016; Kapasa et al., 2017). Since the osteoblasts and osteoclasts activities are controlled by a variety of cytokines and hormones, such as oestrogen. The lack of this hormone contributes for an unbalanced

remodelling, increasing bone loss and risk of osteoporosis (Lerner, 2006). The stimulation of oestrogen receptors (ER) in terminally differentiated osteoclasts inhibits their bone-resorbing activity while, in osteoclast progenitor cells, causes inhibition of osteoclast formation (Taranta et al., 2002; Deroo et al., 2014). Thus, the lack of oestrogen leads to enhanced expression of macrophage colony stimulating factor (M-CSF), RANKL and tumour necrosis factor α (TNF-α) and fails to express OPG. Consequently, there is an enhancement of osteoclasts formation and increase of bone resorption due to lack of osteoclasts apoptosis performed by oestrogen (Lerner, 2006). Moreover, and regarding the osteocytes, oestrogen deficiency decreases the response of bone remodelling process to mechanical load, sensed by the osteocytic network and increases production of sclerostin that inhibits bone formation (Klein-Nulend et al. 2015; Eastell et al., 2016). Conversely, the stimulation of ER in osteoblasts triggers their activities and represses the pathway by which osteoblasts can activate osteoclasts. Therefore, the lack of oestrogen suppresses osteoblast differentiation and activity by mesenchymal stem cells (MSCs) and consequently, inhibits the production of insulin-like growth factor 2 (IGF-2) and transforming growth factor β (TGF-β) (Klein-Nulend et al. 2015; Eastell et al., 2016).

Mortality, morbidity and costs of osteoporosis and associated fractures are one of the most important burdens faced by healthcare systems in Europe and the United States of America. Effectively, in the year of 2010, approximately 158 million people across the world suffered a bone fracture, from which 137 million were women and 21 million were men (Oden et al., 2015). Moreover, with demographic changes in population, it is expected an increase to 319 million over the next 30 years (Óden et al., 2015). Burge et al. (2007) refers that by the year of 2025, the annual estimative surpasses 3 million of fractures occurring associated with incidence of osteoporosis just in the USA population, causing $25 billion of cost resulting in a public health issue (Burge et al., 2007). In particular, hip fractures are the most common ones, contributing to increased morbidity and mortality, with 26.9% of the patients dying 2 years after the fracture incident due to the hip fracture event itself or any comorbidity (Kanis et al., 2003).

An increasing demand for suitable orthopaedic experimental animal models is required, specially in pre-clinical small animal models, to understand the pathophysiological mechanisms involved in the osteoporotic process helping with an early diagnosis and development of new therapies. The USA Food and Drug Administration accepts small ruminants (sheep and goats) as large animal models for osteoporosis research and specially sheep has been used mostly in the last decades in

numerous *in vivo* studies in this field (Reinwald and Burr, 2008; Oheim et al., 2012). Despite sheep exhibit relevant differences to humans on bone tissue, that are noticeable in both macro- and microstructure, composition, biochemical properties and bone mineral density (BMD) (Aerssens et al., 1998; Pearce et al., 2008), as well as in bone metabolism, although influenced by seasonality (Arens et al., 2007), this small ruminant has been described as an efficient osteoporosis animal model. The justification is due to availability, handling and housing, as well as low cost compared with large animal species. Another aspect concerns its acceptance as an experimental animal model by the public opinion in many societies (Turner, 2007). Also, Andreasen et al. (2015) concluded that glucocorticoid (GC) treated ovariectomized (OVX) aged sheep induced a significant bone loss, promoted by an arrest of the reversal phase, resulting in an uncoupling of bone formation and resorption, as demonstrated in postmenopausal women with GC-induced osteoporosis (Jensen et al., 2011; Andersen et al., 2013). These aspects support the importance of this animal model for the study of the pathophysiology of this disorder and as a pre-clinical model for orthopedic implant and biomaterial research. Another very recent study elucidates the osteocyte regulation of OPG/RANKL in the sheep model of osteoporosis, concluding that in the late progressive phase of the osteoporosis induced by steroids, the RANKL expression is stimulated in osteocytes (El Khassawna et al., 2017). Therefore, the most frequent osteoporotic small ruminant model used is by far the OVX sheep with 12 months post-operatively, or more, as a surgical model to perform biomaterial research, bone augmentation, efficacy of implant fixation, fracture-healing improvement or bone defect repair studies in the osteopenic or osteoporotic bone, or by the combined treatment of OVX sheep associated to calcium/vitamin-D deficient diet and GC applications for 6 months (Dias et al., 2018).

Concerning GCs, these pharmacological agents are used to help in the osteoporosis induction in this animal model. An excess of GCs production due to elevated hormonal adrenal secretion is associated to diseases, such as pituitary tumours and Cushing's syndrome, namely in older age (Sato et al., 2016). As consequence, GCs alter the bone metabolism, decreases BMD and strength of cortical and trabecular bone, increasing the prevalence of traumatic fractures and osteonecrosis and also causing muscle weakness (Mellibovsky et al., 2015; Sato et al., 2016). Within the first 3-6 months occurs the highest rate of bone loss which is more apparent in trabecular bone and the fracture risk is higher with higher daily doses of GCs (Buckley et al., 2017). Thus, GCs are often used to induce

bone diseases, including osteoporosis, and Zhang et al. (2016) describes in detail the underlying mechanism of osteoporosis induced by GCs in OVX animals.

Recent mechanical evidence of how GCs act proved that these hormones act via nuclear hormone receptor locally in skeletal cells, and modulate their proliferation, differentiation, and cell death, and to a lesser extent through the mineralocorticoid receptor, which displays a restricted expression pattern (Hachemi et al., 2018). Numerous studies demonstrated that the mechanism of GC-induced osteoporosis affects the physiological bone remodelling process via accelerating bone resorption, decreasing bone formation and increasing apoptosis of osteocytes and osteoblasts (Dalle Carbonare et al., 2001; Jensen et al., 2011; Weinstein, 2011; Sato et al., 2016; Siddiqui and Partridge, 2016), and also impairing differentiation of MSCs into mature osteoblasts (Canalis et al., 2005). In an initial phase there is a reduction of osteoblasts, an increased generation of new osteoclasts and prolongation of their lifespan (Weinstein, 2011). In relation to increased osteoclasts, GCs inhibit synthesis of OPG and stimulate RANKL, with the reduction in the OPG-to-RANKL ratio contributing to osteoclast differentiation and bone resorption (Siddiqui and Partridge, 2016). Furthermore, GCs negatively regulate secretion of oestrogen and calcium balance (Siddiqui and Partridge, 2016).

Although several studies have been published on the sheep osteoporotic model, the authors of this work intend to determine a comprehensive analytical panel composed by several bone turnover markers (BTMs) all along the osteoporosis induction process in this large animal model. The BTMs are proteins that indicate bone metabolism employed in diagnostic clinical application in osteoporosis, generally divided in bone formation markers (total and bone-specific alkaline phosphatase (ALP and BALP, respectively), osteocalcin (OC) and procollagen type I propeptides (PICP and PINP)), bone resorption markers (the products of collagen breakdown, such as the C-terminal (CTX) and N-terminal (NTX) telopeptides of collagen type I, the CTX – matrix metalloproteinase - (CTX-MMP or ICTP), hydroxyproline (HYP) and the collagen cross-links (pyridinoline (PYD), deoxypyridinoline (DPD)), and the enzymes secreted by the osteoclasts, namely the tartrate-resistant acid phosphatase (TRAP) 5b isoform) and osteoclast regulatory protein markers (OPG, RANKL, RANK) (Leeming et al., 2006; Cremers et al., 2008; Sousa et al., 2015).

Therefore, the present study aimed to contribute for the evaluation of osteoporosis induction during the first six months after OVX and posterior GC administration in sheep for the assessment of the pathophysiological significance of the variation pattern of BTMs

formation and resorption, namely ALP and BALP, intact OC, CTX and TRAP, but also the estradiol and the serum minerals. Additionally, it was performed the evaluation of BMD, mechanical properties and microarchitectural characteristics of the body of lumbar vertebrae and femoral heads that were acquired by micro-computed tomography (micro -CT), biomechanical tests and histology and histomorphometry, respectively.

2. Material and Methods

2.1. Experimental animals

Eleven healthy female sheep (Portuguese Serra-da-Estrela breed) with approximately 3 to 4-years-old (mean weight of 55.95±4.5 kg) were acclimatized for 4 weeks before the first blood drawn and surgical protocol procedure. The animals were housed indoors under natural influence of seasonal variation and photoperiod. The barn was spacious, dry, well-drained, ventilated and bedding composed by hay and straw regularly changed. The feed was composed by grass hay, supplemented with 0.250 kg of concentrate feed per animal per day and water provided *ad libitum*. Dry matter and chemical composition of grass hay is made up of dry matter per kg feed (88.5 g), ash per kg dry matter (5.9 g), neutral detergent fibre per kg dry matter (73.3 g) and crude protein per kg dry matter (6.1 g). Dry matter and chemical composition of feed concentrate is made up of dry matter per kg feed (90.4 g), ash per kg dry matter (8.5 g), neutral detergent fibre per kg dry matter (31.6 g) and crude protein per kg dry matter (20.7 g).

The animals were divided in control group (n=6) and experimental group (n=5). Experimental group was bilaterally OVX in July and posteriorly received dexamethasone injections weekly (1 mg/kg IM) as described by Zarrinkalam et al. (2009). For OVX realization, the anesthesic protocol was composed by premedication with acepromazine maleate (0.1 mg/kg EV, Calmivet; Univete, Lisbon, Portugal). The anaesthetic induction was carried out with butorphanol tartrate (0.06 mg/kg EV, Torbugesic; Fort Dodge Veterinaria, S.A., Vall de Vianya, Girona, Spain) and propofol 2% (3 mg/kg EV, Propofol-Lipuro; B.Braun, Melsungen, Germany) and maintained with 1.5% isoflurane in oxygen. The experimental group received 1 mg/kg dexamethasone injections weekly (0.6 mg/kg IM, Dexafort; MSD Animal Health, Portugal and 0.4 mg/kg IM, Oradexon, N.V. Organon, The Netherlands).

During the last four weeks the tapering of steroids was performed (3/4, ½, ¼ and 0 of the

initial steroids dose), since the complete removal of glucocorticoids will be necessary for the subsequent use of this animal model in the study of anti-osteoporotic drugs or for their submission to anaesthetic protocols and development of surgical techniques for orthopedic implant and biomaterial research in the osteopenic and osteoporotic skeleton.

All animals were euthanized at the 24th postoperative week in December, with a lethal EV injection of pentobarbital sodium (Eutasil; Sanofi Veterinária, Miraflores, Algés, Portugal). All animal handling practices followed Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes (Authorization DGV Of. n° 0420/000/000/2009).

2.2. Collection of blood samples

Blood samples were collected from both control and experimental groups preoperatively (T0) and at the 4th (T1), 8th (T2) 12th (T3), 16th (T4), 20th (T5) and 24th (T6) postoperative weeks. The blood samples were drawn from the jugular vein and placed into serological tubes (S-Monovette®, SARSTEDT, Nümbrecht, Germany). Samplings were performed between 9:00 a.m. and 10:00 a.m. and they were carried in a thermal box to laboratory facilities immediately. Blood samples were centrifuged at 3000 rpm for 10 min and the serum stored in Eppendorf tubes at -20°C for serum total ALP, TRAP, estradiol and mineral analyses and at -80°C for the other BTMs analyses.

2.3. Biochemical analysis

The serum BALP activity was determined with an immunocapture method in a microtiter strip format using a monoclonal anti-BALP antibody adsorbed onto strips that captured the BALP in the sample. Para-nitrophenylphosphate (p-NPP) substrate was used for determining the BALP enzymatic activity (Ref. 4660; EIA kit; QUIDEL Corporation, CA, USA). The serum levels of intact OC were determined by a competitive method that uses OC coated onto strips, a mouse anti-OC antibody, an anti-mouse IgG-ALP conjugate and a p-NPP substrate (Ref. 8002; EIA kit; QUIDEL Corporation, Santa Clara, CA, USA). Two highly specific monoclonal antibodies determined the CTX (ACP, Ref. O2F1; ELISA kit; IDS, Boldons, UK) against the amino acid sequence of EKAHD-ß-GGR, where the aspartic acid residue (D) is ß-isomerised, to obtain a specific signal in the ELISA, two chains of EKAHD-ß-GGR must be cross-linked. The TRAP was

performed via an enzymatic method and molecular absorption spectrophotometry using commercially available kits (ACP, Ref. 17304; Sentinel Diagnostics, Milan, Italy).

The measurement of ALP serum activity (ALP, Ref. 6004; Beckman Coulter, CA, USA), calcium (Ca) (Ca-Arsenazo; Ref. 60117, Beckman Coulter, CA, USA), phosphorus (P) (Inorganic Phosphorus, Ref. 6122; Beckman Coulter, CA, USA) and magnesium (Mg) (Magnesium, Ref. 6189; Beckman Coulter, CA, USA) were also determined using commercially available kits, via chemical method and molecular absorption spectrophotometry. All molecular absorbance spectrophotometry test used the same automated biochemistry analyzer (Olympus AU400; Olympus America Inc., PA, USA).

Serum estradiol levels (E_2) (eE2 Ref. 10490889; ADVIA Centaur-Siemens Healthcare Diagnostics, Frimley, UK) were determined by automated direct competitive chemiluminescent immunoassay where monoclonal anti-estradiol antibody was labeled by acridium ester. The manufacturer's protocol was followed as described and samples were assayed in duplicates. The sensitivity of this assay was 19 pg/ml and intra- and inter-assay coefficients of variation were 2.3%-11.1% and 0.9%-2.6%, respectively.

2.4. Micro-CT

Samples from the body of the lumbar vertebrae L1 to L7 were scanned using X-ray scan micrograph (micro-CT; SkyScan 1272; Bruecker, Kontich, Belgium). The samples were maintained in wet conditions by wrapping them with filter paper soaked in saline. Series of two dimensional projections, with a resolution of 7 μm were acquired over a rotation range of 180°, with a rotation step of 0.45°, by cone-beam acquisition and using a copper 0.35 mm + aluminium 0.15 mm filter.

The data were reconstructed using the software NRecon (Version: 1.6.6.0, Skyscan) and analysed in a CT analyser (Version: 1.17.0.0, Skyscan). The region of interest (ROI) was defined as a 4.5 mm diameter circle centred over the specimen. By auto-interpolation of manually-defined ROI with the inner and outer limits of trabecular bone, it was yielded a volume of interest (VOI) with a shape of a cylinder representative of the sample, which was the essential basis for the quantitative analyses. The BMD (g/mm^3) of each sample was determined using the 8 mm phantom calibrators of 0.25 and 0.75 g/mm^3, scanned at the same conditions as the vertebrae samples. For the three-dimensional analysis, the bone region of each section was automatically defined (Ridler-Calvard method) and the

resulting binarised image despeckled to remove the background (for bright speckles <40 voxels). Three-dimensional (3D) reconstructions were produced using the CTVOX software.

Through μ-CT it was acquired the following parameters of each bone sample obtained from the lumbar vertebrae L1 to L7: percent bone volume (BV/TV, %), bone surface/volume ratio (BS/BV, 1/mm), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, 1/mm), trabecular separation (Tb.Sp, mm), closed porosity (Po(cl), %), open porosity (Po(op), %), total porosity (Po(tot), %) and BMD (g/cm_3).

2.5. Biomechanical tests

Vertebrae segments were carefully desiccated by removing evolving soft tissues. Bone samples were then cut from the body vertebrae centre using a 6 mm diameter trephine tool, with 9 mm height (on average). Extremities were flattened against sand paper (180 and 320 grade) in a grinding machine, while keeping bone samples within a prismatic slab previously drilled to guarantee the parallelism of specimen extremities. This procedure was adopted to assure the production of cylindrical specimens with the same dimensions. A 0.9% saline solution was continuously sprayed onto bone samples to keep bone tissue permanently hydrated throughout the cutting operations. Following these processes samples (hereafter designated bone specimens) were duly cleaned, measured (height and cross-area, A_0) and immersed in a saline solution inside Eppendorf tubes, and then frozen at -20°C.

Bone thawing occurred at room temperature (25°C) for no less than 5 hours before conducting compressive tests. A servo-electrical testing system (MicroTester INSTRON® 5848) was used to induce compressive loading, setting a displacement rate to 1.0 mm/min. Biomechanical tests were not performed in femoral heads.

2.6. Histology

Biopsies taken from the lumbar vertebrae (L1 to L7) and femoral heads, 6 mm and 10 mm diameter cylinders respectively, were fixed in formalin 10% (NBF-neutral buffered formalin, Thermo Scientific, USA) and stored at 4ºC. For histological preparations, the bone samples were decalcified by incubation in a solution of TBD-2 (Thermo Scientific, USA) with mechanical stirring during 7 days. The decalcification end-point was defined

as two consecutive days with negative tests for the presence of calcium in the decalcification solution supernatant. In brief, to 0.5 mL of supernatant were added 1.0 mL of citrate-phosphate buffer (0.20 M citric acid and 0.16 M dibasic potassium phosphate, pH 3.2-3.6) and 2.5 mL of saturated ammonium oxalate. After 20 minutes a calcium precipitate in the test tube is formed when the decalcification is still occurring. The decalcification was further confirmed by puncturing the decalcified bone biopsies with a needle to test the resistance.

The decalcified bone samples were then dehydrated in ascending alcohol concentrations before embedding the specimens in paraffin. Sections of 5 µm were cut in the anteroposterior plane on an automate microtome (HM 355S Automatic Microtome, Thermo Scientific, USA) and mounted in glass slides.

Lastly, the histological slices were deparaffinized through downward alcohol concentrations and stained with Hematoxylin & Eosin (H&E) (Thermo Scientific, USA) and Masson Trichrome (Bio-Optica Milano S.p.a, Italy) using standardized protocols.

2.7. Histomorphometry

The bone volume (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm) and trabecular number (Tb.N, number/mm) of the lumbar vertebral and femoral head biopsies were quantified using the BoneJ (Doube et al., 2010) plugin of ImageJ software. For that, all micrographs of the histological cuts stained with H&E were split in the RGB channels. A bitwise operation was performed to subtract the green channel, strongly staining the bone marrow area, to the red channel, roughly corresponding to the bone area and the bone marrow, rendering an image of the bone area. These resulting representations of the bone area were properly treated to remove noise and binarized for the histomorphometric evaluation.

2.8. Statistical analysis

To determine statistical differences between experimental and control groups an analysis of variance (ANOVA) was performed. The baseline values obtained in each group were compared with a Wilcoxon test using the values observed during the 24 post-operative weeks and also a Student's t-test to compare means differences. The degree of correlation between the different biochemical parameters was assessed using a Spearman

correlation test. All statistical analyses were performed with SPSS statistical software (version 23.0, SPSS, Inc., IBM Company, NY, USA). The p-values and r-values of correlations were considered significant at $p<0.05$.

3. Results

Mean body weight during the experiment was 53.9±3.8 kg for control group and 57.0±4.3 kg for experimental group. There were no serious reported complications with the animals during the surgical procedure and the postoperative period. Nevertheless, on the 10th post-operative week, the experimental group started to present some degree of *alopecia disseminate*, a behavioural change represented by laziness and time sleeping, but sheep have always shown normophagia. Our election method had the goal of decreasing the harmful side effects and preserving animal welfare as much as possible, although dramatic bone loss has not been achieved.

3.1. Serum biochemical evaluation

The results for biochemistry analyses are shown in Figures VI.1 and VI.2.
Total ALP presented statistical significant difference between both groups in study along time in the whole model of analysis of variance ($p<0.05$), with the values of the experimental group showing lower levels relatively to the control group after osteoporosis induction. Statistical significant differences were observed for ALP between groups at the 16th post-operatively week but no statistical significant differences were observed along time within each study group. Relatively to the serum minerals analysis, Ca did not show statistical significant differences between both groups in study along time in the whole model analysis of variance ($p>0.05$), nevertheless the values of the experimental groups were always below the values of the control group similarly to ALP for each time point. For P and Mg also statistical differences were obtained between groups in study along time in the whole model of analysis of variance ($p<0.05$ and $p<0.01$, respectively). The P presented statistical differences between groups at the 8th and 16th post-operative weeks with the values of experimental group superior to those of the control group and for Mg statistical differences were observed at the 12th and 16th post-operative weeks with the values of experimental group inferior to those of the control group. For P, just within the experimental group, it was observed a statistical significant increase in values between

the pre-operative time and the 16th post-operative week. The Mg showed statistical differences in control group between the 8th and the 24th post-operative weeks and in the experimental group between the pre-operative time and the 20th and 24th post-operative weeks. The whole model of ANOVA also did not present statistical significance for estradiol ($p>0.05$), nevertheless after the pre-operative period it was possible to observe in the experimental group a decrease in values of this hormone until the 24th post-operative week, while control group just presented a slight fluctuation during the study.

For the BTMs, the BALP and TRAP did not present statistical significant differences between both groups in study along time in the whole model of analysis of variance ($p>0.05$), but OC and CTX presented this statistical difference between both groups in study ($p<0.0001$). Nevertheless, serum BALP showed in the experimental group lower values than those obtained in the control group until the 16th post-operative week. The OC levels were markedly higher in control group in the 4th, 8th and 12th postoperative weeks compared to experimental group and the CTX levels also presented significant differences between study group at the 8th, 16th, 20th and 24th post-operative weeks with the experimental group presenting the highest values. For the OC values it was possible to verify that within the control group there were significant differences between the pre-operative time and from the 16th post-operative week in which a decrease of the OC values could be observed. In the experimental group the OC showed statistical differences between the pre-operative time and the 4th, 8th, 20th and 24th post-operative weeks, which presented lower values. For the CTX it was possible to verify that no statistical differences were observed in the control group along time but in the experimental group there was a statistical difference between the pre-operative time and the 20th post-operative week with this last one value presenting a marked increase.

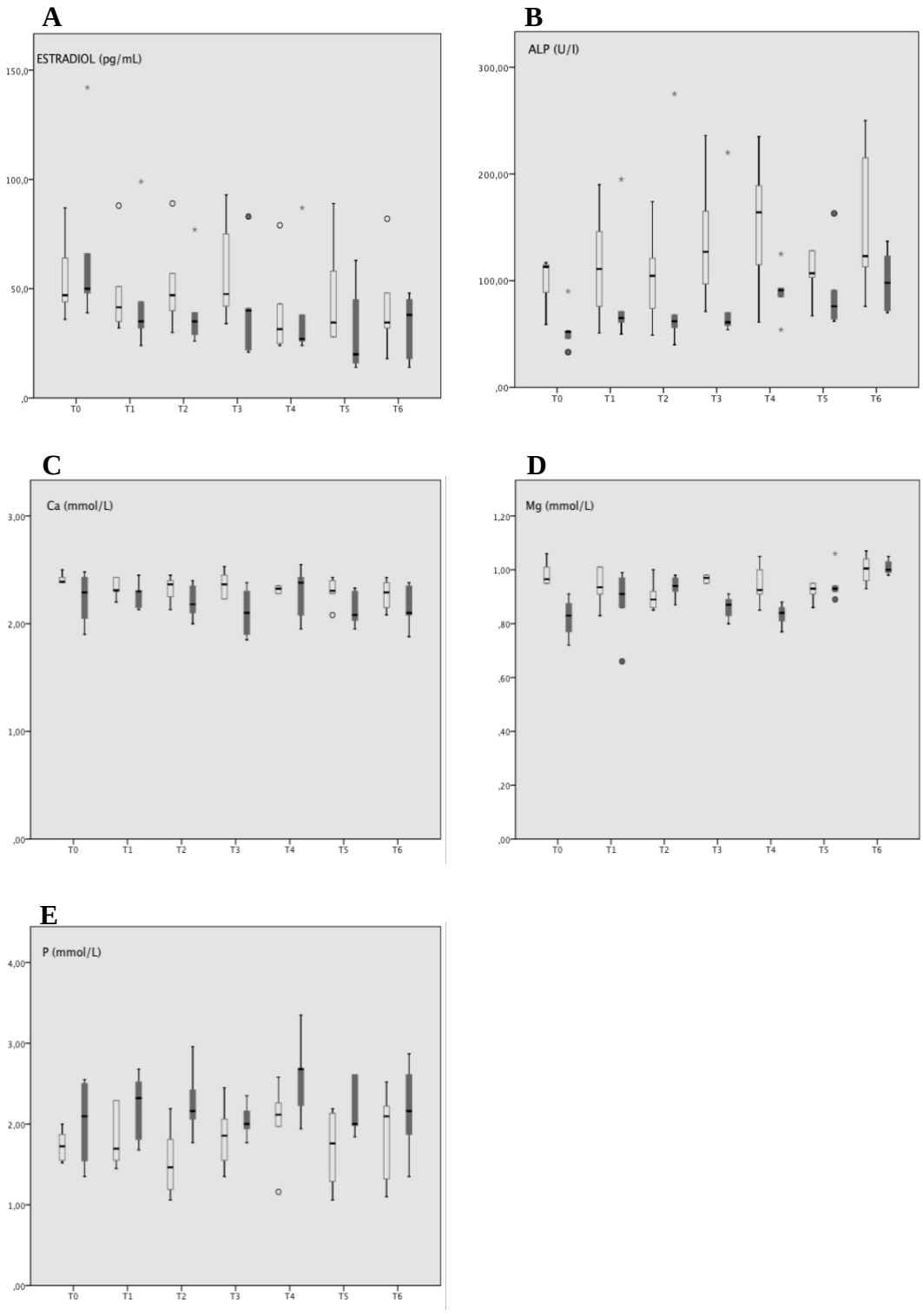

Figure VI.1 - Serological course of estradiol, total bone alkaline phosphatase and serum minerals at the preoperative time and during the post-operative period (Mean ± SD; white columns: control group; grey columns: osteoporosis induction group. Outliers are identified with small circle for out-values and star for extreme values.

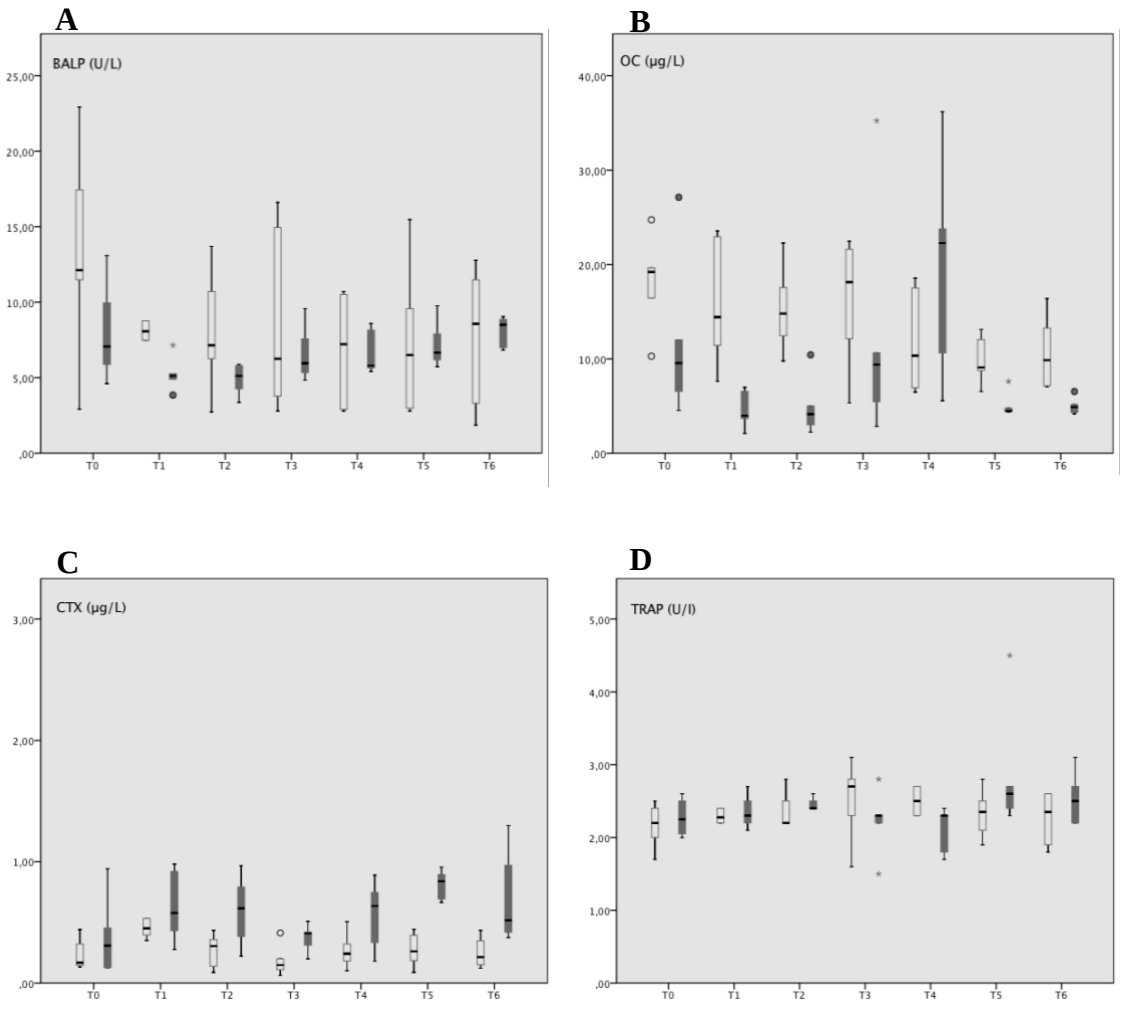

Figure VI.2 - Serological course of bone turnover markers at the preoperative time and during the post-operative period (Mean ± SD; white columns: control group; grey columns: osteoporosis induction group. Outliers are identified with small circle for out-values and star for extreme values.

In Table VI.1 the correlation coefficients between the biochemical parameters in study are presented.

Table VI.1 - Correlation between serum BTMs, estradiol and minerals in the control and experimental groups.

	ALP	BALP	OC	CTX	TRAP	Ca	P	Mg	Estradiol
Control group									
ALP	r=1.00	-	-	-	-	-	-	-	-
	p=1.00								
BALP	r=-0.3558	r=1.00	-	-	-	-	-	-	-
	p=0.0262	p=1.00							
OC	r=-0.2606	r=0.4184	r=1.00	-	-	-	-	-	-
	NS	p=0.0077	p=1.00						
CTX	r=-0.4741	r=0.2452	r=0.2463	r=1.00	-	-	-	-	-
	p=0.0023	NS	NS	p=1.00					
TRAP	r=0.5072	r=-0.5083	r=-0.3237	r=-0.6182	r=1.00	-	-	-	-
	p=0.0010	p=0.0010	p=0.0444	p<0.0001	p=1.00				
Ca	r=-0.0566	r=0.2500	r=-0.0336	r=-0.0322	r=-0.0056	r=1.00	-	-	-
	NS	NS	NS	NS	NS	p=1.00			
P	r=0.3796	r=-0.5196	r=-0.1238	r=-0.2592	r=0.3947	r=-0.2031	r=1.00	-	-
	p=0.0132	p= 0.0007	NS	p=0.0129	NS	NS	p=1.00		
Mg	r=-0.1516	r=0.1237	r=0.1852	r=0.1281	r=-0.0429	r=-0.4135	r=-0.1665	r=1.00	-
	NS	NS	NS	NS	NS	p=0.0065	NS	p=1.00	
Estradiol	r=-0.5299	r=0.6864	r=0.3942	r=0.3116	r=-0.4489	r=0.4489	r=-0.6057	r=0.0698	r=1.00
	p=0.0003	p<0.0001	p=0.0118	NS	p=0.0042	p=0.0029	p<0.0001	NS	p=1.00
Experimental group									
ALP	r=1.00	-	-	-	-	-	-	-	-
	p=1.00								
BALP	r=0.1231	r=1.00	-	-	-	-	-	-	-
	NS	p=1.00							
OC	r=-0.3453	r=0.3312	r=1.00	-	-	-	-	-	-
	NS	NS	p=1.00						
CTX	r=0.3131	r=0.0594	r=-0.1652	r=1.00	-	-	-	-	-
	NS	NS	NS	p=1.00					
TRAP	r=0.1120	r=0.2243	r=-0.2366	r=0.0912	r=1.00	-	-	-	-
	NS	NS	p=0.0490	NS	p=1.00				
Ca	r=-0.3688	r=0.1518	r=0.2844	r=0.0611	r=-0.0943	r=1.00	-	-	-
	p=0.0347	NS	NS	NS	NS	p=1.00			
P	r=0.5079	r=-0.2100	r=0.0561	r=0.0102	r=0.0657	r=-0.4934	r=1.00	-	-
	p=0.0025	NS	NS	NS	NS	p=0.0026	p=1.00		
Mg	r=0.1415	r=0.0979	r=-0.2811	r=0.1917	r=0.4687	r=-0.0373	r=0.1546	r=1.00	-
	NS	NS	NS	NS	p=0.0052	NS	NS	p=1.00	
Estradiol	r=-0.0961	r=0.0289	r=-0.0272	r=-0.0078	r=-0.0055	r=-0.0847	r=0.0403	r=-0.0456	r=1.00
	NS	NS	NS	NS	NS	NS	NS	NS	p=1.00

3.2. Micro-CT

Figure VI.3 illustrates 3D reconstructions of consecutive μ-CT images harvested from L4 vertebrae of the control and experimental sheep groups.

A **B**

Figure VI.3 - 3D reconstructions of consecutive μ-CT images of L4 from the control (A) and experimental (B) groups at the 24th post-operative week.

The whole model of analysis of variance did not present statistical differences during the comparison of the micro-architectural vertebrae parameters and BMD between the study groups ($p>0.05$). Nevertheless, in regards to the BMD, it could be observed that all the vertebrae from the experimental group presented higher values of BMD compared to the ones obtained for the control group. However, non-statistically significant variations were noticed among the analyzed groups or vertebrae (Table VI.2).

Table VI.2. Micro-architectural parameters and BMD obtained by micro-CT from the lumbar vertebrae L1 to L7 of study groups (mean ± SD).

	Lumbar vertebrae L1	L2	L3	L4	L5	L6	L7
Control group							
BV/TV (%)	46.6±8.1$_{a}$	43.7±5.7$_{a}$	43.9±5.9$_{a}$	44.1±5.8$_{a}$	44.3±4.7$_{a}$	43.6±4.1$_{a}$	43.3±8.8$_{a}$
BS/BV (1/mm)	17.3±2.3$_{ab}$	18.5±2.5$_{ab}$	19.3±1.8$_{a}$	18.1±1.5$_{ab}$	16.6±1.7$_{b}$	17.4±0.8$_{ab}$	17.8±2.0$_{ab}$
Tb.Th (mm)	0.143±0.017$_{ab}$	0.132±0.018$_{ab}$	0.127±0.028$_{b}$	0.134±0.019$_{ab}$	0.151±0.014$_{a}$	0.141±0.020$_{ab}$	0.139±0.027$_{ab}$
Tb.N 1/mm	3.29±0.59$_{a}$	3.33±0.40$_{a}$	3.26±0.86$_{a}$	3.39±0.88$_{a}$	2.97±0.48$_{a}$	3.17±0.69$_{a}$	2.84±0.67$_{a}$
Tb.Sp (mm)	0.36±0.05$_{a}$	0.37±0.02$_{a}$	0.37±0.05$_{a}$	0.37±0.05$_{a}$	0.38±0.04$_{a}$	0.38±0.05$_{a}$	0.36±0.02$_{a}$
Po(cl) (%)	0.24±0.14$_{a}$	0.26±0.12$_{a}$	0.25±0.21$_{a}$	0.18±0.13$_{a}$	0.19±0.12$_{a}$	0.24±0.20$_{a}$	0.18±0.13$_{a}$
Po(op) (%)	53.3±8.2$_{a}$	56.2±5.7$_{a}$	55.9±6.1$_{a}$	55.7±5.9$_{a}$	55.7±4.8$_{a}$	56.3±4.1$_{a}$	56.6±9.0$_{a}$
Po(tot) (%)	53.4±8.1$_{a}$	56.3±5.7$_{a}$	56.1±5.9$_{a}$	55.9±5.8$_{a}$	55.7±4.7$_{a}$	56.4±4.1$_{a}$	56.7±8.8$_{a}$
BMD (g/cm$_{3}$)	0.574±0.120$_{a}$	0.584±0.103$_{a}$	0.587±0.105$_{a}$	0.592±0.109$_{a}$	0.612±0.139$_{a}$	0.578±0.098$_{a}$	0.572±0.127$_{a}$
Experimental group							
BV/TV (%)	43.1±2.5$_{a}$	45.6±6.4$_{a}$	42.3±4.0$_{a}$	42.5±3.2$_{a}$	43.2±5.5$_{a}$	43.9±5.8$_{a}$	41.7±4.8$_{a}$
BS/BV (1/mm)	18.2±0.9$_{ab}$	18.4±2.8$_{ab}$	19.6±1.6$_{a}$	18.6±0.6$_{ab}$	17.8±1.9$_{ab}$	18.1±1.7$_{ab}$	18.4±1.1$_{ab}$
Tb.Th (mm)	0.137±0.008$_{ab}$	0.139±0.018$_{ab}$	0.132±0.015$_{ab}$	0.140±0.007$_{ab}$	0.142±0.008$_{ab}$	0.140±0.011$_{ab}$	0.143±0.012$_{ab}$
Tb.N 1/mm	3.14±0.10$_{a}$	3.31±0.64$_{a}$	3.25±0.54$_{a}$	3.05±0.37$_{a}$	3.05±0.50$_{a}$	3.17±0.63$_{a}$	2.93±0.57$_{a}$
Tb.Sp (mm)	0.38±0.05$_{a}$	0.35±0.07$_{a}$	0.36±0.03$_{a}$	0.38±0.02$_{a}$	0.39±0.08$_{a}$	0.37±0.03$_{a}$	0.38±0.05$_{a}$
Po(cl) (%)	0.18±0.07$_{a}$	0.20±0.09$_{a}$	0.21±0.10$_{a}$	0.17±0.11$_{a}$	0.17±0.08$_{a}$	0.22±0.18$_{a}$	0.15±0.13$_{a}$
Po(op) (%)	56.8±2.4$_{a}$	54.3±6.4$_{a}$	57.6±4.1$_{a}$	57.4±3.3$_{a}$	58.2±2.9$_{a}$	56.0±5.9$_{a}$	58.3±4.8$_{a}$
Po(tot) (%)	56.9±2.5$_{a}$	54.4±6.4$_{a}$	57.7±4.0$_{a}$	57.5±3.2$_{a}$	56.8±5.5$_{a}$	56.1±5.8$_{a}$	58.3±4.8$_{a}$
BMD (g/cm$_{3}$)	0.599±0.011$_{a}$	0.639±0.072$_{a}$	0.621±0.080$_{a}$	0.619±0.094$_{a}$	0.635±0.107$_{a}$	0.634±0.112$_{a}$	0.610±0.106$_{a}$

$_{a,b}$Different letters represent significant differences between values, $p<0.05$.

3.3. Biomechanical compressive testing

Figure VI.4 illustrates the load-displacement curves obtained in the mechanical test, from which the initial stiffness (R_0) and ultimate load (P_u) were obtained. Table VI.3 and Figure VI.5 show the values of R_0 and the ultimate stress obtained in these tests ($\sigma_u=P_u/A_0$). Also, for R_0 and σ_u it was verified that the whole model of analysis of variance did not present statistical differences during the comparison of these parameters between the study groups ($p>0.05$). However, elevated values of R_0 and σ_u were presented in the experimental group compared to the control group in all vertebrae with

exception for L1. It was not verified statistical differences for R_0 or for σ_u between different vertebrae of the same group in study, though it was observed a statistical difference in L3 and L4 among the study groups for R_0.

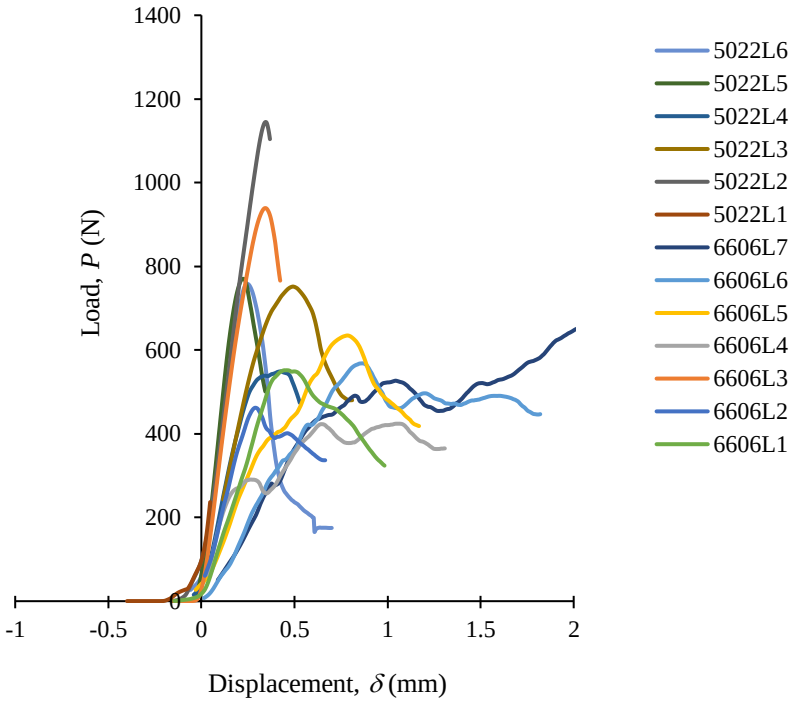

Figure VI.4 - Load-displacement curves obtained in the lumbar vertebrae samples of two sheep from the experimental group through compressive tests (negative values of load are reported in the *y*-axis).

Table VI.3- Initial stiffness (R_0) and ultimate stress ($\sigma_u=P_u/A_0$) obtained in the lumbar vertebrae of study groups (mean ± SD).

	Control group				Experimental group			
Lumbar vertebrae	**$R_{0\ control}$ (N/mm)**	**Standard deviation**	**$\sigma_{u\ control}$ (MPa)**	**Standard deviation**	**$R_{0\ exp}$ (N/mm)**	**Standard deviation**	**$\sigma_{u\ exp}$ (MPa)**	**Standard deviation**
L1	1747	798	27.4	16	2300	1779	21.7	9
L2	2206	1005	24.8	8	2996	1344	26.5	10
L3	1829	792	24.0	15	3000	1275	31.1	8
L4	1695	682	21.9	8	2960	1120	26.4	11
L5	1915	1032	24.3	11	2789	1297	26.5	5
L6	1872	572	23.7	8	2548	1500	26.9	12
L7	1651	471	22.2	7	2035	1514	26.0	12

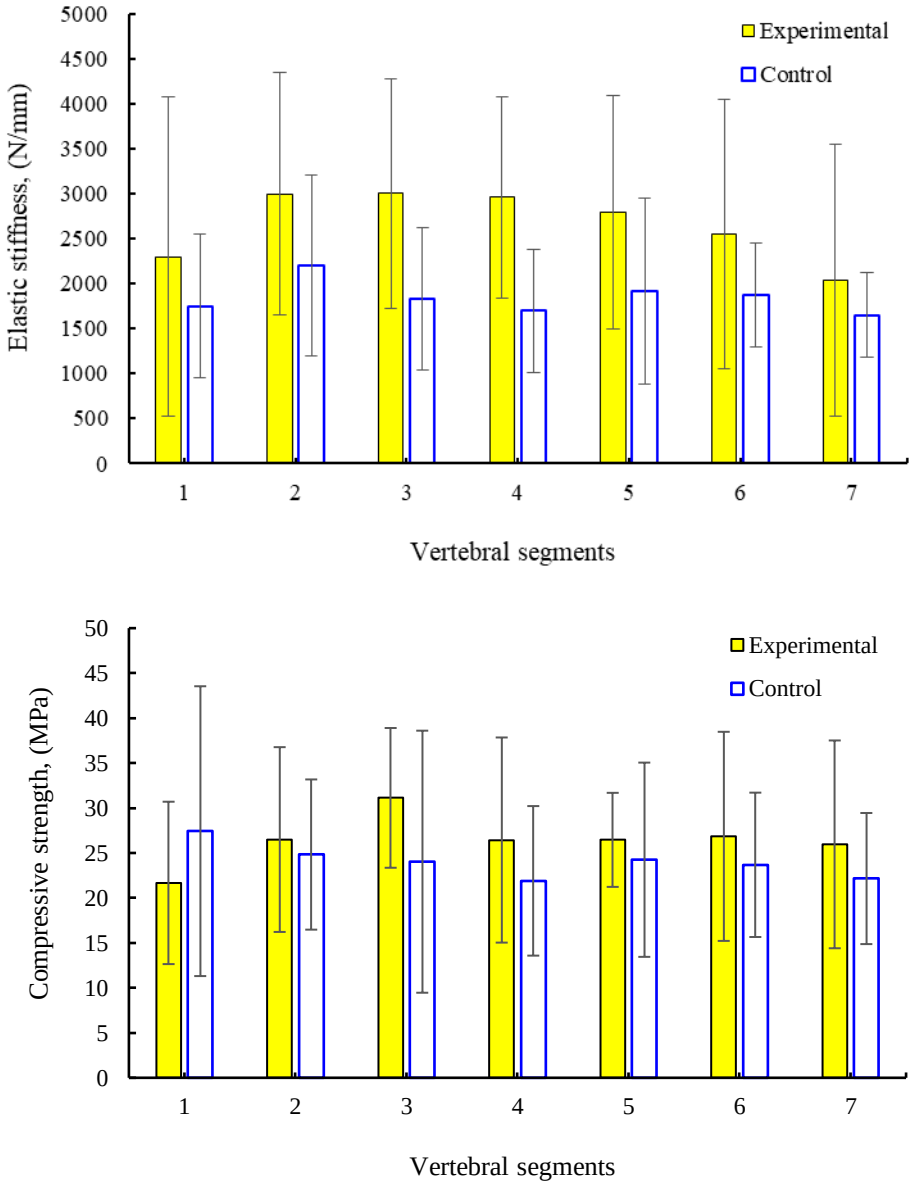

Figure VI.5 - Initial stiffness (R_0) and ultimate stress ($\sigma_u=P_u/A_0$) of lumbar vertebrae (L1-L7) in the study groups (negative values of strength are reported in the *y*-axis).

3.4. Histology

The H&E and Masson trichrome stained histological slides allowed to identify the general bone morphology aspects of both samples of the study groups. It was possible to observe two morphologically different bone regions: 1) a dense rigid outer shell of compact bone, i.e., the cortex; and 2) a central medullary or trabeculae of thin interconnecting narrow (Figure VI.6). The larger pores observed in cortical bone correspond to cross-sections of the neurovascular Haversian canals, around which are

visible concentric bony layers or lamellae (Figure VI.6B, H). The smaller pores in the matrix correspond to lacunae (Figure VI.6C, I) and the collagenous outer periosteal surface, the so-called periosteum (Figure VI.6A, G). At the cellular level, mainly osteoprogenitor periosteal cells (Figure VI.6A, G) and osteocytes located within lacunae (Figure VI.6C, I) were observed. It was also identified within the marrow space both adipose tissue and hematopoietic marrow (Figure VI.6E, K). Interestingly, in femoral heads only adipose tissue was observed histologically in the marrow space (FigureVI.6L).

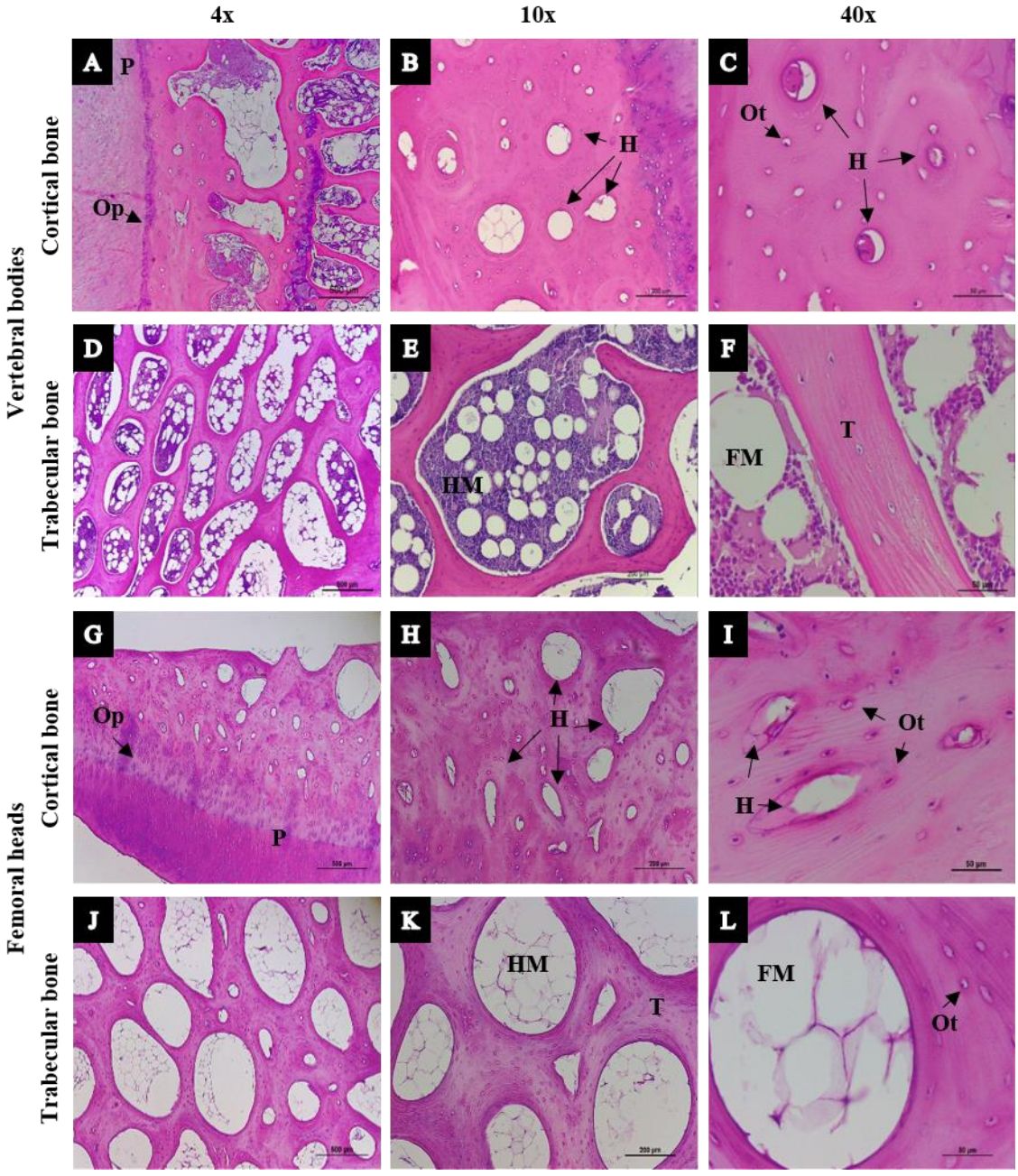

Figure VI.6 - Bone tissue morphology, H&E staining. Representative micrographs of cortical and trabecular bone in vertebrae and femoral head biopsies. FM) Fatty marrow; H) Haversian systems; HM) Hematopoietic marrow; Op) Osteoprogenitor cells; Ot) Osteocytes; P) Periosteum; T) Trabeculae.

Regarding cortical bone of vertebral bodies, differences were detected within bone tissue of animals that were subjected to the combined treatment, OVX and GC administration, presenting greater porosity than healthy animals of the control group (Figure VI.7A, B). Regarding trabecular bone of the vertebral bodies and comparing control and experimental groups there were no evident morphological differences between them (Figure VI.7C, D). However, in some samples of vertebral bodies subjected to OVX associated with GC administration, pathological necrosis in micro-fracture areas in bone tissue was observed (Figure VI.8A, B).

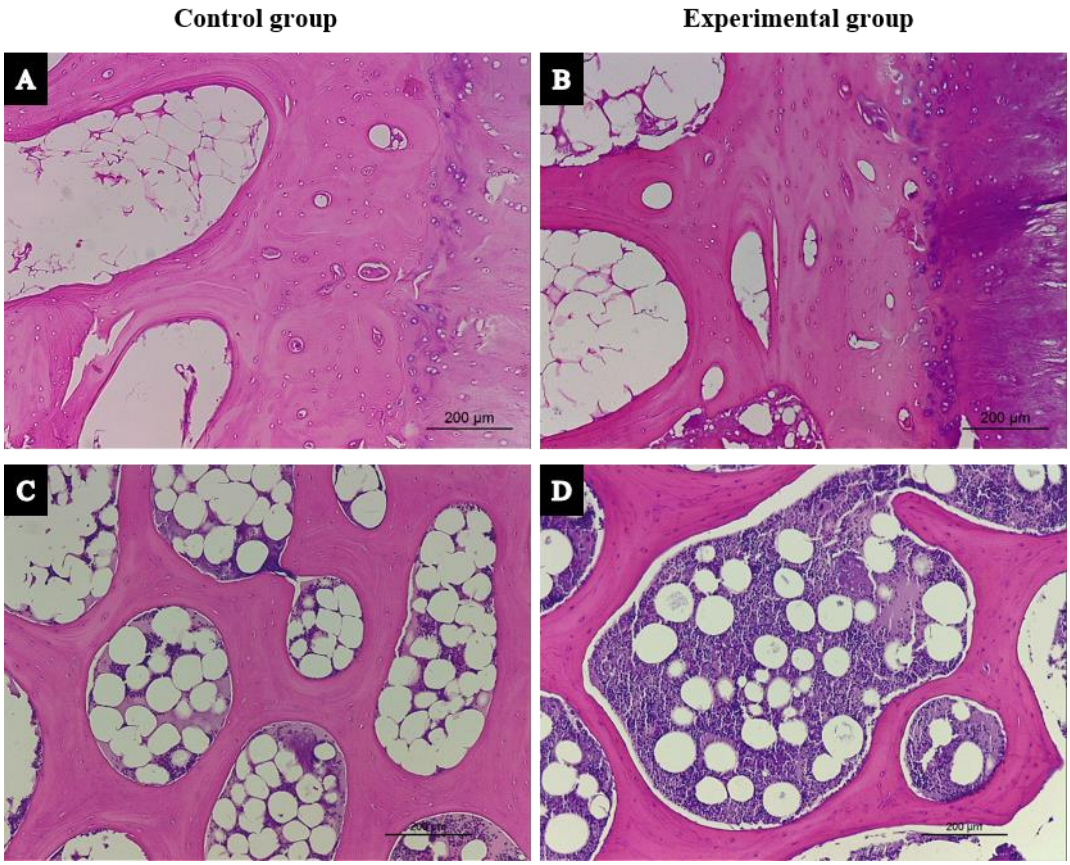

Figure VI.7 - Histological differences between study groups in the cortical (A, B) and trabecular bone (C, D) in vertebral biopsies (x10).

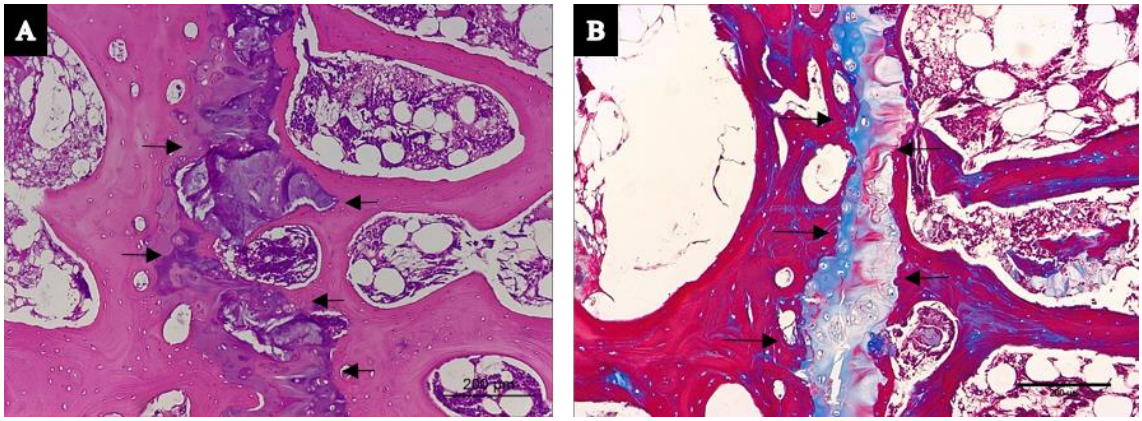

Figure VI.8 - L4 vertebrae biopsy with H&E staining (A) and Masson trichrome staining (B) (x10). Identification of osteonecrosis process (arrows) in vertebra of experimental group.

As in vertebral biopsies, bone tissue of femoral heads from the experimental group presented greater porosity than the control group (Figure VI.9A, B). However, there were no clear differences in the morphology of the trabecular bone (Figure VI.9C, D).

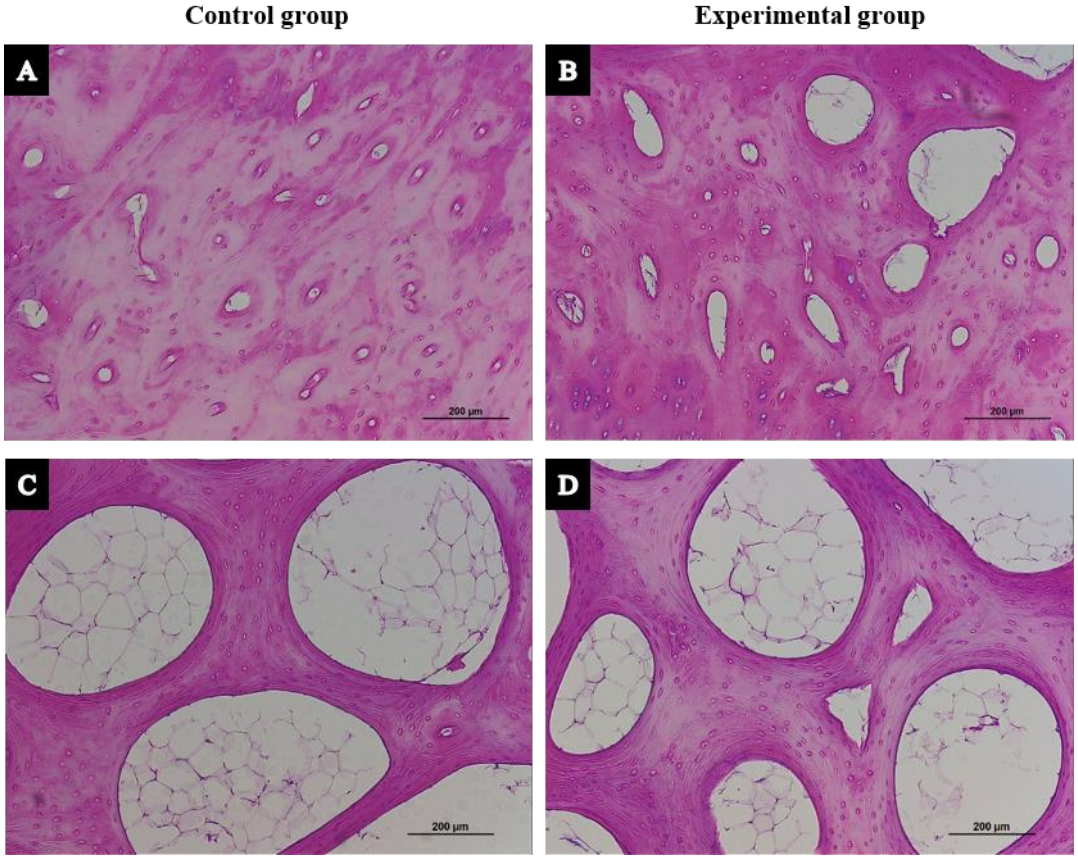

Figure VI.9 - Histological differences between untreated and treated groups, in cortical (A, B) and trabecular bone (C, D) of femoral heads (x10).

3.5. Histomorphometry

The cortical porosity (Ct.Po) and cortical thickness (Ct.Th) were assessed in the cortical bone and the ratio between bone volume/total volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N) were assessed in the trabecular bone of both femoral heads and vertebral bodies.

Comparing the bulk control group and the experimental group, it was verified that the OVX and GC treatment significantly affected both Ct.Po and Ct.Th (Figure VI.10A, B). The combined treatment promoted an overall Ct.Po increase from 3.8±0.4% to 8.6±0.7% ($p<0.0001$) (Figure VI.10A) and the Ct.Th decrease from 757.6±162.1 µm to 623.5±134.0 µm ($p<0.001$) in lumbar vertebrae (Figure VI.10B). In particular, it was observed that, concerning the Ct.Po values, the most affected vertebrae were the L6 and L7 which significantly increased from 3.0±2.0% to 9.9±1.9% and from 4.6±2.6% to 10.6±2.9% ($p<0.05$), respectively (Figure VI.11). Regarding the Ct.Th, only in the L6 vertebra significant differences were observed in which there was a decrease in thickness from 908.6±201.7 µm to 618.4±96.0 µm ($p<0.05$) (Figure VI.12). Regarding the trabecular bone, comparing the bulk control and experimental groups, it was observed that, for all assessed parameters, there were no significant differences (Figure VI.10C, D, E, F). Likewise, there were no significant differences in the evaluated parameters for each lumbar vertebra individually (Figures VI.13, VI.15 and VI.16) except L4 vertebra in which Tb.Sp significantly increased from 366.7±17.6 µm to 430.8±64.2 µm ($p<0.05$) (Figure VI.14).

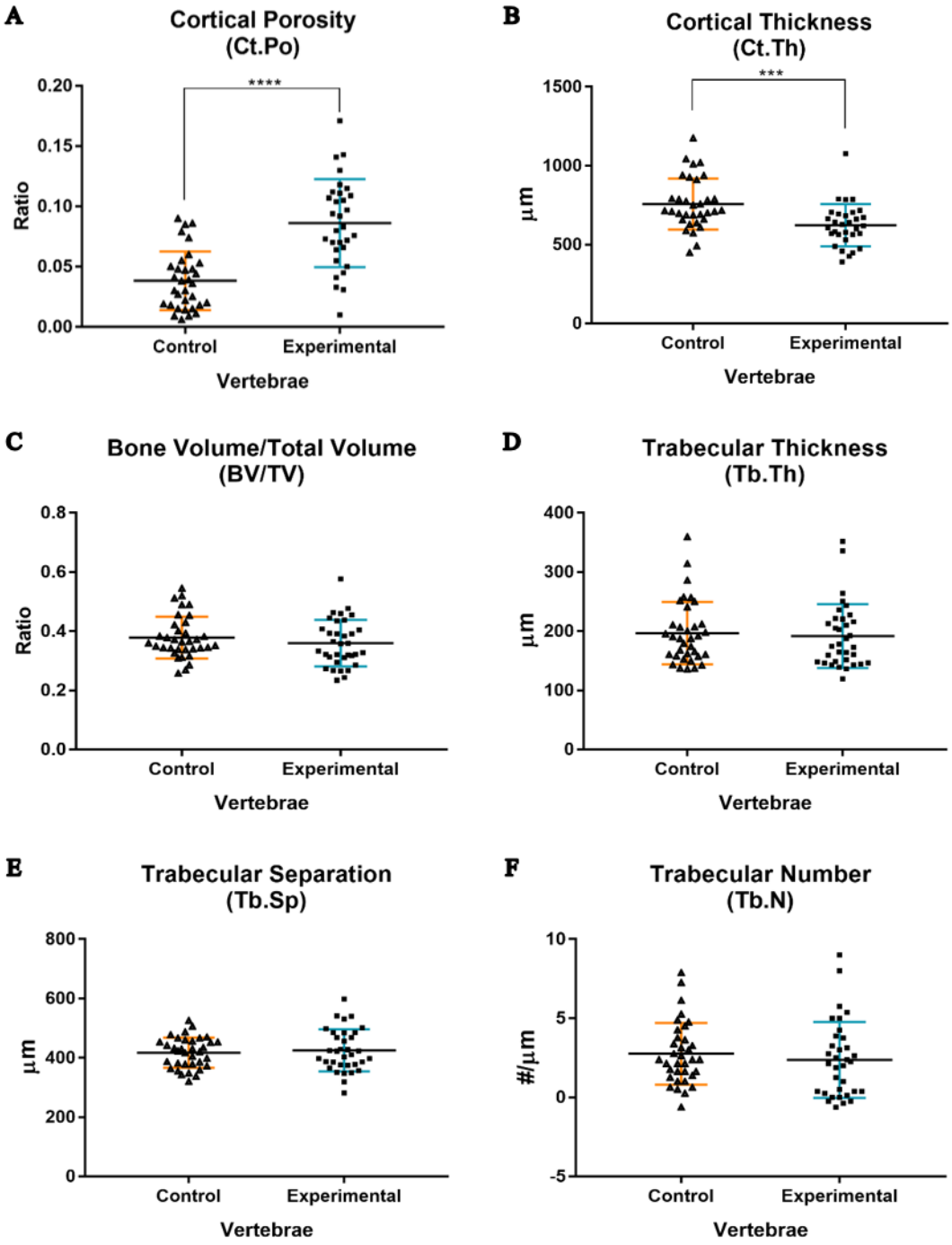

Figure VI.10 - Histomorphometry analysis performed to the cortical and trabecular bone of lumbar vertebral samples. Graphical representation, mean ± SD, of the Ct.Po, Ct.Th, BV/TV, Tb.Th, Tb.Sp and Tb.N differences between the study groups. Each triangle (▲) represent a control individual sample and each square (■) represent an experimental individual sample. Symbols denote significant differences for $p<0.001$ (***) and $p<0.0001$ (****) by Wilcoxon test.

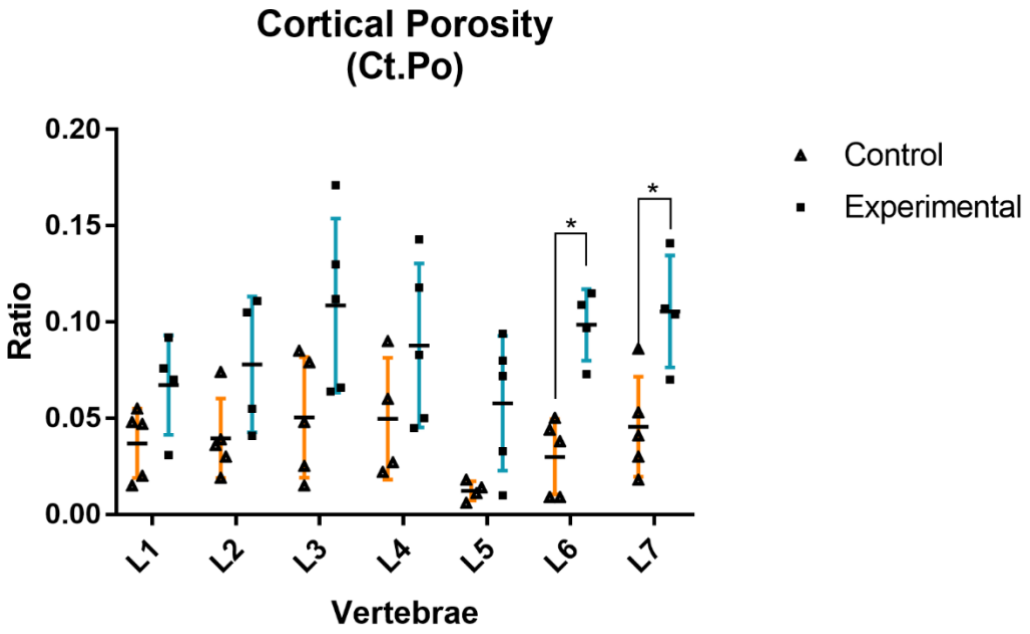

Figure VI.11 - Histomorphometry analysis performed to the cortical bone of lumbar vertebral samples. Graphical representation, mean ± SD, of the Ct.Po of the vertebrae (L1 to L7) from study groups. Each triangle (▲) represent a control individual sample and each square (■) represent an experimental individual sample. Symbol denote significant differences for $p<0.05$ (*) and by Wilcoxon test.

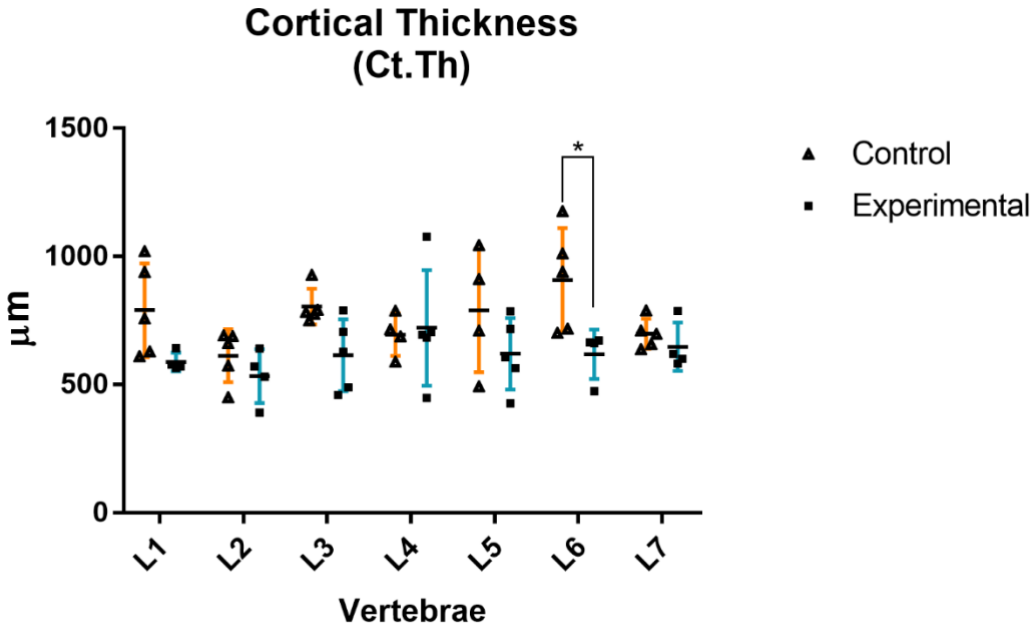

Figure VI.12 - Histomorphometry analysis performed to the cortical bone of lumbar vertebral samples. Graphical representation, mean ± SD, of the Ct.Th of the vertebrae (L1 to L7) from study groups. Each triangle (▲) represent a control individual sample and each square (■) represent an experimental individual sample. Symbol denote significant differences for $p<0.05$ (*) and by Wilcoxon test.

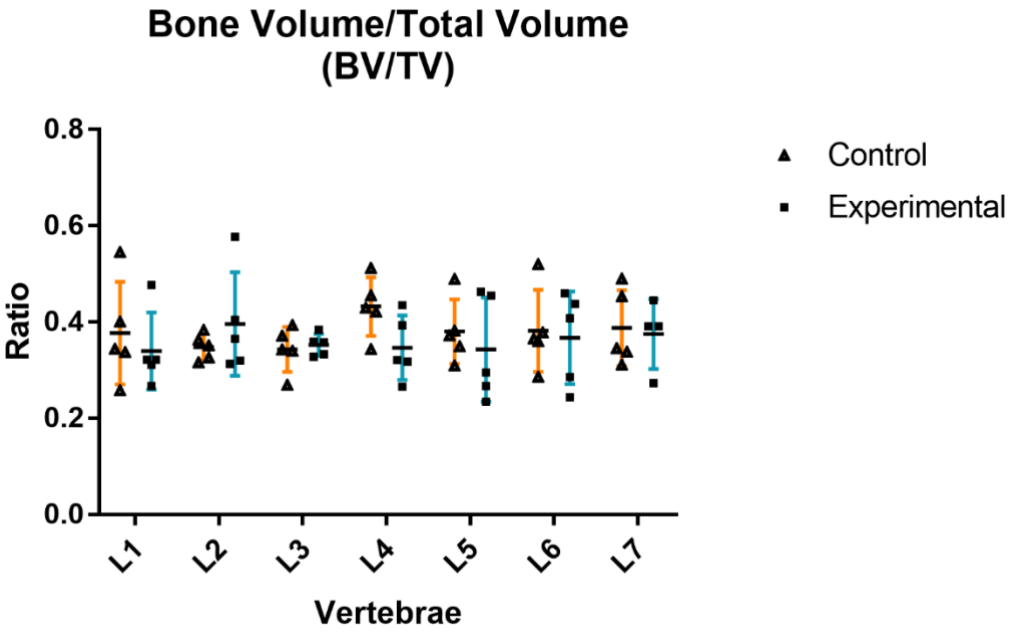

Figure VI.13 - Histomorphometry analysis performed to the trabecular bone of lumbar vertebral samples. Graphical representation, mean ± SD, of the BV/TV of the vertebrae (L1 to L7) from study groups. Each triangle (▲) represent a control individual sample and each square (■) represent an experimental individual sample.

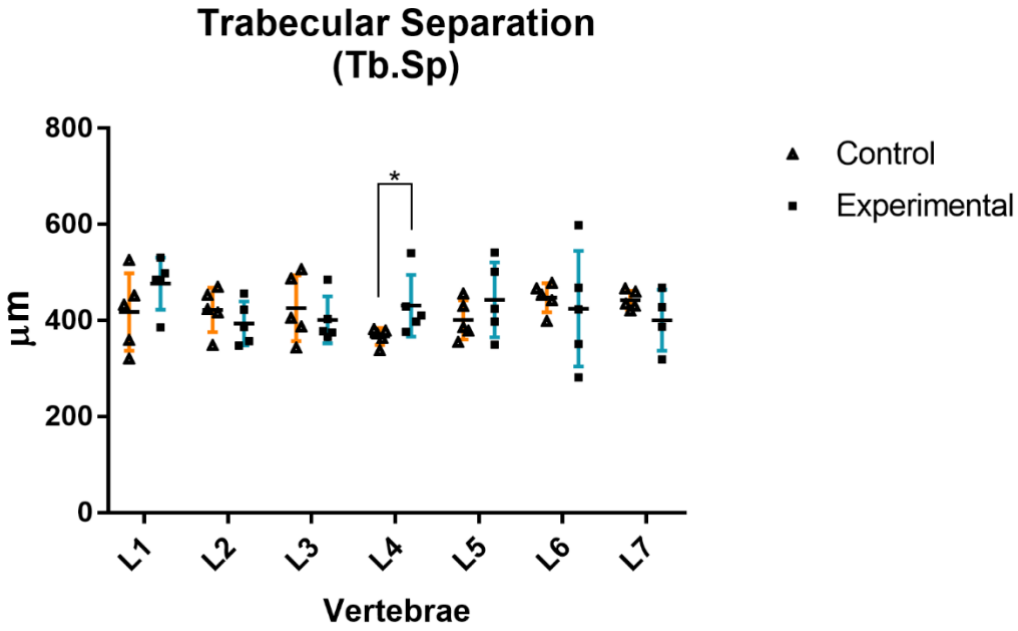

Figure VI.14 - Histomorphometry analysis performed to the trabecular bone of lumbar vertebral samples. Graphical representation, mean ± SD, of the Tb.Sp of the vertebrae (L1 to L7) from study groups. Each triangle (▲) represent a control individual sample and each square (■) represent an experimental individual sample. Symbol denote significant differences for $p<0.05$ (*) and by Wilcoxon test.

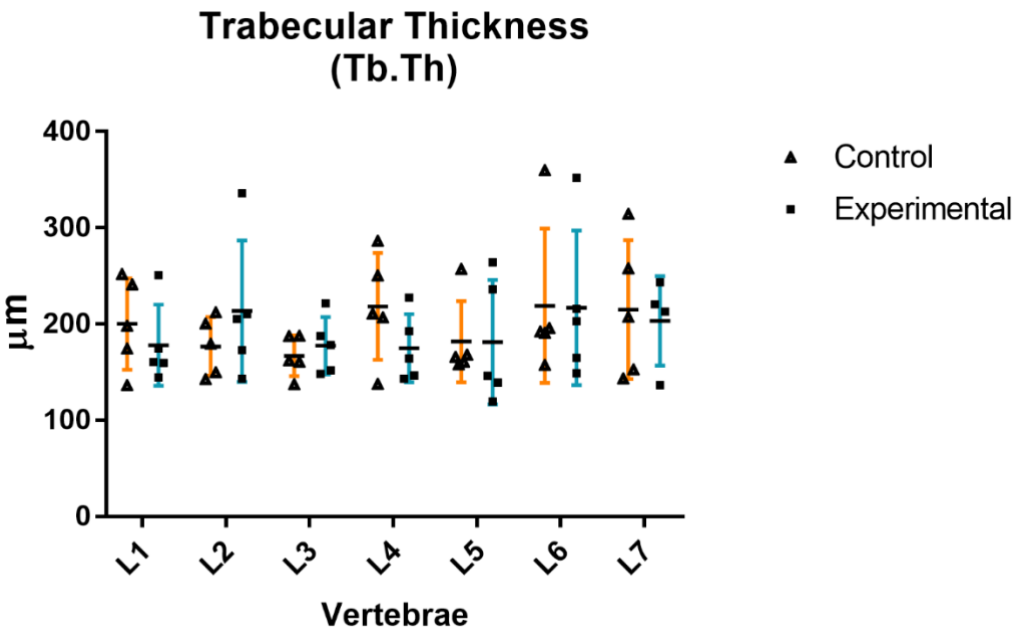

Figure VI.15 - Histomorphometry analysis performed to the trabecular bone of lumbar vertebral samples. Graphical representation, mean ± SD, of the Tb.Th of the vertebrae (L1 to L7) from study groups. Each triangle (▲) represent a control individual sample and each square (■) represent an experimental individual sample.

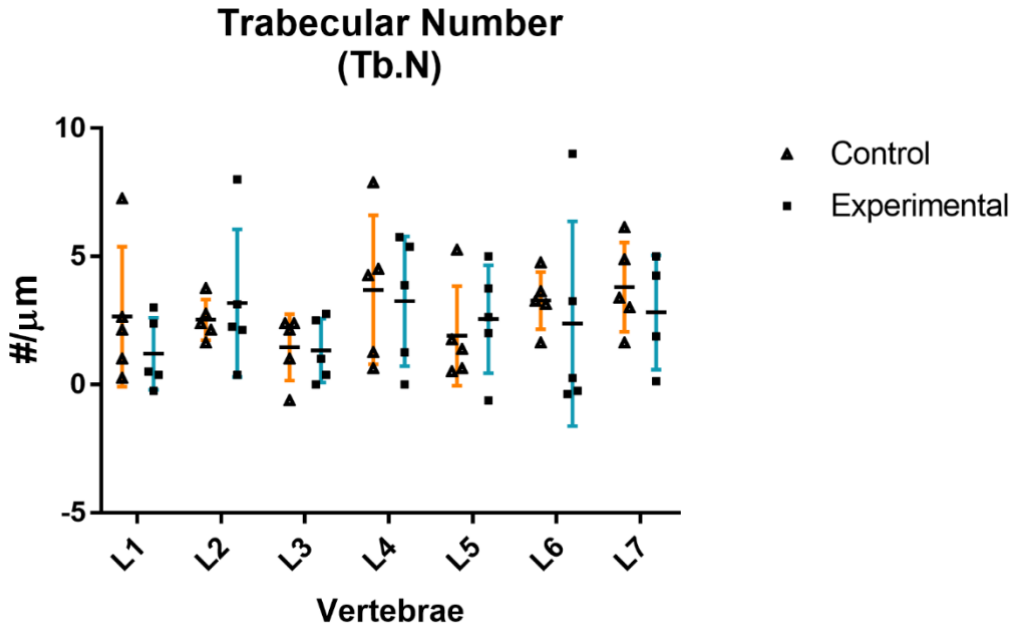

Figure VI.16 - Histomorphometry analysis performed to the trabecular bone of lumbar vertebral samples. Graphical representation, mean ± SD, of the Tb.N of the vertebrae (L1 to L7) from study groups. Each triangle (▲) represent a control individual sample and each square (■) represent an experimental individual sample.

For the cortical bone of femoral heads it was observed a Ct.Po increased from 2.2±0.4% to 7.5±1.2% ($p<0.01$) in the group with the combined treatment (Figure VI.17A). On the

other hand, the Ct.Th decreased from 1238.7±114.4 μm to 772.6±45.1 μm ($p<0.01$) (Figure VI.17B). In the trabecular bone analysis, BV/TV, Tb.Th, Tb.Sp and Tb.N were assessed and in all parameters there were significant differences (Figure VI.17C, D, E, F). Combined treatment promoted a reduction in bone volume from 48.1±3.8% to 36.1±8.1% ($p<0.05$) (Figure VI.17C), a reduction in Tb.Th from 305.4±21.2 μm to 244.2±28.1 μm ($p<0.05$) (Figure VI.17D) and a decrease in the Tb.N from 2.6±1.6 #/μm to 0.025±1.0 #/μm ($p<0.05$) (Figure VI.17F). Moreover, the Tb.Sp increased from 448.9±32.6 μm to 553.0±109.6 μm ($p<0.05$) (Figure VI.17E).

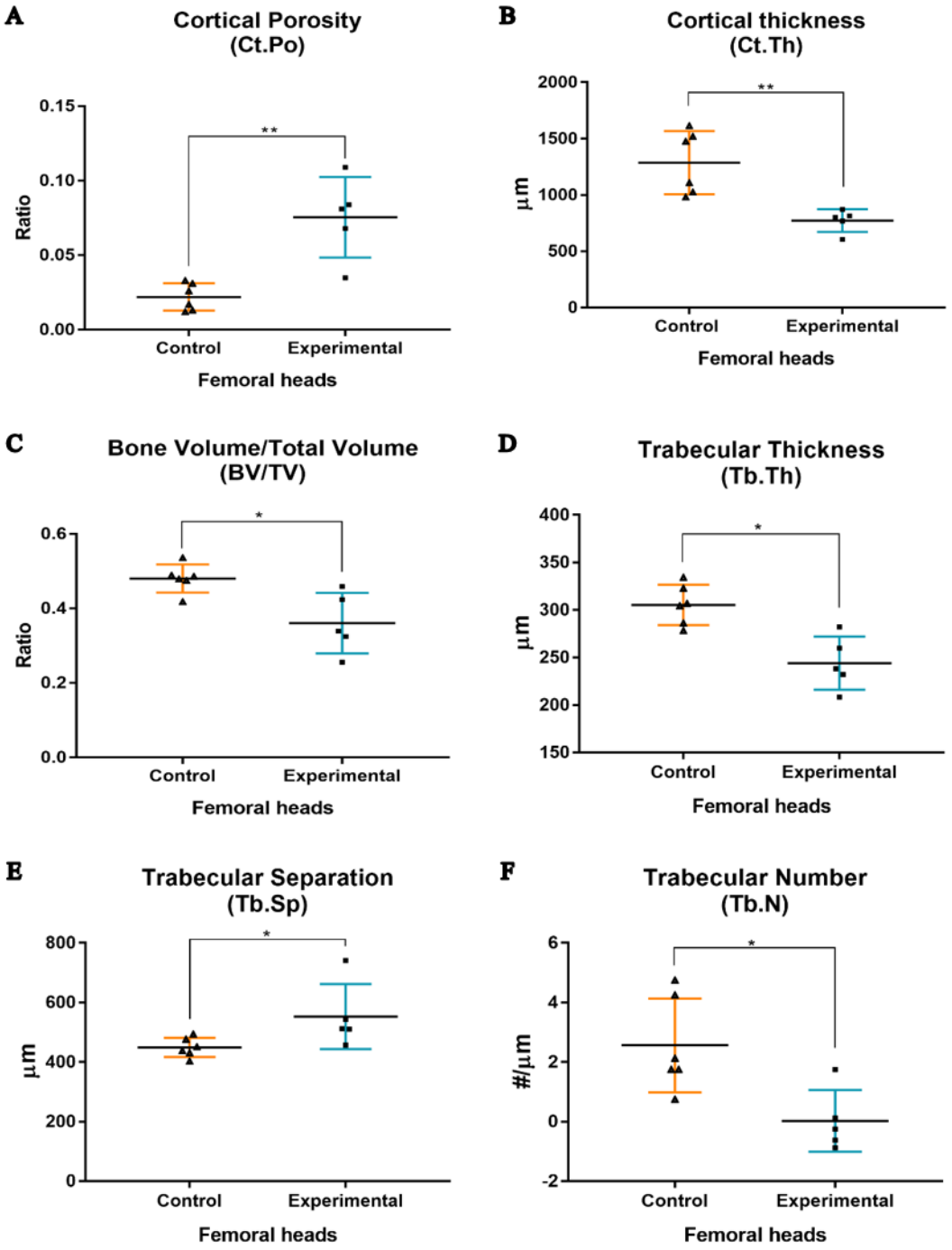

Figure VI.17 - Histomorphometry analysis performed to the cortical and trabecular bone of femoral heads samples. Graphical representation of the Ct.Po, Ct.Th, BV/TV, Tb.Th, Tb.Sp and Tb.N differences between the study groups. Each triangle (▲) represent a control individual sample and each square (■) represent an experimental individual sample. Symbols denote significant differences for $p<0.05$ (*) and $p<0.01$ (**) by Wilcoxon test.

4. Discussion

The present work aimed at thoroughly evaluating one of the most frequently used model of osteoporosis in sheep regarding the main characteristics of bone loss, such as in osteoporotic humans. For that purpose, experimental sheep were bilaterally OVX and posteriorly submitted to steroid therapy for 24 weeks (Zarrinkalam et al., 2009), with proved similarities between the presented osteoporosis induction animal model with the pathophysiological mechanisms that occur in both postmenopausal and GC-induced osteoporosis in humans by deficient bone formation resulting from an uncoupling of the bone formation and resorption during the reversal phase (Jensen et al., 2011; Andersen et al., 2013; Andreasen et al., 2015). In this way, a complete analytical panel was determined referring to BTMs, whose existing commercial kits have reactivity to ovine species, relative to that published in the scientific literature. The evaluation of the BTMs variation, prior to OVX and then in a monthly pattern over 6 months post-OVX, in which GC were administered weekly, was complemented by bone tissue composition and properties evaluation through the analysis of bone biopsies obtained at the body level of the lumbar vertebrae and femoral heads by means of micro-CT, mechanical compression tests and bone histology and histomorphometry.

During bone loss in osteoporosis, BTMs are released into the blood stream and/or excreted by kidneys in urine, namely BALP, OC and CTX (Seibel, 2005). The BALP is a glycoprotein and a membrane-bound enzyme synthesized and expressed by osteoblasts (Millan, 2006) and it is involved in the calcification of bone extracellular matrix (ECM) through the hydrolysis of phosphate esters on the osteoblast cell surface, resulting in a high extracellular inorganic phosphate concentration (Whyte, 1994). The OC is also a non-collagenous bone protein synthesized by mature osteoblasts, deposited in bone ECM during bone formation, with a small amount released to blood stream during this process and also during bone resorption process (Seebeck et al., 2005; Cremers et al., 2008), which influences bone mineralization by binding calcium and consequently hydroxyapatite formation (Neve et al., 2013). The CTX is a type I collagen molecule breakdown product, composed by a short peptide sequence from the non-helical domain of this molecule, which is released during bone resorption process (Cremers et al., 2008). Otherwise, TRAP is a glycoprotein produced by osteoclasts, activated macrophages and dendritic cells (Leeming et al., 2006). These last two markers are reliable and sensitive indicators of the bone resorption process. These BTMs have been evaluated as potential

tool for predicting impaired quality of bone and monitoring treatment of osteoporosis (Vasikaran et al., 2011) through rapid and non-invasive method such as blood or urine analyses. The normal values of BTMs in sheep were assessed in previously published studies (Klein et al., 2004; Seebeck et al., 2005; Dias et al., 2008; Sousa et al., 2014).

As markers of bone formation, it is understandable and expected that total ALP, BALP and intact OC almost always (except for intact OC at the 16th post-operative week) showed higher values over time in the control group than in the experimental group (Figures 1 and 2). Just CTX, representing a bone resorption marker, always presented higher values in the experimental group than control (Figure 2). From the hemodynamic point of view, these results confirm the reliability of the determination of these BTMs in sheep, proving the decreased bone formation by decreased levels of BALP and intact OC, which indicate delay or disruption in the process of bone tissue mineralization and increased resorption through increased CTX in the group. These results are in line with those presented by Andreasen et al. (2015), Kielbowicz et al. (2015) and Cabrera et al. (2018) who also determined intact OC and CTX in similar animal model, with those of Ding et al. (2010) for intact OC and also with the CTX results obtained in the study of Zarrinkalam et al. (2009). Thereby, following BTMs it is possible to consider that sheep model reaches the highest remodelling after 8 weeks of induction. Other authors support the same hypothesis that the sheep model undergoes the highest remodelling in short term and might be not suitable for long term studies (Sigrist et al., 2007; Zarrinkalam et al., 2009). However, the significant reduction of intact OC associated with an increase of CTX (Figure 2) could reflect a second wave of remodelling after the 20th post-operative week. Therefore, the seasonal influence might be a reasonable justification for that abrupt variation extended until the 24th post-operative week. The TRAP did not present statistical differences between groups which could be justified by the fact that this enzyme represents the set of two known isoforms – the TRAP 5a and 5b, and not just of the 5b isoform, which is the specific biomarker of osteoclastic resorption activity. Thus, the TRAP result might represent the metabolic expression of different organic tissues, and not just of bone. In the control group, serum Ca always showed higher levels over the post-operative period compared to the experimental group, although the analysis of variance of this parameter was not statistically significant, and serum P values were lower in the control group compared to the experimental group along time. This last parameter, according to the analysis of variance, is statistically significant (Figure 1). These differences allow us to perceive the involvement of these minerals in the process of bone

turnover and the negative correlation of the variation between these two parameters. Positive correlations in physiologic normal conditions between the formation marker BALP with serum Ca, although this one without a statistically significant result, and negative with serum P, support a possible involvement of this BTM with the mineralization process. Also, the significant positive correlation between BALP and OC and negative correlation between these two bone formation markers and the bone resorption marker TRAP, could help to confirm their role in the osteoblastic activity.

Although the analysis of variance of estradiol did not yield statistically significant results, it is noteworthy that its serum level remained in the experimental group always with values below those of the control group, declining until 24 weeks postoperatively (Figure 1), as expected, and as reported by Sigrist et al. (2007) and Kielbowicz et al. (2015). Also, regarding estradiol values, the result with high statistical significance should be highlighted in the study of correlations obtained in the control group in which this parameter varies positively with the bone formation markers BALP and OC and serum Ca and negatively with the marker of bone resorption CTX and serum P, which is extremely elucidative about the role of this hormone in the process of bone turnover. It should also be noted that all correlations obtained between estradiol and BTMs and serum minerals under normal physiological conditions change and disappear in the experimental group.

Regarding the results obtained by μ-CT analysis and the biomechanical compression test, we found that they are in agreement with each other, confirming that the results of BMD (Table 2), stiffness and ultimate stress (Table 3 and Figure 5) of bone tissue at vertebral level presented in general higher values in the experimental group compared to the control group, although the differences were not statistically significant. This seemingly unexpected result may be explained by the glucocorticoid stepwise reduction until zero in the last 4 weeks, since gradual removal of corticosteroids is required to avoid symptoms related to suprarenal gland atrophy and due to the later use of this animal model for testing of new anti-osteoporotic drugs or for its subjection to anaesthetic and surgical protocols, would promoted an imbalance in favor of bone formation process. Another aspect to be mentioned in the justification of these results will be the fact that BMD determination and mechanical tests were performed only at the level of the axial skeleton (lumbar vertebrae body) and not in the appendicular skeleton, the first location less subjected to the process of bone remodelling (Schorlemmer et al., 2003; Osterhoff et al., 2016). It should also be noted that in the present study there was no

comparison with preoperative values within the same group, but only between the experimental and the control groups at the end of 6 months of the osteoporosis induction protocol, with the control group representing a normal physiological condition. So, the possibility of a slight decrease in these parameters within the experimental group relatively to the preoperative values should not be totally excluded.

Although in the scientific literature numerous studies refer that similar OVX sheep model, associated with different GC treatments (GC type, dose and frequency, administration route and least duration), resulted in effective induction of osteopenia or osteoporosis 6 to 7 months after initiation of the induction protocol (Schorlemmer et al., 2003; Zarrinkalam et al., 2009; Andreasen et al., 2015; Kielbowicz et al., 2015, 2016; Cabrera et al., 2018), there also are studies reporting results analogous to those obtained in the present study. For instance, Turner et al. (1995) did not observe significant differences in BMD in OVX old sheep during 6 months treatment period at calcaneous, distal radius and lumbar vertebrae. Wu et al. (2008) also observed a slight increase in BMD at lumbar vertebral body level and of the bone mechanical properties at vertebral and femoral condyle levels 6 months after OVX in sheep compared to the control group, with significant differences in these parameters only observable postoperatively after 12 months. These authors also did not observe significant changes in the micro-architectural parameters from femoral neck and vertebral body specimens, which could only be observed at 12 months after OVX. In another study, Ding et al. (2010) also verified that a prolonged GC treatment in OVX sheep, associated with a low calcium and phosphorus intake, was needed for a long-term observation (7 months) to keep osteopenic bone at lumbar vertebrae and that the microarchitecture and bone strength, after 3 months without GC treatment, recovered to a similar level of the controls. Cabrera et al. (2018) also observed, after cessation of GC administration in aged-OVX sheep, a recovery of bone mass in an experimental group treated with GC for only 2 months.

Osterhoff et al. (2016) describe, regarding changes in the trabecular bone tissue during early osteoporosis, that there is an associated reduction in bone mass and in strength of this type of bone tissue. However, they also refer that trabecular properties changes are variable depending on time and anatomical site, generally with trabecular bone loss being more evident in extremities than in central regions. Regarding the later changes in the cortical bone tissue with osteoporosis progression and ageing, Osterhoff et al. (2016) refer an increase in mineral content, in porosity and in diameter of the bone cortex associated with a degradation of mechanical properties and a decrease of cortical

bone thickness. The review of Osterhoff et al. (2016) confirmed the present study results with histological analysis showing an increase of porosity of cortical bone tissue at vertebral and femoral head levels, proving that BMD values assessed by imaging techniques and/or the biomechanical results should be associated with bone tissue composition and morphological analysis for an accurate diagnostic of the osteopenic/osteoporosis degree. The evidences of osteoporosis are firstly at histomorphometric level, before the biomechanical symptoms initiate (e.g. noticed fractures) star (Felsenberg and Boonen, 2005), since the bone response to mechanical loads depend on loading direction (compression, tension, rotation), but also on the density, stiffness, and microarchitecture and composition of cortical and trabecular bone tissues (Osterhoff et al., 2016).

Regarding morphometric evaluation of bone tissue, the vertebral cortical bone of the healthy sheep presented an average of 3.8% and 757.6 μm for the Ct.Po and Ct.Th, respectively (Figure VI.10), and the values reported for healthy human bones are Ct.Po<5% (Clarke, 2008) and a Ct.Th average around 641 μm (Roux et al., 2010). Which concerns the histomorphometric analysis at the vertebral trabecular bone level, the sheep from control group presented BV/TV of 37.8%, Tb.Th of 196.8 μm, Tb.Sp of 417.5 μm and Tb.N of 2.575 (Figure VI.10) and the values reported for humans are 28.8%, 228 μm, 543 μm and 1.46 for BV/TV, Tb.Th, Tb.Sp and Tb.N, respectively (Hans et al., 2011). These results confirm the studies of Aerssens et al. (1998) and McLain et al. (2002) referring that the forces generated by large and sizable musculature supporting the spine of quadruped domestic mammals increase axial compressing stresses enhancing vertebral bone mass, which exceed that of humans. In the present study, histomorphometric analysis also confirmed the above mentioned by Felsenberg and Boonen (2005) with porosity and thickness of cortical bone tissue surrounding the body of the vertebrae to be significantly affected in the experimental group with respect to control group, although there were no significant differences based on micro-TC and mechanical tests. Histomorphometry analysis proved that the greatest influence on the morphological characteristics in the vertebral cortical bone tissue, which constitutes the outer envelope of the vertebrae body, showing a significant decrease in terms of its porosity and thickness, was observed at L6 and L7 segments. Similar results were observed by Zarrinkalam et al. (2009) in L2-L4 for cortical bone thickness in GC-treated (6 months) OVX sheep. However, the L4 showed up the most influenced vertebra by treatment, which concerns the morphometric evaluation of trabecular bone tissue, based on the

increase of trabecular separation, with L5 presenting a similar pattern although without statistical confirmation. Also, the results of the vertebral trabecular bone histomorphometry analysis in general confirm the results obtained through micro-TC and biomechanical tests just performed at lumbar vertebral body samples.

Sheep have quadruped locomotion and the generated forces over each of the lumbar spine vertebrae are difficult to determine. However, considering that there is an increase of the torsion in the last vertebrae in the locomotion of quadruped animals (Boszczyk et al., 2001), this could justify why L6 and L7 vertebrae are the most likely to be affected concerning the cortical bone tissue. On the other hand, vertebral trabecular bone has a complex and inhomogeneous 3D microstructure, adapted to support large deformations due to flexion, extension and rotation of the whole skeleton (Seeman, 2003). Thus, variations in vertebral trabecular bone microstructure is crucial for assessing vertebrae functional status, being Tb.Sp, Tb.Th and Tb.N major parameters influencing the mechanical strength of a bone (Felsenberg and Boonen, 2005). These parameters are usually altered with osteoporosis in humans (Osterhoff et al., 2016), and in the study of Zarrinkalam et al. (2009) significant differences in trabecular bone were observed in the lumbar spine of sheep with a decreased BV/TV by 29.2% and a Tb.Th decreased by 33.4% after 6 months of OVX + GC treatment ($p<0.01$). In the present study no significant differences were observed after the OVX + GC treatment however, there was a slight decrease in BV/TV, Tb.Th and Tb.N and a slight increase in Tb.Sp in the experimental group relative to the control (Figure VI.10).

In relation to femoral heads, the histomorphometric values between sheep and humans are more disparate than the vertebrae but could also be compared. Sheep presented BV/TV of 48.1%, Tb.Th of 305.4 μm, Tb.Sp of 448.9 μm and Tb.N of 2.6 in the control group (Figure VI.17). Comparing with the values reported in humans, 26.1%, 194 μm, 638 μm and 1.595 are the values for BV/TV, Tb.Th, Tb.Sp and Tb.N, respectively (Hildebrand et al., 1999). This disparity of results can be explained by the difference in the type of locomotion between sheep and humans. In primates – *Pan paniscus*, in terms of kinematics and dynamics, the trunk is around 37° more erect and plantar pressure distributions are different during bipedal locomotion (D'Août et al., 2004). In the evaluation of bone samples obtained at the femoral head level, all measured morphometric parameters presented statistically significant changes induced by treatment, OVX associated with the administration of CG. Thus, in the cortical bone tissue occurred increase of porosity and decrease of thickness, and in trabecular bone

tissue decreased of bone volume, thickness and number of bone trabeculae, and increased trabecular separation, thus confirming the greater sensitivity of the cortical and trabecular bone tissue at limb level as referred by Osterhoff et al. (2016).

5. Conclusions

Ewe have been one of the most widely used large animals in the study of postmenopausal osteoporosis pathophysiology, as well as in preclinical testing of new pharmacologic strategies for their treatment, research in biomaterials and in the development of orthopedic implants and prostheses for fixation of fragility fractures and joints replacement in the osteopenic/osteoporotic skeleton, respectively. Thus, this study used a large variety of methods to evaluate the effect of GC-treated OVX sheep for extensive characterization of this large animal model of osteoporosis. The BTMs determined in this study proved to be very sensitive parameters in determining changes in the bone turnover process by the association of the influence of estrogen lowering and the effects of GCs at the cellular level. However, in the present study it was found that the combination of GC-treated OVX sheep may not show explicit results of osteoporosis induction at the level of trabecular vertebral tissue with regard to decreased BMD and alteration of the parameters obtained by the mechanical tests, due to possible recovery of preoperative values after cessation of GC administration. Possibly, the utilization of GC-treated aged-OVX sheep, associated with a calcium/vitamin-D deficient diet, would have allowed more obvious results of osteoporosis induction at the vertebral level.

However, the results were more relevant at the cortical and trabecular bone femoral level, considering the results obtained by histologic and histomorphometric analysis that shown evidences of osteoporosis-related bone degeneration in the GC-treated OVX sheep. This result also reinforced the fact that the isolated use of imaging techniques for BMD determination may be insufficient for the diagnosis of early-stage osteopenia/osteoporosis. Thus, it is increasingly important to develop new/combined diagnostic techniques to evaluate early osteoporotic bone changes. At this particular point, a very recent study recommends the marrow adiposity and cortical porosity indices determination through high-resolution peripheral quantitative TC, associated to BMD measurement, to improve the fragility fracture risk evaluation in women (Zebaze et al., 2019).

Another original aspect of this study has to do with the analysis of trabecular bone tissue individually in the body of all lumbar vertebrae – L1 to L7, on the influence of OVX associated with GC administration in sheep by micro-CT analysis, mechanical tests and histomorphometric. This analysis aimed to verify whether if significant differences would occur between different vertebrae, allowing to conclude that significant differences arose at the cortical tissue level at L6-L7 and at the trabecular bone tissue level at L4, showing these vertebrae as the most suitable for further pre-clinical and translational studies of vertebral augmentation or spinal fusion in this animal model.

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Chapter VII – Final Conclusions

1. Final Conclusions

- Literature presents different methods to induce osteoporosis in small ruminants, isolated OVX is the method chose for the majority of the authors. However, OVX associated with steroids showed a significant bone loss promoted by an arrest of the reversal phase in previously studies.

- Bone turnover biomarkers represent a promising field to clinic human medicine however biological and analytical variability remain as first impediment to clinic use. On the other hand, its use in research is admittedly wide and proven efficient by this study.

- Bone mineral density is a recognized method of osteoporosis diagnosis however our study presented a low decreased of BMD at the level of trabecular vertebral tissue also verified by mechanical test. This fact may be associate to a cessation of glucocorticoids administration over the last 4 weeks of the study which can be lead a recovery of the bone tissue.

- Histology and histomorphometry results clarified the variation of cortical and trabecular bone of the femoral head and lumbar vertebrae. Where femoral head obtained a higher variation compared to lumbar vertebrae. While the analysis of lumbar veterbrae allowed to conclude that L4, L6 and L7 are the most suitable vertabrae for orthopedics stuidies due to highest variation of trabecular tissue by the first one and cortical tissue by last two.

- Analysis of BMD results associate with histologic and histomorphometric results lead to conclude that isolated imaging techniques can be insufficient method to assess early osteoporotic changes. Thus, further studies are necessary to develop a reliable method of assessment.

- The knowledge about patophysiological mechanism during osteoporosis induction in sheep have been increasing recently, therefore this animal model holds similar features to developed by humans during osteoporosis.

- This study consider that further investigation is required in order to obtain an intense bone loss similar which mimic the human osteoporosis. However, the sheep model is feasible and applicable model for different areas of research such as development of biomaterials and orthopedic implants.