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UNIVERSIDADE DO PORTO

EFFECT OF DIFFERENT LANTHANIDES ON HUMAN BONE CELLS DIFFERENTIATION

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DISSERTAÇÃO DE MESTRADO APRESENTADA
À FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO EM
ENGENHARIA BIOMÉDICA

U.PORTO
FEUP FACULDADE DE ENGENHARIA UNIVERSIDADE DO PORTO

MEB - MESTRADO EM ENGENHARIA BIOMÉDICA 2014/2015

A Dissertação intitulada

"Effect of different lanthanides on human bone cells differentiation"

foi aprovada em provas realizadas em 15-07-2015

o júri

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Resumo

Os lantanídeos são substitutos isomórficos dos iões de cálcio, na medida em que possuem raios iónicos, preferência pelos átomos doadores e números de coordenação análogos. Devido a esta similaridade, estes elementos exibem uma acentuada atividade biológica e têm sido investigados devido às suas potenciais aplicações médicas. A afinidade dos lantanídeos pelo tecido ósseo já é reconhecida há décadas, contudo, só recentemente foi sugerido o seu potencial para substituir o cálcio ósseo e afetar o ciclo de remodelação óssea, através da modelação do desenvolvimento das células ósseas. O efeito dos lantanídeos em populações celulares relevantes para o metabolismo ósseo têm sido analisado em diversos trabalhos. Contudo, apenas um número limitado de trabalhos reportou o efeito destes elementos em células ósseas humanas cultivadas *in vitro*.

Neste contexto, analisaram-se os efeitos celulares e moleculares dos lantanídeos no desenvolvimento de células humanas precursoras de osteoblastos e osteoclastos. As culturas celulares de osteoblastos foram obtidas a partir de cabeças de fémur de pacientes (25-45 anos) submetidos a cirurgia ortopédica e foram caracterizadas para o conteúdo de ADN, apoptose, atividade da fosfatase alcalina (ALP), coloração histoquímica da ALP e colagénio, e coloração da F-actina. As culturas celulares de osteoclastos foram estabelecidas a partir de células percursoras isoladas de sangue periférico humano (PBMCs) e caracterizadas para o conteúdo de ADN, apoptose, atividade da fosfatase ácida resistente ao tartarato (TRAP), número de células multinucleadas TRAP positivas e pela presença de células com anéis de actina e que expressam recetores de vitronectina e calcitonina. Adicionalmente, foi também analisado o envolvimento de algumas vias de sinalização associadas ao processo de osteoblastogénese e osteoclastogénese, na resposta celular induzida pelos lantanídeos.

As culturas celulares estudadas foram cultivadas na presença de zinco e magnésio (como catiões de referência) e três lantanídeos diferentes (cério, lantânio e praseodímio) em concentrações compreendidas entre 10^{-7} e 10^{-3} M e foram caracterizadas após 14 e 21 dias em cultura.

Os resultados obtidos indicaram que o cério (na maioria das concentrações testadas) não afetou significativamente a proliferação dos precursores osteoblásticos. Adicionalmente, foi observado que este catião, nas concentrações mais baixas testadas, induziu a diferenciação dos precursores osteoblásticos e que este efeito foi perdido nas concentrações mais altas testadas.

A densidade dos precursores osteoclásticos foi regulada positivamente pelas concentrações mais baixas de lantânio, contudo, este efeito foi-se perdendo ao longo do tempo. Por outro lado, a diferenciação dos precursores osteoclásticos foi inibida pelas concentrações mais altas de lantânio em ambos os dias.

O praseodímio, independentemente da concentração testada, pareceu actuar como um modulador positivo da proliferação dos precursores osteoblásticos, enquanto a influência oposta foi observada relativamente à densidade dos precursores osteoclásticos. Embora este catião tenha mostrado a capacidade de regular negativamente a diferenciação tanto dos precursores osteoblásticos como dos precursores osteoclásticos, nas culturas dos precursores osteoclásticos este efeito só foi observado nas concentrações mais altas.

Todos os lantanídeos testados modularam o desenvolvimento das células osteoclásticas e osteoblásticas, embora tenham revelado diferentes perfis de modulação dos processos de osteoblastogénese e osteoclastogénese. Adicionalmente, foi observado que o efeito promovido pelos lantanídeos depende da sua concentração e do tempo de cultura. Foi ainda observado que o mesmo lantanídeo pode afetar os dois tipos celulares de forma inversa. Em termos gerais as vias de sinalização testadas foram afetadas de forma diferente pelos lantanídeos, sugerindo que estes elementos afetam os processos de osteoblastogénese e osteoclastogénese através de diferentes mecanismos.

Em suma, este trabalho demonstrou a capacidade dos lantanídeos de modular o desenvolvimento de células ósseas humanas e estabeleceu algumas das vias de sinalização que poderão estar envolvidas na resposta celular induzida pelos mesmos. Os resultados obtidos reiteraram a necessidade de compreender e manipular o comportamento dual destes elementos de forma a induzir o efeito pretendido. Deste modo, é necessário investigar com mais detalhe o efeito dos lantanídeos na atividade das células ósseas humanas e clarificar os seus mecanismos de ação.

Abstract

Lanthanides are isomorphic replacements of calcium ions, as they possess analogous ionic radius, donor atom preferences and coordination numbers. By virtue of their resemblance to calcium, lanthanides exhibit a pronounced biological activity and have attracted intensive research interest for medical applications. Although the affinity of lanthanides for bone has been known for decades, their potential to exchange with calcium in bone and affect bone remodeling cycle, by modulating osteoblast and osteoclast development, was only suggested a few years ago. Since then, several works have addressed the effect of lanthanides in cellular populations with relevance to bone metabolism. Nevertheless, only a limited number of studies have reported the effect of lanthanides on human bone cells cultured in vitro. Furthermore, the signaling pathways involved on the cellular response have not yet been elucidated.

In this context, the aim of this study was to investigate the cellular and molecular effects of lanthanides on human osteoblast and osteoclast precursor cells development. Osteoblast precursor cell cultures were obtained from femur heads of patients (25-45 years old) undergoing orthopedic surgery procedures and were then studied for DNA content, apoptosis, alkaline phosphatase (ALP) activity, histochemical staining of ALP and collagen, and F-actin staining. Osteoclastic cell cultures were established from precursor cells isolated from human peripheral blood (PBMCs) and were characterized for DNA content, apoptosis, tartrate-resistant acid phosphatase (TRAP) activity, number of TRAP-positive multinucleated cells, and the presence of cells displaying F-actin rings and expressing vitronectin and calcitonin receptors. Also the involvement of some osteoblastogenesis and osteoclastogenesis-related signaling pathways on cellular response were addressed.

Cell cultures were treated with zinc, magnesium (as reference physiological cations) and three different lanthanides (cerium, lanthanum and praseodymium) at concentrations of 10^{-7} M to 10^{-3} M, and were characterized at days 14 and 21.

The results showed that cerium (in the majority of the concentrations tested) was not able to significantly affect the proliferation of osteoblast precursor cells. Moreover, it was observed that this cation induced the differentiation of osteoblast precursor cells at the lowest concentrations tested, and that this effect was lost at the highest concentrations.

The density of osteoclast precursor cells was up-regulated by the lowest concentrations of lanthanum, nevertheless, this effect was also showed to be lost over time. On the other hand, the differentiation of osteoclast precursor cells was inhibited by the highest concentrations of lanthanum at both days.

Praseodymium, regardless of the concentration tested, appeared to act as a positive modulator of osteoblast precursor cells proliferation, while the opposite influence was observed concerning the density of osteoclast precursor cells. Although, this cation was shown to be able to regulate negatively the differentiation of both osteoblast and osteoclast precursor cells, this effect on osteoclast cultures was only observed at the highest concentrations tested.

Hence, all lanthanides were shown to be able to affect osteoclastic and osteoblastic precursor cells development although with different profiles on their osteoblastogenic and osteoclastogenic modulation properties. Moreover, it was observed that the effect of the lanthanides is closely related to the concentration and culture time. Also, it was observed that the same lanthanide may affect osteoclastic and osteoblastic precursor cells development in an antagonistic manner.

The signaling pathways involved in the process were overall affected differently by the different lanthanides tested, suggesting that they affect osteoblastogenesis and osteoclastogenesis processes through different mechanisms.

In sum, the present study underlined the ability of lanthanides to modulate the development of human bone cells and outlined some of the signaling pathways involved in this cellular response. Furthermore, the results obtained reiterate the need to understand and to manipulate the dual behavior of these elements to attain the required effect. Accordingly, further experiments are required to fully understand the effect of lanthanides on human bone cells activity and their mechanism of action.

Acknowledgments

This work was only possible thanks to the contribution of several people to which I want to express my gratitude:

To Professor João Rodrigues, my supervisor, for giving me the opportunity to undertake this work, and for his constant support, availability, incentive and knowledge transmitted.

To my co-supervisor, Professor Maria Helena Fernandes, who provided all the necessary conditions for the development of this thesis.

To Dra. Mónica Garcia for all the training, availability, support and patience.

Finally, I would like to thank my parents and sister for providing me with the conditions to take this course and for all the encouragement on the difficult days, and to João for the unconditional love, support and patience.

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Abbreviations and acronyms

Arp	Actin-Related Proteins
Akt	Protein Kinase B
ALP	Alkaline Phosphatase
AP-1	Activator Protein-1
BAD	Bcl-2-Associated Death Promoter
BSP	Bone Sialoprotein
BMP	Bone Morphogenetic Proteins
BMU	Bone Multicellular Unit
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CASR	Calcium - Sensing Receptor
CTR	Calcitonin Receptors
CLSM	Confocal Laser Scanning Microscopy
Dlx5	Distal-Less Homeobox-5
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Signal-Regulated Kinase
FGF	Fibroblast Growth Factor
Gla	Gamma-Carboxyglutamic Acid
HAp	Hydroxyapatite
IGF	Insulin-Like Growth Factor
IL	Interleukin
IκB	Inhibitory κB
JNK	c-Jun N-terminal kinase
LPR	Lipoprotein Receptor-Related Protein

LDH	Lactate Dehydrogenase
Ln	Lanthanides
MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage Colony-Stimulating Factor
Mcl	Myeloid Cell Leukemia
MEK	Methyl Ethyl Ketone
MITF	Microphthalmia-Associated Transcription Factor
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cell
MTT	3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MV	Matrix Vesicle
NFATc1	Nuclear Factor Of Activated T-Cells Cytoplasmic 1
NF-κB	Nuclear Factor-Kappa ß
OC	Osteocalcin
ON	Osteonectin
OPG	Osteoprotegerin
OPN	Osteopontin
ON	Osteonectin
Osx	Osterix
PBS	Phosphate-Buffered Saline
PBMC	Peripheral Blood Mononuclear Cell
PDGF	Platelet-Derived Growth Factor
PG	Prostaglandin
PI3K	Phosphoinositide 3-Kinase
PKC	Protein Kinase C
pNPP	p-nitrophenylphosphate
PTH	Parathyroid Hormone
RANK	Receptor Activator of Nuclear Factor Kappa-ß
RANKL	RANK Ligand
RGD	Arginine-Glycine-Aspartate
ROS	Reactive Oxygen Species
Runx2	Runt-Related Transcription Factor 2
Tcf	T-cell factor
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TRAF	TNF Receptor-Associated Factor

TRAP	Tartrate-Resistant Acid Phosphatase
TNFR	Tumor Necrosis Factor Receptor
VNR	Vitronectin
WASp	Wiskott-Aldrich syndrome protein
α-MEM	α-Minimal Essential Medium

Chapter 1

General Introduction

1.1 Bone Tissue

Bone is a dynamic and specialized connective tissue, with a unique capacity to heal and remodel without leaving a scar (Salgado, Coutinho, & Reis, 2004). One of the primary functions of this tissue is to provide the mechanical integrity for both locomotion and protection of the vital organs. Metabolically, bone maintains the mineral homeostasis, regulates the acid-base balance, acts as a reservoir of growth factors and cytokines, and stores energy reserves as lipids in areas filled with yellow bone marrow. Furthermore, bone tissue encloses the red bone marrow thus providing the environment for haematopoiesis to occur (Kini & Nandeesh, 2012).

This tissue is renewed continuously in the adult skeleton in response to a variety of stimuli by the process of bone remodelling (Datta, Ng, Walker, Tuck, & Varanasi, 2008). This process allows each bone to adapt to changes in biomechanical forces and guarantees the removal of old and microdamaged bone, replacing it with new and mechanically stronger bone which ensures the preservation of bone strength (Clarke, 2008). Bone remodelling involves the interaction between different cell phenotypes and is regulated by a variety of biochemical and mechanical factors (Hadjidakis & Androulakis, 2006). A disruption or imbalance in these processes can lead to either an increase or decrease in bone mineral density that may be detrimental to skeletal strength. Accordingly, the process of bone turnover must be carefully regulated in order to maintain bone strength (Datta *et al.*, 2008).

1.2 Macroscopic Morphology

On a macroscopic level, bone exists in one of two forms: cortical bone, also called dense or compact bone, and trabecular bone, also called cancellous or spongy bone (Bancroft & Mikos, 2002; Sandhu, 2003). Overall the adult human skeleton is constituted of 80% cortical bone and 20% trabecular bone (Buckwalter, Glimcher, Cooper, & Recker, 1995; Hadjidakis & Androulakis, 2006). However, different types of bone and skeletal sites within the bone present different ratios of cortical to trabecular bone. For example, the ratio of cortical to trabecular bone in

the vertebra is 25 to 75 percent, in the femoral head 50 to 50 percent and 95 to 5 percent in the radial diaphysis (Clarke, 2008).

Cortical bone presents two types of surfaces. The surface on its inner side, facing the bone marrow, is known as the endosteum, and the surface facing the surrounding soft tissues, is known as the periosteum (Morgan, Barnes, & Einhorn, 2010). Cortical bone is dense and organized, providing protection and mechanical support. It is only 10% porous, and therefore provides space for only a low concentration of cells and blood vessels (Suárez-González & Murphy, 2008).This tissue makes up the outer tubular shell of long bones and the outer surface of small and flat bones in the skeleton (Bancroft & Mikos, 2002).

Trabecular bone is loosely organized and highly porous (50-90% porosity) and contains a functional vasculature and a bone marrow space (Suárez-González & Murphy, 2008). Due to its characteristics, trabecular bone is more elastic, has a higher turnover rate and performs a more active metabolic function than cortical bone (Downey & Siegel, 2006). Trabecular bone is found near the ends of long bones, in the interior of small bones, and between the surfaces of flat bones (Bancroft & Mikos, 2002).

1.3 Microscopic Morphology

At the microscopic level, bone is described as either woven or lamellar bone, depending on the organization of the collagen fibres (Becken, Hilger, & A.E., 2005).

Woven bone, also designated as primary bone, is characterized by a random organization of the collagen fibres. This type of bone exists mainly during embryonic and fetal development. In later years, woven bone is found at sites of fracture healing and in some metaphyseal regions of the growing skeleton since it is laid down more quickly than lamellar bone (Downey & Siegel, 2006; Kini & Nandeesh, 2012; Morgan *et al.*, 2010). Lamellar bone, which is the major portion of both cortical and trabecular bone in adults, is more highly organized and specialized than woven bone. Collagen fibres in the lamellar bone are aligned in thin sheets, called lamellae, which are stacked in a plywood-type arrangement (Morgan *et al.*, 2010).

Both woven and lamellar bones contain small cavities called lacunae which are connected to each other by means of tubular canals called canaliculi. The lacunae are arranged along the interfaces between the lamellae in the lamellar bone. Osteocytes, a specific type of bone cells, remain entrapped within these lacunae and receive and transmit nutrients and stimuli from and to the bone through the canaliculi (Cowin, 2007; Kamioka, Honjo, & Takano-Yamamoto, 2001).

Lamellar bone is also commonly arranged in smaller cylindrical structures, called secondary osteons or Harversian systems (Gore, Unnikrishnan, Hussein, & Morgan, 2012). Osteons make up approximately two-thirds of cortical bone volume. Each osteon consists of 10 to 30 concentric rings of lamellae, which surround a central cavity, the Haversian canal, in which blood vessels and nerves are contained. Each osteon is in direct contact with the periosteum, the bone marrow and with other osteons through horizontally oriented canals, called Volkmann's canals. The osteons have a typical diameter of approximately 200 μm and lengths of 1 to 3 mm. The outer border of each osteons contains a cement line that consists of a layer of mineralized matrix attaching adjacent osteons together (Gore *et al.*, 2012; Morgan *et al.*, 2010).

In trabecular bone, the counterpart to the osteon is the trabecular packet (Sandhu, 2003). Although, trabeculae are primarily composed of lamellar bone arranged in packets, ticker tra-

beculae can also contain secondary osteons (Gore *et al.*, 2012). Trabecular osteons are semilunar in shape, normally approximately 35 mm thick, and composed of concentric lamellae. It is estimated that there are 14 x 10^6 trabecular osteons in healthy human adults, with a total trabecular area of approximately 7 m^2 (Clarke, 2008).

1.4 Microscopic Organization

Bone is a composite material containing an inorganic and an organic phase. By weight, approximately 60% of bone tissue is inorganic matter, 8 to 10% water and the remainder, is organic matter (Gong, Arnold, & Cohn, 2005).

The mineral (or inorganic) phase of bone is mainly formed by a calcium-deficient, carbonate-containing, poorly-crystalline analogue of the naturally occurring mineral hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$ or HAp) (A.L. Boskey, 2005). The small plate-shaped (20-50 mm long, 15 nm wide, and 2-5 nm thick) apatite crystals contain impurities, most notably small amounts of carbonate in place of the phosphate groups. Other noticed substitutions are magnesium, potassium, strontium and sodium in place of the calcium ions and chloride and fluoride in place of the hydroxyl groups. These impurities reduce the crystallinity of the apatite resulting in the change of certain physical properties, such as the solubility (Zhu, Robey, & Boskey, 2010). Hence, bone mineral crystals are more soluble than geologic apatite, which facilitates bone to act as a reservoir for calcium and phosphate and other ions (Hammett-Stabler, 2004).

The organic matrix (or osteoid) is mainly composed of type I collagen that constitutes up to 85-90% of the total protein in the bone matrix. The remaining 10-15% in composed of noncollagenous proteins, whereas the cellular content comprises less than 1% of the bone mass (Robey & Boskey, 2008). Type I collagen is the most abundant protein in vertebrates. Besides being expressed in bone, type I collagen is also expressed in most of the other connective tissues, especially in tendons, ligaments and dermis. The repetitive nature of the amino acid sequences of collagen, which consists of Gly-X-Y, where X and Y are often proline and hydroxyproline residues, allows the protein to assemble into triple helical structures. Type I collagen is secreted as a propeptide, but the globular ends are cleaved rapidly by specific proteases, in order that shorter molecules assemble to form fibrils (Bou-Gharios & Crombrugghe, 2008). These fibrils are gathered in bundles to form collagen fibres. Noncollagenous proteins and mineral are found within gaps, named hole zones, that exists at the ends of collagen molecules and within pores formed between the sides of parallel molecules (Gore *et al.*, 2012; Morgan *et al.*, 2010).

Noncollagenous proteins present in bone matrix appear to regulate the organization, turnover, and mineralization of the bone matrix. In fact, most of these proteins appear to have more than one function (A.L. Boskey, 2005).

Osteocalcin (OC) is the major non-collagenous protein of bone, accounting for 10 to 20% of the noncollagenous protein content (van Gaalen *et al.*, 2008). OC is exclusively synthesized by osteoblasts, odontoblasts, and hypertrophic chondrocytes and is only marginally detectable during the earlier phases of proliferation and matrix maturation (Seibel, 2001). This protein contains three vitamin-K dependent gamma-carboxyglutamic acid (Gla) residues, which are responsible for the calcium-binding properties (van Leeuwen, van der Eerden, van de Peppel, Stein, & Lian, 2013). Due to its interaction with HAp it is believed that OC regulates the growth and maturation of bone crystals (Gundberg, 2003). Moreover, some investigators have suggested that OC may act as a chemoattractant for osteoblasts (Chenu *et al.*, 1994).

Osteonectin, osteopontin and bone sialoprotein are multifunctional phosphorylated glycoproteins with high affinity for bone mineral carrying one or more arginine-glycine-aspartate (RGD) sequences, thus capable to linking cells, mineral and matrix (A.L. Boskey, 2005). Osteonectin (ON) is a glycoprotein secreted by the osteoblast that binds collagen, calcium and Hap (van Leeuwen *et al.*, 2013). It is assumed that this molecule supports bone remodelling, regulates cell proliferation and cell-matrix interactions, and stimulates the production of matrix metalloproteinases (Sroga & Vashishth, 2012). Osteopontin (OPN) is a phosphorylated glycoprotein that is expressed in several tissues, including bone, kidney and in the immune and vascular systems (Lund, Giachelli, & Scatena, 2009). In bone, OPN is produced by osteoblasts, osteocytes, macrophages, and osteoclasts and is found at the late stage of osteoblastic maturation, during matrix formation and mineralization (Robey, 2008; Zhu *et al.*, 2010).OPN promotes osteoclast binding to the extracellular matrix and activates intracellular signalling pathways (McNamara, 2011; Robey, 2008). During normal bone mineralization, osteoclast-derived OPN inhibits the formation of HAp, thus acting as an inhibitor of mineralization (Hunter, 2013). Bone sialoprotein (BSP) comprises 15% of the total noncollagenous proteins in bone (Wuttke *et al.*, 2001). BSP expression marks a late stage of osteoblastic differentiation and an early stage of matrix mineralization (Robey, 2008). This sialoprotein binds to collagen and promotes osteoblast attachment (A.L. Boskey, 2005). Additionally, it is considered that BSP also plays a role in matrix mineralization due to its high affinity for calcium and because of the timing of its appearance in relation to the timing of mineral emergence (Robey, 2008). Although, alkaline phosphatase (ALP) is found mainly distributed on the cell membrane and in matrix vesicles, it is also a bone matrix constituent (Bonucci, 2013). ALP is considered an important regulator of mineralization, due to its ability to enzymatically degrade pyrophosphate, a mineralization inhibitor *(*Gade *et al.*, 2011).

Proteoglycans play important roles in organizing the bone extracellular matrix, taking part in the structuring of the tissue itself as active regulators of collagen fibrillogenesis (Lamoureux, Baud'huin, Duplomb, Heymann, & Rédini, 2007). The two most abundant proteoglycans present in bone matrix are decorin, and biglycan (Zhu *et al.*, 2010). These proteoglycans bind to type I collagen and interact with several growth factors, therefore, it is assumed that they play a part in modulating the activity of growth factors and matrix proteins (Robey, 2002). Furthermore, decorin and biglycan were both found to inhibit bone cell attachment, apparently by binding to fibronectin and inhibiting its cell-matrix binding abilities (Robey, 2002).

Growth factors and cytokines such as, transforming growth factor-ß (TGF-ß), insulin-like growth factor (IGF), osteoprotegerin, tumour necrosis factors (TNFs), interferon-γ and bone morphogenetic proteins (BMPs 2-10) are present in very small quantities in bone matrix. These proteins are responsible for the regulation of bone cell behaviour, namely for, cell differentiation, activation, growth and turnover. Additionally, it is thought that these growth factors act as coupling factors linking bone resorption and formation processes (Zhu *et al.*, 2010).

1.4.1 Matrix Mineralization

Mineralization of bone collagen fibrils occurs in an organized manner. Initially crystal appear in hole zone regions within individual fibrils, separated by unmineralized regions. Gradually they start to appear in an increasing number of hole zone regions. Ultimately, crystal growth, embraces the zone of the collagen fibrils between hole zones and occupies all of the available space within the fibrils (Buckwalter *et al.*, 1995).

One possible mechanism of HAp crystal formation is via extracellular matrix vesicles (MVs) (New & Aikawa, 2013). MVs are small (approximately 100 nm in diameter) membrane particles, which bud off from the plasma membrane of mineralizing cells (chondrocytes, osteoblasts and odontoblasts) and are released into the pre-mineralized organic matrix (Golub, 2011; Vigorita, 2008). The signals that promote MVs release are not well understood, although concentrations of intracellular calcium and extracellular phosphate may be important (Orimo, 2010).

Mineralization occurs in two phases: an initial formation of apatite within the MV, and a subsequent propagation phase in the matrix (Golub, 2011). The first phase starts with the incorporation of calcium into the MV by calcium-binding proteins and calcium-binding lipids present in the MV membrane. Phosphate concentration is also raised during this phase through the enzymatic activity of phosphohydrolases, such as ALP, which reside on the MV membrane (Anderson, 2003; Leonor, Azevedo, Alves, & Reis, 2005; Nair & Jagannathan, 2013). At some point, the elevation of calcium and phosphate within the protective environment of the membrane leads to the precipitation of amorphous calcium phosphate mineral. The amorphous calcium phosphate mineral is later converted into HAp via octacalcium phosphate. Subsequently, HAp crystals penetrate the MV membrane and get exposed to the extracellular fluid leading to the beginning of the second phase of mineralization process. The breakdown of MVs is assisted by the hydrolytic action of phospholipases and proteases (Anderson, 2003; Nair & Jagannathan, 2013). During the second phase the preformed apatite from the MVs migrate to collagen, become inserted into the aligned hole zones of the fibrils, and then undergo maturation (Golub, 2011; Leonor *et al.*, 2005). Rates of mineralization are affected by levels of calcium and phosphate in the extracellular fluid, pH, and by matrix components that are able to accelerate or retard mineral propagation (Vigorita, 2008).

Several families of proteins associated with the collagen matrix are involved in regulation of the mineralization process, as both inhibitors and promoters of mineralization depending on the extent of post-translational modification and/or their concentration (A.L Boskey, 2007). BSP, ON, dentin sialophosphoprotein, and ALP are nucleators or initiators of mineral crystal formation. These proteins bind to calcium and/or phosphate ions, creating a surface similar to the apatite surface that provides the start of nucleation (Clarke, 2008; Zhu *et al.*, 2010). Proteins, such as aggrecan, OPN, dentin matrix protein-1, α_2-HS glycoprotein, and albumin act as inhibitors of mineral crystal formation. These proteins can chelate calcium or phosphate ions creating a protected environment around the crystal nucleus which prevents crystal growth. On the other and OC, vitronectin and matrix Gla protein bind to one or more faces of the growing crystal, blocking crystal growth in one or more directions or blocking growth beyond a specific size. Other proteins, such as decorin, thrombospondin, fibronectin, vitronectin and versican bind to the collagen backbone of the matrix and to other non-collagenous proteins, changing their conformation and their ability to affect crystal nucleation and growth (Zhu *et al.*, 2010). Vitamin D participates indirectly in the promotion of bone matrix mineralization. Calcitriol, of the active form of vitamin D, maintains the appropriate serum calcium and phosphorus concentrations required for passive mineralization, through the stimulation of intestinal absorption of these minerals (Clarke, 2008).

1.5 Bone Cells

The main cell types associated with bone homeostasis are osteocytes, osteoclasts and osteoblasts. These cells account for approximately 90% of all cells in the adult skeleton (Morgan *et al.*, 2010; Sommerfeldt & Rubin, 2001). Bone cells are originated from two particular cell lines lineages: a mesenchymal stem cell lineage and a hematopoietic stem cell lineage. The osteoblasts and osteocytes are derived from mesenchymal stem cells (MSCs), whereas preosteoclasts and osteoclasts are originated from hematopoietic stem cells (Buckwalter *et al.*, 1995; Downey & Siegel, 2006).

1.5.1 Osteoblasts

Osteoblasts are the cells responsible for the synthesis and mineralization of osteoid, the protein component of bone tissue. Moreover, osteoblasts are also involved in the regulation of osteoclast differentiation and activity (Ferrari *et al.*, 2000; Kini & Nandeesh, 2012).

As mentioned formerly, osteoblasts derive from pluripotent MSCs, which prior to osteoblast commitment can also differentiate into fibroblasts, chondrocytes, myoblasts and bone marrow stromal cells depending on the activated signalling transcription pathways (A. Yamaguchi, Komori, & Suda, 2000b).

Osteoblasts are cuboidal cells, usually found in a single layer adherent to bone surface and contain an extensive and oriented secretory organelle apparatus that includes a large Golgi complex near the nucleus, a well-developed rough endoplasmatic reticulum and a high mitochondrial content (Kartsogiannis & Wah Nga, 2004; Mackie, 2003).

The maintenance of osteoblastic function and the ability of these cells to respond to metabolic and mechanical stimuli is sustained by cell-matrix and cell-cell interactions that occur through a variety of transmembranous proteins, such as, integrins, conexins and cadherins and specific receptors for cytokines, hormones and growth factors. In addition, the tight junctions formed between adjacent osteoblasts have regions of the plasma membrane specialized in vesicular trafficking and secretion (Ferrari *et al.*, 2000; Sommerfeldt & Rubin, 2001).

As osteoblasts differentiate from their precursors they begin to secrete bone matrix proteins into the region of unmineralized matrix between the cell body and the mineralized matrix (Gay, Gilman, & Sugiyama, 2000). The lifespan of human osteoblasts it is of approximately 8 weeks, and during this period osteoblasts lay down 0.5 to 1.5 µm of osteoid per day (Sommerfeldt & Rubin, 2001).

As mentioned, besides their role in osteoid synthesis, osteoblasts are also involved in osteoid mineralization. As osteoid becomes mineralized, osteoblasts follow one of three pathways: persist as active osteoblasts and disappear from the site of bone formation; become surrounded by their own calcified matrix and change their phenotype turning into osteocytes or become fairly inactive, decrease their synthetic activity and assume a flatter shape of bone lining cells (Dallas & Bonewald, 2010).

1.5.1.1 Osteoblastogenesis

Osteoblastogenesis can be divided into three stages, namely proliferation, matrix development, and maturation and mineralization (Eglence, Duivenvoorden, Ghert, & Singh, 2009). In the first stage, cells proliferate intensively and produce some extracellular matrix proteins,

such as fibronectin and type I collagen (Przekora & Ginalska, 2015). The second stage is characterized by a modification in the composition of extracellular matrix, a downregulation of proliferation and activation of genes correlated with matrix maturation and organization (Leonor, Gomes, Bessa, Mano, & Reis, 2008). The third stage of differentiation is connected with the mineralization process. During this stage osteoblasts have moderate ALP activity, high mineralization activity and express high levels of proteins that are correlated with mineral deposition (mainly, OPN and OC) (Przekora & Ginalska, 2015).

The markers most frequently used to follow osteoblasts differentiation include type I collagen, ALP and OC. Overall, type I collagen and ALP are thought as early osteoblastogenesis markers, while, OC is considered an advanced marker of osteoblast differentiation due to it's closely association with bone matrix mineralization (Eglence *et al.*, 2009).

Two transcription factors have been demonstrated to be required for osteoblast formation and differentiation, namely, runt-related transcription factor 2 (Runx2) and Osterix (Osx).

Runx2 has been described as a master regulator of osteoblastogenesis (Baek, Choi, & Kim, 2014). Expression of Runx2 is both necessary and sufficient for MSC differentiation towards the osteoblastic lineage. This factor directly stimulates the transcription of osteoblast-related genes, such as those encoding TGF-ß receptor, ALP, OC, OPN, vitamin D receptor, BSP, type I collagen and collagenase, by binding to specific enhancer regions containing the core sequence (Mackie, 2003). Hence, Runx2-deficient mice exhibit a complete lack of both intramembranous and endochondral ossification due to the absence of osteoblast differentiation (Baek *et al.*, 2014). Runx2 can be phosphorylated and thus activated by the mitogen-activated protein kinase (MAPK) cascade via stimulation of α2ß1-integrins on the osteoblast surface. Both expression and activity of Runx2 are tightly controlled by other transcription factors and by protein–DNA or protein–protein interactions. Stat1, Sox9, Sox8, Aj18, MEF, Nrf2, and YAP have been reported to repress Runx2 expression, while, Rb, TAZ, HoxA10, BAPX-1, Smad1&5, CEBP/ß&δ, and Menin actively enhance the function of Runx2 (J.F.L. Chau, Leong, & Li, 2008).

The second required transcription factor for osteoblast differentiation is the zinc finger-containing transcription factor Osx (J.F.L. Chau, Leong, & Li, 2009). It has been demonstrated that Osx is necessary for bone formation and mineralization *in vivo* (Nakashima *et al.*, 2002). Genetic inactivation of Osx in mice results in completely lack of mineralized bone (Baek *et al.*, 2014).

Additional studies indicated that Osx acts downstream of Runx2 in the transcriptional cascade of osteoblast differentiation since Osx knock-out mice express Runx2, but Osx is not expressed in Runx2 knock-out mice (Baek *et al.*, 2014; C. Zhang *et al.*, 2011). The mechanism underlying the regulation of Osx expression in osteoblasts is still unclear. However, it was shown that both BMP-2 and IGF-1 can induce Osx expression in undifferentiated MSCs. Furthermore, ascorbic acid and $1,25(OH)_2$ vitamin D_3, have also been shown to up-regulate Osx expression. On the other hand, some studies emphasized the ability of negative regulators of osteoblastogenesis to inhibit Osx expression. For instance, TNF was demonstrated to inhibit Osx mRNA in pre-osteoblastic cells (C. Zhang 2010).

BMP, Wnt, and Notch signalling pathways play important roles in osteoblast differentiation (Lin & Hankenson, 2011).

The first indication that Wnt signalling plays a critical role in bone formation came from human genetic studies where recessive loss-of-function mutations in lipoprotein receptor-related protein (LRP) 5 were linked to osteoporosis, while, dominant missense LRP5 mutations

were associated with high bone mass diseases (Lin & Hankenson, 2011; Zuo *et al.*, 2012). Canonical Wnt signalling in osteoblastogenesis has been linked to Runx2. The Runx2 gene promoter contains a Wnt-responsive T-cell factor (Tcf) regulatory element, and both ß-catenin and Tcf1 are recruited to the Runx2 locus. Additionally, transient activation of Wnt/ß-catenin signalling in MSCs *in vitro* induces expression of bone lineage genes such as Distal-less homeobox-5 (Dlx5) and Osx (Lin & Hankenson, 2011).

In vitro and *in vivo* studies have established that BMP-Smad signalling regulates osteoblast differentiation from MSC, osteoprogenitor cell expansion, osteoblast bone formation activity, and its coupling to osteoclasts (J.F.L. Chau *et al.*, 2009). BMP2 activates Smad1/5/8 signalling and regulates the transcription of osteogenic genes, including Dlx5, which is a key mediator of BMP2-induced expression of Runx2 (Jang, Kim, Lee, Son, & Koh, 2011). *In vitro* studies with a human marrow stroma derived cell line, demonstrated that BMP2 treatment promotes the increase of both Runx2 gene expression and ALP levels (Gori, Thomas, Hicok, Spelsberg, & Riggs, 1999).

MAPKs, namely, the p38, extracellular signal-regulated kinase (ERK) 1/2 and c-Jun N-terminal kinase (JNK) 1/2 were shown to promote the expression and activation of Runx2 (R. L. Huang, Yuan, Tu, Zou, & Li, 2014). Additionaly, the inhibition of p38 MAPK was shown to downregulate Osx expression and reduce osteoblast differentiation (J.F.L. Chau *et al.*, 2008).

As with BMP and Wnt signalling in osteogenesis, Runx2 function is also influenced by Notch signalling. Runx2 transcriptional activity is physically antagonized by the protein encoded by Notch target gene Hey1 (Lin & Hankenson, 2011). Transient transfection of MSCs with Notch intracellular domain, Hey decreases Runx2 transactivity. In addition, Notch intracellular domain can interact directly with Runx2 protein and repress terminal osteoblastic differentiation *in vitro* (Lin & Hankenson, 2011).

In addition, several hormones, such as, IGF-1, parathyroid hormone (PTH), and glucocorticoids influence osteoblast differentiation. For instance, PTH has been found to regulate the expression and activity of Runx2 and to inhibit Osx expression (Barbuto & Mitchell, 2013). Moreover, IGF-1 has been shown to promote osteoblast differentiation and mineralization *in vitro* (W. Zhang *et al.*, 2012). Lastly, glucocorticoid have been shown to inhibit osteoblast differentiation through the suppression of cytokines such as interleukin (IL)-11 (Rauch *et al.*, 2010).

1.5.2 Osteoclasts

Osteoclasts are highly specialized, multinucleated and terminally differentiated cells generally regarded as the only cells in the body capable of resorbing bone (Chambers, 2000; Clarke, 2008; Itzstein, Coxon, & Rogers, 2011).

Mobilization of osteoclasts precursors involves their release from bone marrow to the blood circulation and their guidance to bone resorption sites, where these cells fuse into multinucleated cells to form mature osteoclasts, a process that is regulated by osteoblasts and stromal cells of the bone marrow (Boyle, Simonet, & Lacey, 2003; Soysa, Alles, Aoki, & Ohya, 2012)

Osteoclasts contain large numbers of mitochondria and acidic vacuoles that carry acid phosphatases and other lysosomal enzymes. Compared to the cytoplasmic volume of the cells, the rough endoplasmic reticulum is scarce and the Golgi apparatus consists of few flattened cisternae around the nuclei, suggesting that mature osteoclasts do not synthesise large amounts of protein (Stenbeck, 2002).

Bone resorbing cells are highly migratory cells with a relatively short lifespan and considered rare in bone with only two to three cell per mm (Miyazaki *et al.*, 2012). Activated osteoclasts are able to resorb 200000 μm^3 of bone per day, an amount of bone formed by seven to ten generations of osteoblasts (Sommerfeldt & Rubin, 2001).

1.5.2.1 Osteoclast Activity

Osteoclast activation is initiated upon cell attachment to bone matrix, an event leading to osteoclast actin cytoskeletal reorganization. Cytoskeletal organization promote osteoclast polarization and the formation of new membrane domains, namely, the sealing zones and the ruffled membrane (Bellido, Plotkin, & Bruzzaniti, 2014).

The sealing zone appears as a ring of filamentous actin (F-actin, known as the actin ring), consisting of dynamic, densely packed small actin punctuate structures, called podosomes. These consist of a core of densely packed actin filaments and F-actin-associated proteins such as cortactin, Wiskott-Aldrich syndrome protein (WASp) and actin-related proteins (Arp) 2/3, surrounded by integrins and attachment-related proteins such as vinculin and talin (Itzstein *et al.*, 2011).

The attachment of osteoclast to bone matrix is controlled by the vitronectin receptor, αvβ3 receptor, which binds to RGD sequences within matrix proteins, such as vitronectin, OPN, and type I collagen (Bellido *et al.*, 2014).

As the osteoclast prepares to resorb bone, it attaches to the bone matrix through the sealing zone and forms another specific membrane domain, the ruffled border (Väänänen, Zhao, Mulari, & Halleen, 2000). The ruffled border is a highly convoluted membrane domain formed by the fusion of targeted transport vesicles with the apical membrane (Bellido *et al.*, 2014). This membrane, together with its underlying resorption lacuna create the bone-resorbing organelle in osteoclasts (Zhao & Väänänen, 2006).

As mentioned, the main physiological function of osteoclasts is to degrade mineralized bone matrix. This involves dissolution of crystalline HAp and proteolytic cleavage of the organic matrix, which is rich in collagen. Before proteolytic enzymes can reach and degrade collagenous bone matrix, tightly packed HAp crystals must be dissolved (Väänänen *et al.*, 2000). Osteoclasts resorb bone in resorption lacunae by generating a pH gradient between the cell and bone surface (Hienz, Paliwal, & Ivanovski, 2014). The low pH in the resorption lacuna is achieved by the action of ATP-consuming vacuolar proton pumps both at the ruffled border membrane and in intracellular vacuoles (Sagalovsky, 2013). Protons are provided to the proton pumps by carbonic anhydrase II, which is highly expressed in osteoclast cytosol (Baron & Horne, 2005). Carbonic anhydrase II converts CO_2 and H_2O into H^+ and HCO_3^- (Nakamura, 2007). The HCO_3^- ions are exchanged for Cl^- through an anion exchanger located in the basolateral membrane, leading to continued availability of Cl^- for acidification of the resorption lacuna (Sagalovsky, 2013). The hydrochloric acid formed in the resorption lacuna dissolves the HAp component of bone matrix and exposes the organic matrix to the enzymatic attack of cysteine proteases and metalloproteinases (Bellido *et al.*, 2014; Hienz *et al.*, 2014). The degradation of the demineralized organic component of the bone matrix is primarily due to the action of cathepsin K, a member of the papain family of cysteine proteases that is highly (although not exclusively) expressed by activated osteoblasts. Cathepsin readily degrades type I collagen, with an optimal activity at approximately pH 6.0 (Arnett, 2013). Thereafter, matrix metalloproteinases (MMPs), such as

gelatinase A (MMP-2), stromelysin (MMP-3), and collagenase (MMP-1), continue with the matrix degradation process (Hienz *et al.*, 2014).

After matrix degradation, the degradation products are removed from the resorption lacuna via a transcytotic vesicle transport process oriented towards the centrally located functional secretory domain of the basolateral membrane (Mulari, Zhao, Lakkakorpi, & Väänänen, 2003).The specific enzyme tartrate-resistant acid phosphatase (TRAP) is located in cytoplasmic vesicles, which fuse to the transcytotic vesicles to destroy the endocytosed material. When the osteoclast moves away from the resorption lacuna, phagocytes clean up the debris, and osteoblasts move in to begin bone formation (Hienz *et al.*, 2014). Additionally, osteoclasts possess some mechanisms to guarantee the maintenance of cytoplasmic pH homeostasis, since the extrusion of large amounts of acid would eventually cause the rise of osteoclast intracellular pH that would lead to detrimental effects on many cellular processes. Hence, osteoclast intracellular alkalinisation is compensated by the action of the HCO_3^-/Cl^- exchanger in the basolateral membrane (Bellido *et al.*, 2014).

1.5.2.2 Osteoclastogenesis

The first step in osteoclastogenesis is the commitment of the hematopoietic stem cell to the myeloid lineage. The transcription factor PU.1 plays a central role in this step. Its absence *in vivo* results in general myeloid lineage deficiencies (Biosse-Duplan, Horne, & Baron, 2012). Another transcription factor that is crucial to early stage of osteoclast development is microphthalmia-associated transcription factor (MITF). MITF-deficient mice display a suitable macrophage differentiation, but an impaired osteoclast differentiation, which might indicate that this transcription factor functions downstream of PU.1 in osteoclast differentiation, and regulates the balance between macrophage and osteoclast fate (Wei & Wan, 2013).

Two cytokines are essential and sufficient for osteoclastogenesis, specifically, receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). M-CSF contributes to proliferation, survival, and differentiation of early osteoclasts precursors (Ross, 2006). In turn, RANKL activates downstream signalling pathways that control osteoclast differentiation and bone resorption (Gupta *et al.*, 2010)

M-CSF is a homodimeric glycoprotein produced by several cell types, such as granulocytes, endothelial cells, fibroblasts, osteoblasts and lymphocytes (Costa-Rodrigues, Teixeira, Sampaio, & Fernandes, 2010). Loss of function mutation in the M-CSF gene originates an osteopetrotic phenotype due to the lack of osteoclasts (Provot, Schipani, Wu, & Kronenberg, 2008). The transcription factor PU.1, mentioned earlier, promotes the expression of the M-CSF receptor, c-Fms, and prepares the cell to respond to M-CSF (Morgan *et al.*, 2010).

M-CSF binding to c-Fms, a member of the receptor tyrosine kinase family, results in its dimerization and auto-phosphorylation. Phosphorylated c-Fms activates ERK via growth factor receptor bound protein 2 (Grb-2) son of sevenless (Sos) complex (Grb2/Sos) and protein kinase B (Akt) via phosphoinositide 3-kinase (PI3K) (Kikuta & Ishii, 2013). PI3K/Akt signalling pathway has been shown to be involved in osteoclasts survival and differentiation (Moon *et al.*, 2012). Akt activity was demonstrated to be necessary and in some cases sufficient to promote cell survival. Akt target the apoptotic machinery by phosphorylating downstream molecules like Bcl-2-associated death promoter (BAD), caspase-9, glycogen-synthase kinase and forkhead family members (Glantschnig, Fisher, Wesolowski, Rodan, & Reszka, 2003). The mechanism by which Akt regulates the differentiation of osteoclasts was associated to the ability of Akt to

regulate nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) signalling cascade, a master regulator of RANKL-induced osteoclast differentiation (Moon *et al.*, 2012). The ERK pathway has also insightful effects on apoptosis regulation through the post-translational phosphorylation of apoptotic regulatory molecules such as Bim, myeloid cell leukemia 1 (Mcl-1), and B-cell lymphoma 2 (Bcl-2) (Longo *et al.*, 2008)

Most importantly, M-CSF induces receptor activator of NF-κB (RANK) expression in osteoclast precursor cells, promoting an efficient response to the RANKL-RANK signalling pathways (Morgan *et al.*, 2010).

RANKL is a type II homotrimeric protein of the TNF superfamily. It is expressed at highest levels in bone and bone marrow, but is also found in lymphoid tissues (Provot *et al.*, 2008). RANKL expression is strongly stimulated by $1,25(OH)_2$ vitamin D3, prostaglandin (PG) E2, interleukin-1 alpha (IL-1α), and TNF-α (Chambers, 2000). RANKL is a critical cytokine for the final stages of osteoblast differentiation, accordingly, the genetic deletion of RANKL leads to an osteopetrotic phenotype characterized by a complete absence of osteoclasts (Morgan *et al.*, 2010; Weitzmann, 2013). RANKL initiates a cascade of events that leads to the activation of a nuclear genetic program orchestrated by the transcription factors NF-kB, c-Fos/activator protein 1 (AP-1) and NFATc1 (Indo *et al.*, 2013).

The sole receptor for RANKL, found on osteoclast progenitors and their precursors, is RANK (Provot *et al.*, 2008). Binding of RANKL to RANK initiates a sequence of signal transduction that lead to the differentiation of the early osteoclast precursor into a preosteoclasts. Preosteoclasts ultimately fuse with each other into mature multinucleated bone resorbing osteoclasts recognized by the expression of key osteoclast markers including TRAP, calcitonin receptors, cathepsin K, pp60c-src, MMP9, and the alpha V beta 3 integrin chains (Weitzmann, 2013).

The soluble receptor, osteoprotegerin (OPG), a member of the tumor necrosis factor receptor (TNFR) superfamily, was shown to act as a decoy receptor, preventing association of RANKL with RANK receptor. Knock-out and transgenic overexpression studies in mice demonstrated that the deletion of the OPG gene leads to severe bone erosions. OPG–RANKL complex counterbalances the effect of the RANK–RANKL complex in order to regulate bone resorption and density (Boyle *et al.*, 2003).

RANKL activates RANK receptor through the interaction with an adaptor molecule TNF receptor-associated factor (TRAF). To date, six TRAF proteins have been identified but only TRAF6 seems to have a critical role in osteoclastogenesis (Yavropoulou & Yovos, 2008). The binding of TRAF6 to RANK induces TRAF6 trimerization and leads to the activation of NF-κB and MAPKs (Takayanagi, 2008).

In human, the NF-κB transcription factor family is composed of five members, named p50, p52, RelA, c-Rel and RelB. Among NF-κB members, p50 and 52 are crucial for osteoclastogenesis, since, animals lacking both the p50 and p52 members develop severe osteopetrosis (Roodman, 1999). In resting cells, NF-κB are kept silent in the cytosol through their binding to members of the inhibitory κB (IκB) family (Remouchamps & Dejardin, 2015). NF-κB activation involves the degradation of IκB and the release of NF-kB dimers, which translocate into the nucleus and bind to specific DNA sequences triggering the transcription of specific genes (Yavropoulou & Yovos, 2008).

The transcription factor c-Fos, a component of AP-1, is essential for osteoclast differentiation (Matsuo & Ray, 2004). Hence, c-Fos-deficient mice exhibit severe osteopetrosis as a result of a complete block of osteoclasts differentiation (Faccio, Choi, Teitelbaum, & Takayanagi, 2011). On the other hand, the role of the Jun family proteins of AP-1 has been shown to be

redundant. Mice lacking Jun proteins are embryonically lethal, yet, although deficiencies in JunB or c-Jun lead to a significant decrease in osteoclast formation, the blockage of osteoclastogenesis doesn't occur, suggesting that Jun members can substitute each other (Faccio *et al.*, 2011).

RANKL-induced activation of NF-κB and c-Fos has been shown to be essential for the induction of NFATc1 (Choi *et al.*, 2013). The role of NFATc1 in osteoclastogenesis was proposed after *in vitro* observations that NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts, and that ectopic expression of NFATc1 in the absence of RANKL promotes bone marrow-derived precursor cell differentiation in osteoclasts (Takayanagi, 2008).

The majority of MAPKs are activated downstream of RANK and can relay RANK stimulation to the cellular response (Wada, Nakashima, Hiroshi, & Penninger, 2006). MAPKs (particularly JNK) were shown to be involved in the activation of AP-1 components and therefore perform a role in osteoclastogenesis, however the molecular mechanisms involved are not clear to date (Faccio *et al.*, 2011; Wada *et al.*, 2006). Hotokezaka and his co-workers used p38-MAPKs and ERKs specific inhibitors to study the involvement of MAPKs in osteoclastogenesis. The authors suggested that osteoclastogenesis is regulated under a balance between ERK and p38 pathways and that the ERK pathway negatively regulates osteoclastogenesis while the p38 pathway does so positively (Hotokezaka *et al.*, 2002).

1.6 Bone Modelling and Remodelling

The coordinated actions of osteoblasts, osteocytes and osteoclasts occur within two biological contexts, namely, bone modelling and remodelling (Baron & Horne, 2005).

Bone modelling is defined as either the formation of bone by osteoblasts or resorption of bone by osteoclasts on a given surface (Allen & Burr, 2014). Thus, this process is responsible for the gain in skeletal mass and the changes in skeletal size and shape taking place during the growth period (Delgado-Calle, Garmilla, & Riancho, 2012). Although the processes of formation and resorption during bone modelling are locally independent, they are not globally independent since both processes occur simultaneously and must be coordinated to shape bone (Allen & Burr, 2014).

The coupling of osteoclast and osteoblast activities at one specific bone area is known as bone remodelling (Sims & Martin, 2014). Bone remodelling replaces old bone by new bone tissue, throughout life, and specifically in the adult skeleton, to maintain bone mass, repair bone microfractures, sustain mineral homeostasis, and ensure mechanical competence by modifying the microarchitecture (Delgado-Calle *et al.*, 2012)

Bone remodelling can be classified as targeted or stochastic. Targeted remodelling is characterized by a local signalling event that directs osteoclasts to a specific location to begin remodelling. The most accepted signalling events are bone microdamage and osteocyte apoptosis, which have been demonstrated to be connected (Allen & Burr, 2014). In fact, it has been proposed that osteocytes are able to sense bone deformation and to detect microdamage in old bone, and transmit signals of an unknown nature to recruit osteoclast precursors to a specific bone site and trigger the remodelling process (Feng & McDonald, 2011). In contrast, stochastic remodelling is not site-dependent, although probably is not also a completely random process (Burr, 2002). This process occurs in response to systemic hormones, like PTH, thyroxine, growth hormone and estrogen (Eriksen, 2010). While, targeted remodelling plays an important

role in the maintenance of skeletal integrity, stochastic remodelling enables bone to fulfil its metabolic requirements (Fuchs, Warden, & Turner, 2009).

Remodelling takes place within temporary anatomical structures, known as bone multicellular units (BMUs), which consist of clusters of bone-resorbing osteoclasts and bone-forming osteoblasts (Raggatt & Partridge, 2010). The remodelling cycle encompasses four sequential phases, respectively, activation, resorption, reversal, and formation.

The activation phase is characterized by the recruitment of osteoclast precursors from blood circulation to the bone surface. This phase involves the interaction of osteoclast and osteoblast precursor cells, which leads to the differentiation, migration, and fusion of the large multinucleated osteoclasts (Kini & Nandeesh, 2012; Raisz, 1999).

Once mature osteoclasts are present, bone lining cells retract from the bone surface exposing the mineralized matrix to bone-resorbing cells (Bellido *et al.*, 2014; Raisz, 1999). This process appears to be stimulated either by the osteoclasts as they approach to the surface or by the same signals that initiate the remodelling process (Bellido *et al.*, 2014). Subsequently, osteoclasts adhere to bone and resorb this tissue by means of acidification and proteolytic digestion (Manolagas, 2000; Raisz, 1999). The regulatory mechanisms that arrest osteoclastic activity are poorly understood. It was suggested that since osteoclasts have a limited lifespan, they eventually undergo apoptosis after an extensive resorptive activity. Additionally, it was also considered that TGF-ß or related peptides released from the matrix during the resorption phase could inactivate osteoclasts (Hill, 1998).

When resorption has been completed, the reversal phase begins. In this phase, mononuclear cells of an undetermined lineage remove the undigested demineralized collagen matrix from the resorption lacunae and prepare bone surface for subsequent osteoblast-mediated bone formation (Raggatt & Partridge, 2010). The reversal phase couples bone resorption to bone formation (Delaisse, 2014). Bone matrix-derived factors released during bone resorption, such as, TGF-ß, IGF-1, IGF-2, BMPs, platelet-derived growth factor (PDGF), and fibroblast growth factors (FGF) were suggested to link these two phases, namely by recruiting osteoblasts into the reabsorbed area (Kini & Nandeesh, 2012; Raggatt & Partridge, 2010).

During formation phase of the remodelling cycle, the cavity created by resorption can be completely filled in by successive layers of osteoblasts, which differentiate from their mesenchymal precursors and deposit a mineralizable matrix (Raisz, 1999).

Imbalances in bone remodeling can result in severe perturbations in skeletal structure and function, leading to conditions such as osteoporosis, osteosclerosis, and osteopetrosis (Boyle *et al.*, 2003).

1.7 Bone and Calcium Homeostasis

The total amount of calcium in the human body ranges from 1000 to 1200 g (Blaine, Chonchol, & Levi, 2014). Calcium accretion begins throughout the third trimester of fetal life and increases during childhood, adolescence, and adulthood before peaking in early adulthood and declining thereafter (about 1 to 2% per year) (Unnanuntana, Gladnick, McArtur, McCarthy, & Lane, 2010).

Over 99% of total body calcium is found as HAp crystals in bone. Bone calcium contributes to the mechanical weight-bearing properties of the skeleton and, provides a dynamic store to maintain the intra- and extracellular calcium pools (Peacock, 2010; Review, 2011). Non-bone

calcium, which accounts for less than 1% of total body calcium is found in the intracellular and extracellular fluid compartments, including blood (Favus, Bushinsky, & Lemann, 2006).

Approximately 0.9% of total body calcium content is intracellular (Shahay, 2013). Intracellular calcium is a crucial regulator of numerous cellular events, including muscle contraction, hormone secretion, glycogen metabolism, and cell division (E.M. Brown, 1991). The intracellular concentration of calcium is approximately 0.15 μM (Hruska, Levi, & Slatopolsky, 2007). At the intracellular level, calcium homeostasis is maintained through the influx of extracellular calcium or from release of intracellular calcium stores (Comission, 2003).

Extracellular calcium accounts for roughly 0.1% of the total body calcium content (Calvi & Bushinsky, 2008; Shahay, 2013). Extracellular calcium not only provides a steady supply of calcium for intracellular use but also plays an important role in clotting and membrane integrity (E.M. Brown, 1991). Serum calcium concentration is held in a very narrow range, usually from 2.2 to 2.6 mM (or 8.8 to 10.4 mg/dl) (Peacock, 2010). Extracellular calcium exists in three forms, protein-bound, complexed, and ionized. Approximately 40% of serum calcium is bound to proteins, namely albumin. Another 10% is complexed with various chelators, such as citrate, phosphorus and sulfate. The remaining 50% of serum is found in the ionized form (Calvi & Bushinsky, 2008; Favus *et al.*, 2006).

The biological activity of calcium is attributed to the ionized fraction, which is readily exchangeable with pools of calcium in bone, blood, and intracellular sites (Favus *et al.*, 2006). To avoid calcium toxicity, the concentration of serum ionized calcium is tightly maintained within a physiologic range of 1.10 to 1.35 mM (4.4 to 5.4 mg/dl) (Peacock, 2010).

The extracellular ionized calcium concentration is tightly controlled by the concerted action of calcium absorption in the intestine, reabsorption in the kidney, and exchange from bone, which are all under the control of the calciotropic hormones, such as PTH, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$; calcitriol) and calcitonin (Blaine *et al.*, 2014; Flynn, 2003; Mundy & Guise, 1999).

During bone remodeling, osteoclasts and osteoblasts function in local environments characterized by dramatic fluctuations in extracellular calcium concentrations (Silver, Munflls, & Etherington, 1988). Elevation of extracellular calcium has been shown to inhibit osteoclastic bone resorption and to stimulate proliferation and chemotaxis of osteoblasts. Therefore, calcium released by bone resorption may perform an important role in the coupling of bone resorption and bone formation (Sugimoto *et al.*, 1993).

1.7.1 Parathyroid Hormone, Calcitriol and Calcitonin

Parathyroid hormone (PTH) is secreted by the parathyroid glands as a polypeptide (Juppner, Gadella, Brown, Kronenberg, & Potts, 2010). Secretion of PTH is highly dependent on the ionized calcium concentration and represents a simple negative feedback loop. Calcium level is sensed by the calcium-sensing receptor (CASR) in the parathyroid chief cells. Hence, low calcium levels stimulate PTH secretion, whereas, high calcium levels supress, although not entirely, PTH secretion (V. David & Quarles, 2011; Mundy & Guise, 1999).

Elevated levels of PTH target the PTH receptor on kidney to increase renal reabsorption of calcium and to promote the conversion of 25(OH)D into the active state of $1,25(OH)_2D$, by increasing the activity of the renal 1-α-hydroxylase (Blaine *et al.*, 2014; V. David & Quarles, 2011; Moe, 2008). An elevated level of $1,25(OH)_2D$ increases intestinal absorption of calcium and phosphate (V. David & Quarles, 2011). In bone, both PTH and $1,25(OH)_2D$ promote bone

resorption and a calcium and phosphate efflux by increasing the number of osteoclasts, indirectly by binding initially to osteoblasts and stimulate RANKL expression on their membrane (Lieben & Carmeliet, 2013; Takahashi, Udagawa, & Suda, 2014). Finally, the increased serum calcium acts on CASR to reduce PTH secretion at the level of the parathyroid glands, thus completing the endocrine feedback loop (V. David & Quarles, 2011; Moe, 2008).

Calcitonin is a 32-amino acid peptide that is synthesized and secreted by the parafollicular cells of the thyroid gland. Increases in ionized calcium produce an increase in calcitonin secretion, and conversely, a fall in the ambient calcium concentration inhibits calcitonin secretion (Mundy & Guise, 1999). The principal target organ for calcitonin is bone, however renal excretion of calcium and phosphate are also affected. Calcitonin reduces blood calcium by supressing bone resorption and increasing osteoid mineralization. This peptide hormone binds to its receptor on the surface of osteoclasts and inhibits osteoclastic resorption by affecting their actin cytoskeleton (Chiappelli, 2011).

1.8 Lanthanides

The lanthanides (Ln) are an exceptional group of fourteen elements, with unique and distinctive physicochemical and magnetic characteristics, ranging from Cerium (Ce_{58}) to Lutetium (Lu_{71}) (Madan & Prakash, 2001; Raj, 2008).

These elements were originally called rare earths due to their natural occurrence as metal oxides, which in some ways resemble calcium, magnesium an aluminium oxides known as common earths. The name rare earths is a misnomer since these elements are neither rare or earths (Sastri, Perumareddi, Rao, Rayudu, & Bünzli, 2003). In fact, lanthanides are more common than gold, silver and platinum (Leonard, Nolan, Stomeo, & Gunnlaugsson, 2007).

The elements of this series are called lanthanides since they follow lanthanum (La_{57}) in the periodic table. Although lanthanides are confined to the fourteen elements following La in the periodic table this element is generally included in this group since it presents the main features of the other elements of the series (Madan & Prakash, 2001; Raj, 2008).

Lanthanides are elements in which the *f* orbitals are partly or completely filled, while the outermost *p* and *d* orbitals are empty. Since the *f* orbitals do not have as much effect on the chemical properties as the *p*, and *d*, they are chemically very similar (Abdelrahman, 2011).

Lanthanides generally favour tripositive oxidation state (Ln^{3+}). Hence, they are highly electropositive and predominantly ionic in nature. Even thought, tetravalent and divalent forms exist as well, only cerium (Ce^{4+}) and europium (Eu^{2+}) are stable enough to persist in aqueous solutions (C.H. Evans, 1990).

A particular feature of these elements consists on the decrease in the atomic size and radius along with the increase in the atomic number (La 1.06 Å and Lu 0.85 Å) (Fricker, 2006).

This phenomenon, called lanthanide contraction, is attributed to the shielded 4f-orbital, which cannot compensate the effect of increased nuclear charge. Thus enhanced nuclear attraction is exerted upon the whole electron cloud which finally shrinks and leads to the contraction of the entire ionic structure (Madan & Prakash, 2001).

The preferred coordination number of all lanthanides is 8–9, but complexes of 6–12 coordination are quite stable. In aqueous solution, the hydration shell of lanthanides is between 9–12 water molecules, but this number has not been unequivocally determined. The ionic character

of complexes of trivalent lanthanide ions is very high, with a typical order in strength of bonding of O > N > S (Jakupec, Unfried, & Keppler, 2005).

1.8.1 Biochemistry of Lanthanides

The research on the biochemical aspects of lanthanides began when the ability of these elements to substitute for a large number of metallic ions, such as calcium (Ca^{2+}), magnesium (Mg^{2+}), iron (Fe^{2+}), and manganese (Mn^{2+}) was first stated. Among these metallic ions, calcium (Ca^{2+}) is of particular interest due to its importance in cellular metabolism (C.H. Evans, 1983).

Lanthanides ions (Ln^{3+}), as shown in Table 1.1, have similar ionic radii to calcium, but by virtue of possessing a higher charge-to-volume-ratio, they have a stronger binding to water molecules, an increased stability of analogous complexes, and a preference for higher coordination numbers (Fricker, 2006; Jakupec *et al.*, 2005). Even though, lanthanide ions are able to replace calcium in many biomolecules they not necessarily substitute its functionality. For example, the substitution of lanthanide ions for calcium ions in enzymes generally results in an inhibition of the enzyme process (Jakupec *et al.*, 2005). In addition, there are also some calcium binding sites that totally refuse lanthanide ions binding, for instance, lanthanide ions cannot replace calcium in scallop myosin or concanavalin A (Madan & Prakash, 2001).

Since larger lanthanide ions are generally closer in ionic radius to calcium ions, a relationship between ionic radius and activity has been described. Yet, in some cases these relationship is not observed. For instance, the inhibition of Ca^{2+}/Mg^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum decreases from La^{3+} to Lu^{3+}, while the activation of α-amylase tends to increase from La^{3+} to Lu^{3+}. These behaviours are thought to occur due to the decrease of free energy upon binding interaction (Sastri *et al.*, 2003).

Lanthanide ions are generally unable to penetrate cellular membranes of healthy cells, however they can still exert influence on transmembrane processes (Korenevskii, Sorokon, & Karavaiko, 1997). Binding of lanthanides to membrane proteins stabilizes the phospholipid membranes, increases their rigidity and alters their surface charge towards higher electropositivity. This effect further leads to increasing membrane potential and resistance and consequently to a dysfunction of the voltage-dependent calcium channels. Calcium entrance may also occur by receptor operated calcium channels (C.H. Evans, 1990).

Accordingly, lanthanide ions were used in several studies to explore the mechanism of calcium influx and calcium-related downstream events. Although the reported results are discrepant, depending on the experimental conditions, overall it was observed that lanthanide ions suppress several agonist-induced calcium influxes by blocking the calcium channels (K. Wang, Cheng, Yang, & R., 2003). Mlinar and Enyeart showed that lanthanide ions block the T-type calcium channels by pore occlusion with a potency that varies inversely with ionic radius (Mlinar & Enyeart, 1993). In contrast, lanthanide ions inhibit L-type calcium channels with a potency that varies directly with ionic radius (Lansman, 1990).

Moreover, lanthanide ions were also shown to be able to induce the increase of calcium concentration in a variety of cells, such as, hepatocytes (by La^{3+}) and osteoblasts (by Gd^{3+}), by activating a membrane-bound CaSR which responds to extracellular calcium and releases calcium from the intracellular pool (K. Wang *et al.*, 2003).

However, lanthanides can also exert their influence on cellular functions by other means than inhibiting calcium fluxes. Lanthanide ions have been shown to coordinate with further membrane structures, such as receptors for acetylcholine and Na^{+}/K^{+}-ATPase (P. David & Karlish,

1991; Rübsamen, Hess, Eldefrawi, & Eldefrawi, 1976). Moreover, the cyclic adenosine monophosphate (cAMP) content of hepatocytes was increased upon incubation with Ce^{3+} in a concentration-dependent manner, wich suggested that lanthanide ions can also trigger cAMP signaling system (K. Wang *et al.*, 2003).

The duality and diversity are common features in the biological effects of lanthanides. In many cases, they affect a biological event from opposite sides (K. Wang *et al.*, 2003). This dual behaviour points toward the use of the positive effects indorsed by lanthanides in the intervention of pathologic events. However, the clinical success of these approaches will depend on the ability to modulate the opposing effects (K. Wang *et al.*, 2003).

Table 1.1 — Properties of calcium and lanthanides ions (Sastri *et al.*, 2003).

Property	**Ca^{2+}**	**Ln^{3+}**
Coordination number	6-12 reported 6 or 7 favored	6-12 reported 8 or 9 favored
Coordination geometry	Highly flexible	Highly flexible
Donor atom preference	O >> N >> S	O >> N >> S
Ionic radius (Å)	1.00 – 1.18 (CN 6-9)	0.86 – 1.22 (CN 6-9)
Type of bonding	Ionic	Ionic
Hydration number	6	8 or 9
Water exchange constants (s^{-1})	≈ 5 x 10^8	≈ 5 x 10^7
Diffusion coefficient (cm^2/s x 10^5)	1.34	La^{3+}, 1.3
Crystal-field stabilization	None	Negligible

1.8.2 Lanthanide Induced-Proliferation, Apoptosis and Differentiation

Likewise other hormetic agents, cell proliferation is promoted by lanthanide ions only in a narrow range of low concentration. Beyond an upper limit the effect becomes inhibitory. This concentration dependency has been reported in different cell types, such as, fibroblasts, and hepatocytes (Preeta & Nair, 1999; Rai *et al.*, 1997).

Cell proliferation promoted by lanthanide ions is associated with enhanced DNA, RNA and protein synthesis (K. Wang *et al.*, 2003). Furthermore, Sarkander and Brade highlighted that different effects of lanthanides on RNA synthesis are due to variations in their ionic radius and that those effects depend on species, cells and conditions examined (Sarkander & Brade, 1976).

Since intracellular calcium act as mediator of cell proliferation, lanthanides may exert their influence on cell proliferation by increasing intracellular calcium concentrations. Gadolinium, a stretch-activated channel blocker, inhibited strain-induced $^{45}Ca^{2+}$ influx and suppressed strain-enhanced DNA synthesis in rat fibroblasts (M. Liu, Xu, Tanswell, & Post, 2005). Moreover, some studies indicated that lanthanide ions, as agonists of CaR, are able to induce cell proliferation through the inositol trisphosphate (IP_3) signalling pathway (E. M. Brown, Fuleihan, Chen, & Kifor, 1990).

Aside from promoting cell proliferation, lanthanides were also documented to have the ability to inhibit cell proliferation. Weiss *et al.* demonstrated the ability of lanthanides to block store-operated calcium entry and inhibit human colon carcinoma cells proliferation (Weiss, Amberger, Widschwendter, Ofner, & Dietl, 2001). The effect of lanthanides on cell proliferation had been previously demonstrated by Sato and its co-workers. In this work, the growth rates of

melanoma cells in the presence of 1 mM of metal ions (La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, Gd^{3+}, Yb^{3+}, Al^{3+}) were significantly lower than that of control cells. Measurements of cell cycle indicated that the metal ions arrested the transitions from G0/G1 to S state (Sato, Hashizume, Hotta, & Okahata, 1998).

Effects of lanthanides on cell apoptosis was described in several cell types, such as rat skin fibroblasts, HeLa and PC12 cells (K. Wang *et al.*, 2003). Macrophages apoptosis was shown to be induced by Gd^{3+} in a concentration-dependent manner. Higher concentrations of Gd caused more rapid and widespread cell death than the lower concentrations (Mizgerd, Molina, Stearns, Brain, & Warner, 1996).

The mechanisms underlying lanthanides induced-apoptosis are not completely stablished, but the increase of intracellular calcium concentration is known to play an important role in this event, since it activates the endonucleases and protein kinases and mediates DNA cleavage and apoptosis-related gene expression, respectively (K. Wang, Li, Cheng, & Zhu, 1999).

The interactions of lanthanides with mitochondria was also proposed to explain lanthanide induced-apoptosis. Incubation of mitochondria isolated or in whole cell with La^{3+}, Gd^{3+} and Yb^{3+} resulted in mitochondria swelling, increased membrane fluidity, decrease of mitochondrial membrane potential and release of cytochrome c, all characteristics of pre-apoptotic events. Cellular reactive oxygen species (ROS) level was shown to increase in the presence of La^{3+}, Gd^{3+} and Yb^{3+}, suggesting that perhaps lanthanide-induced apoptosisis is related with ROS induction (H. Liu, Yuan, Yang, & Wang, 2003).

Although poorly documented, influence of lanthanides on cell differentiation is thought to result of the interference with calcium-depending processes. La^{3+} and Gd^{3+} inhibit electrically stimulated differentiation of PC12 cells. The differentiation was explicated with electrically induced calcium influx (Kimura, Yanagida, Haruyama, Kobatake, & Aizawa, 1998). Another study suggested that La^{3+} inhibit calcium mediated keratinocyte differentiation. According to the authors, La^{3+} enters cells and inhibits calcium mediated keratinocyte differentiation both by blocking calcium influx and by blocking calcium regulated intracellular processes, such as transglutaminase directed cornified envelope formation (Pillai & Bikle, 1992).

1.8.3 Lanthanides Distribution in the Skeleton

The distribution of lanthanides in bone was studied by Durbin in 1962, using rat models. Although this work has already been developed a few decades ago, it still epitomizes the utmost complete systematic comparative study of lanthanides in the same animal strain, under the same conditions. The results obtained point out to an increased bone accumulation by dint of a decrease of the ionic radius (Durbin, 1962).

The development of biokinetic models based on this work, and on later works, made possible the realization of studies to predict the behaviour of the lanthanides in the human body. Using this models it was observed that both the initial deposition pattern and the further redistribution of the metals within the skeleton depends on the metal charge. In addition, it was noticed that lanthanides show no initial diffuse distribution into the bone volume (as with other metals, such as, uranium or neptunium), in contrast they tend to be more concentrated on resorbing and resting bone surfaces, such as, Haversian canals, endosteum, and periosteum. This work showed also an equal initial distribution of lanthanides in the trabecular and the cortical surfaces (Taylor & Leggett, 2003).

Lanthanides show a prolonged skeletal retention, with a biological half-life of almost two decades. Throughout bone turnover process, lanthanides are removed from bone and are laid down on new bone surfaces which supports their homogeneous distribution within the bone volume and, accordingly, within the skeleton. Since this process occurs gradually, only a small proportion of the element is released into the circulation and eliminated from the body (Vidaud, Bourgeois, & Meyer, 2012)

Lanthanum carbonate was strongly studied because it presents phosphate-binding properties, being used for the treatment of hyperphosphatemia. Hence, lanthanum distribution in bone was mapped, using scanning X-ray microfluorescence, in rats treated with high doses (>1000 mg/kg/day) of lanthanum carbonate. Lanthanum was found at the edge of mineralized bone, on both actively mineralizing and quiescent sites of bone turnover, and also deep inside the trabecular bone and in cells in close proximity to the resorption lacunae (Behets *et al.*, 2005).

1.8.4 Lanthanides Interaction with Bone Matrix

The outstanding affinity of lanthanides for bone results from the ability that both the mineral and the organic components of the bone matrix have to intervene in the binding of multivalent cations to bone. Indeed, *in vitro* studies have shown that the collagen and the glycoproteins present in the bone matrix are very efficient binders for lanthanides (Vidaud *et al.*, 2012). Nouisiainen *et al.* investigated calcium, magnesium and lanthanum binding to bovine bone OC. The results indicated that lanthanum ion bound to OC (although in a lesser extensive manner than either calcium or magnesium) and did not induce the protein dimerization *(Nousiainen et al.*, 2002).

Both natural and synthetic HAp present the ability to undergo cationic and anionic substitution (Cawthray, Creagh, Haynes, & Orvig, 2015). Accordingly, lanthanides are able to replace calcium ion sites within HAp, without altering the hexagonal apatite structure, due to their similarity to calcium (Vidaud *et al.*, 2012).

A systematic study of the isomorphous substitution of lanthanides in synthetic HAp showed that the maximum incorporation of lanthanides decreases along the element series, contradicting the deposition of these elements in the skeleton (which increases along the series) (Ardanova *et al.*, 2010). Furthermore, *in vivo* studies on rats with lanthanum carbonate demonstrated that lanthanum replaces no more than 1 out of 2000 calcium atoms within the HAp lattice (Behets *et al.*, 2005). Thus, although lanthanides are able to simply replace calcium ions in the apatite crystal, the mechanism of their incorporation in bones is apparently more complex (Vidaud *et al.*, 2012).

1.8.5 Lanthanides Interaction with Bone Cells

Due to the affinity of lanthanides for bone, the biological activity of these elements has been investigated in order to understand their ability to modulate bone tissue metabolism and the bone regeneration process (J. Coelho *et al.*, 2012).

Several experimental *in vivo* tests were conducted to assess the biological effects of this elements. In one of this works, the effects of lanthanum on the femur bone mineral of male Wistar rats after oral administration of lanthanum nitrate over a 6-month period was investigated. Lanthanum was found to accumulate in the bone tissue of male Wistar rats. Moreover, a decrease in the mineral-to-matrix ratio and an increase in carbonate content was shown by

thermogravimetric analysis. Additional methodologies revealed a smaller mean thickness of the mineral crystals in the bone of lanthanum nitrate-treated rats. Accordingly to the results obtained, the authors suggested an obstruction of bone maturation prompted by lanthanum (J. Huang *et al.*, 2006).

Behets *et al.* investigated the effect of lanthanum carbonate on bone histology in rats with normal renal function and with chronic renal failure, after oral administration of the compound for 12 weeks. The results obtained indicated that the administration of lanthanum carbonate did not cause noticeable effect on bone in rats with normal renal function, which contradicts the results mentioned above. Nevertheless, at higher doses this compound was shown to cause an impairment of bone mineralization in rats with chronic renal failure (Behets *et al.*, 2004).

Due to the conflicting results obtained in the *in vivo* studies, the effect of these elements on bone cells *in vitro* started to be also addressed by several authors. One of the most studied lanthanides is lanthanum, which due to its pronounced similarity to calcium, has been recognized as a "bone-seeking element" (X. Wang, Yuan, Huang, Zhang, & Wang, 2008).

Liu *et al.* studied the effect of lanthanum on osteoblastic differentiation of primary rat MSCs. Lanthanum inhibited the osteoblastic differentiation in the early and middle stages of culture, as demonstrated by the decrease in the ALP activity and OC expression. Furthermore, MAPK kinase inhibitor was shown to completely block the inhibitory effect of lanthanum on ALP activity of MSCs suggesting that the response induced by lanthanum occurred by a MAPK-kinase dependent mechanism (H. Liu, Zhang, Xu, & Wang, 2006).

The enhancement of osteoblastic differentiation of calvaria-derived rat osteoblasts by lanthanum was shown in another report by Wang *et al.* The authors observed an increased expression of osteoblast-specific genes (Cbfa-1, OPN, and BSP) upon lanthanum treatment, yet, no changes were observed concerning the expression of type I collagen. Also, they suggested that lanthanum effect on osteoblasts was, at least, partially mediated by the enhanced phosphorylation of ERK pathway, since the inhibition of this pathway was shown to suppress the effects perceived (X. Wang *et al.*, 2008).

The formation and bone resorbing activity of rabbit osteoclast-like cells was also shown to be affected by lanthanum. The promotion or inhibition of osteoclasts formation and activity was shown to be dependent on the assayed concentration (J. Zhang , Xu, Wang, & Yu, 2003).

The biological effects of other lanthanides rather than lanthanum were already studied (such as, neodymium, samarium, and gadolinium) and the majority of the literature reports corroborate the ability of lanthanides to modulate the behaviour of bone-relevant cellular populations (J. Coelho *et al.*, 2012).

1.9 Chapter Insights and Research Objectives

Bone is the dynamic tissue that comprises functionally distinct and unique cell populations that support the biochemical, mechanical and structural integrity of the skeleton (Fitzpatrick, 2004). Bone undergoes continuous remodeling throughout life, an active and dynamic process that depends on the balance between bone resorption by osteoclasts and bone deposition by osteoblasts (Rucci, 2008). An imbalance in these processes can lead to either an increase or decrease in bone mineral density, causing the structural deterioration of bone (Datta *et al.*, 2008).

Lanthanides are a group of metal ions with similar properties, which have been recognized for their diversity of biological effects and outstanding potential in medical applications (Wang,

Li *et al*. 1999). More recently, these elements have been the focus of several investigations related to their potential to modulate bone tissue metabolism and the bone regeneration process due to their high affinity for bone and their ability to exchange with calcium in bone and, consequently, affect the remodeling cycle, by modulating osteoblast and osteoclast development (Pidcock and Moore 2001; Webster, Massa-Schlueter *et al*. 2004; Barta, Sachs-Barrable *et al*. 2007).

Although the biological effect of lanthanides has been extensively studied, few reports have focused on the effect of these elements on human bone cells. Accordingly, the aim of this study was to investigate the cellular and molecular effects of three different lanthanides (cerium, lanthanum and praseodymium) at different concentrations (10^{-7} to 10^{-3} M) on the proliferation and differentiation of human osteoblasts and osteoclast precursor cells. Zinc and magnesium were used as reference physiological cations, since their effect on bone cells development is already well-stablished. Moreover, it was also assessed the involvement of some signaling pathways important for osteoblastogenesis and osteoclastogenesis in cellular responses of bone cells cultured in the presence of these elements.

Chapter 2

Materials and Methods

2.1 Materials

All cell culture chemicals and supplies were acquired from Merck and Sigma Aldrich (St. Louis, MO) unless otherwise noted. All tissue culture flasks and plates were obtained from Corning (Corning, NY).

2.2 Methods

2.2.1 Osteoblastic Cell Cultures

Osteoblast precursor cells were obtained from femur heads of patients with ages comprised between 25 and 45 years old, undergoing orthopaedic surgery procedures, after informed consent, as previously described (Costa-Rodrigues *et al.*, 2010). Briefly, bone was broken in small pieces, which were maintained in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum, 100 IU/mL penicillin, 2.5 µg/mL streptomycin, 2.5µg/mL amphotericin B and 50 µg/mL ascorbic acid. At approximately 70-80% confluence, cells were enzymatically detached with 0.05% trypsin and 0.5 mM ethylenediamine tetraacetic acid (EDTA).

Cells, seeded at 2 x 10^4 cells/cm^2, were maintained in α-MEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 2.5 µg/mL streptomycin, 2.5 µg/mL amphotericin B, 50 µg/mL ascorbic acid.

2.2.2 Osteoclastic Cell Cultures

Blood was collected from healthy donors with ages comprised between 25 and 35 years old, after informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated as described previously (Costa-Rodrigues, K.A., Teixeira, & Fernandes, 2012). Shortly, blood was diluted (2:1) with phosphate-buffered saline (PBS) and applied on top of Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences, Little Chalfont, UK). After centrifugation at 400g for 30 minutes, PBMC were collected and washed twice with PBS. On average, for each 100 mL of processed blood about 60 x 10^6 PBMC were obtained.

PBMC, seeded at 1.5 x 10^6 cells/cm^2, were maintained in α-MEM supplemented with 30% autologous human serum, 100 IU/mL penicillin, 2.5 µg/mL streptomycin, 2.5 µg/mL amphotericin B, 2 mM L-glutamine.

2.2.3 Exposure to Lanthanides

Osteoblastic and osteoclastic cell cultures, established as described above, were cultured for 24 hours. Following this, the culture medium was removed and replaced with one containing the the osteogenic enhancers 10 mM ß-glycerophosphate and 10 nM dexamethasone, in the case of osteoblastic cultures, or the osteoclastogenic promoters 25 ng/mL M-CSF (R&D Systems, Minneapolis, USA) and 40 ng/mL RANKL (Insight Biotechnology, London, UK), in the case of PBMC cultures (M. J. Coelho & Fernandes, 2000; Costa-Rodrigues, Fernandes, & Fernandes, 2011).

Moreover, cell cultures were further treated with zinc, magnesium (as reference physiological cations) and lanthanides (cerium, lanthanum and praseodymium) at concentrations of 10^{-7} M to 10^{-3} M. The concentration range tested was based on information regarding the literature. Cell cultures performed in the absence of zinc, magnesium and lanthanides were used as negative controls.

The culture medium was changed once a week, and the cations were renewed at each medium change. Cell cultures were incubated at 37ºC in a 5% CO_2 humidified atmosphere, for 21 days.

2.2.4 Osteoblastic and Osteoclastic Cell Response to Lanthanides

Osteoblast precursor cells cultured in the presence of zinc, magnesium and lanthanides at 10^{-7} M to 10^{-3} M, were analysed at days 14 and 21, for DNA content, apoptosis, ALP activity, histochemical staining of ALP and collagen, and F-actin staining. Osteoblast precursor cells cultured in the presence of the lowest concentration that caused statistically significant inhibition of ALP-related parameters, were further characterized for the involvement of several common osteoblastogenesis-related signalling pathways.

PBMC cultured in the presence of zinc, magnesium and lanthanides at 10^{-7} M to 10^{-3} M, were assessed at days 14 and 21, for DNA content, apoptosis, TRAP activity, number of TRAP-positive multinucleated cells, and the presence of cells displaying F-actin rings and expressing vitronectin (VNR) and calcitonin receptors (CTR). PBMC cultured in the presence of the lowest concentration that caused statistically significant inhibition of TRAP-related parameters, were further characterized for the involvement of several common osteoclastogenesis-related signalling pathways.

2.2.4.1 DNA content

DNA quantification was performed using PicoGreen® dsDNA kit (Molecular Probes, Eugene, USA), according to the manufacturer's instructions. Briefly, PBMC and osteoblast precursor cell cultures, at days 14 and 21, were washed twice with PBS and solubilized with 0.1% (V/V) Triton X-100 for 15 minutes. PicoGreen® dye was diluted by making a 1:200 dilution of the concentrated dye solution in 1xTE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Posteriorly, diluted PicoGreen® was added to each sample in a black 96-well plate and incubated for 2 minutes at

room temperature, protected from the light. Fluorescence was measured at 485/520 nm (excitation/emission) in an ELISA plate reader (Synergy HT; Biotek). The results of DNA content were express in fg_{DNA}.

2.2.4.2 Apoptosis

Apoptosis quantification was performed using EnzCheck® Caspase-3 Assay Kit #2 (Molecular Probes, Eugene, USA), according to the manufacturer's instructions. Briefly, osteoblast precursor cell and PBMC cultures, at days 14 and 21, were washed twice with PBS and solubilized with 0.1% (V/V) Triton X-100 for 15 minutes. 2x Reaction Buffer was prepared with 5X Reaction Buffer (50 mM PIPES, pH 7.4; 10 mM EDTA, 0.5% CHAPS), 1 M Dithiothreitol (DTT) and deionized water. 2X substrate working solution was then prepared by mixing Z-DEVD–R110 substrate with 2X Reaction Buffer. Then, 2X substrate working solution was added to each sample in a black 96-well plate and incubated for 30 minutes at room temperature, protected from the light. Fluorescence was measured at 485/520 nm (excitation/emission) in an ELISA plate reader (Synergy HT; Biotek). Results obtained were normalized with the corresponding DNA content and presented as a percentage relative to control.

2.2.4.3 TRAP and ALP Activities

ALP (osteoblast precursor cell cultures) and TRAP (PBMC cultures) were assessed at days 14 and 21 with the p-nitrophenylphosphate (pNPP) hydrolysis assay. Briefly, osteoblast precursor cell and PBMC cultures were washed twice with PBS and solubilized with 0.1% (V/V) Triton X-100 for 15 minutes. For ALP activity, osteoblast precursor cells were incubated with 22.5 mM pNPP prepared in 0.15 M bicarbonate buffer (pH 10.3), for 1 hour at 37ºC. For TRAP activity, PBMC cultures were incubated with 22.5 mM pNPP prepared in 0.225 M sodium acetate, 0.3375 M KCl, 0,1% Tx-100, 22.5 mM sodium tartrate and 0.225 mM iron chloride (pH 5.8) for 1 hour at 37ºC. After incubation, the reaction was stopped with 5 M NaOH, and the absorbance of the samples was measured at 400 nm in an ELISA plate reader (Synergy HT; Biotek). Results were normalized with the corresponding DNA content and results were expressed in $nmol/min/pg_{DNA.}$

2.2.4.4 Number of TRAP-Positive Multinucleated Cells

At days 14 and 21, PBMC cultures were washed twice with PBS and fixed with 3.7% formaldehyde for 10 minutes at room temperature. Following this, cells were stained for TRAP with the Acid Phosphatase, Leukocyte (TRAP) kit, according to the manufacturer's instructions. Briefly, cells were incubated with 0.12 mg/mL naphthol AS-BI, in the presence of 6.76 mM tartrate and 0.14 mg/mL Fast Garnet GBC, for 1 hour at 37°C, in the dark. After incubation, cell layers were washed and stained with hematoxylin. TRAP-positive (purple/dark red) multinucleated (>2 nuclei) cells were counted under an Inverted Phase Contrast microscope (Nikon TMS, Tokyo, Japan).

2.2.4.5 Histochemical Staining of ALP and Collagen

At days 14 and 21, osteoblast precursor cell cultures were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer for 10 minutes at room temperature and, thereafter, maintained in sodium cacodylate buffer 0.14 M and conserved at 4ºC. For the staining of ALP, fixed cultures were incubated for 1 hour in the dark with a mixture, prepared in Tris buffer (pH 10),

containing 2 mg/mL Na-a-naphthyl phosphate and 2 mg/mL Fast Blue RR salt. The reaction was stopped by rinsing the samples with distilled water. A positive reaction is identified by the presence of a brown to black colour, in accordance with the amount of the enzyme. For the staining of collagen, fixed cultures were incubated with Sirus Red dye for 1 hour at room temperature, and then rinsed with distilled water. Stained cultures were visualized using an Inverted Phase Contrast microscope (Nikon TMS, Tokyo, Japan).

2.2.4.6 F-actin Staining of Osteoblasts Precursor Cells

Osteoblast precursor cell cultures, at day 21, were washed twice with PBS and after being fixed with 3.7% para-formaldehyde for 15 minutes at room temperature, were permeabilized with 0.1% (V/V) Triton X-100 for 5 minutes. Cultures were stained for F-actin with 5 U/mL Alexa Fluor® 647-Phalloidin (Invitrogen, Massachusetts, USA). Cultures were observed by Confocal Laser Scanning Microscopy (CLSM) with a Leica TCP SP2 AOBS confocal microscope (Leica, Wetzlar, Germany).

2.2.4.7 PBMCs Displaying F-actin Rings and Expressing VNR and CTR

PBMC cultures, at day 21, were washed twice with PBS and fixed with 3.7% para-formaldehyde for 15 minutes at room temperature. After fixation, cells were permeabilized with 0.1% (v/v) Triton X-100 for 5 minutes at room temperature. To reduce non-specific staining cells were incubated with 10 mg/mL bovine serum albumin (BSA) for 1 hour at room temperature and then stained for F-actin with 5 U/mL Alexa Fluor® 647-Phalloidin (Invitrogen, Massachusetts, USA), and for VNR and CTR with 50 µg/mL mouse IgGs anti-VNR and IgGs anti-CTR (R&D Systems, Minneapolis, USA), respectively. Detection of IgGs anti-VNR and IgGs anti-CTR was performed with 2mg/mL Alexa Fluor1 488-Goat anti-mouse IgGs. Cultures were observed CLSM with a Leica TCP SP2 AOBS confocal microscope (Leica, Wetzlar, Germany).

2.2.4.8 Intracellular Signalling Mechanisms

The osteoblastic and the osteoclastic cell responses to the lowest concentration that caused statistically significant inhibition of ALP or TRAP-related parameters were characterized for several common signalling pathways involved in osteoblastogenesis and osteoclastogenesis. For this purpose, PBMC and osteoblast precursor cells were treated with the corresponding commercial signalling pathway inhibitor throughout the culture time. The following pathways were tested: MEK (methyl ethyl ketone, inhibitor U0126, 1 lM), NF-kB (nuclear factor kappa-ß, inhibitor PDTC, 10 lM), PKC (protein kinase C, inhibitor GO6983, 5 lM), and JNK (c-Jun N-terminal kinase, inhibitor SP600125, 10 lM). At days 14 and 21, cultures were assessed for ALP (osteoblast precursor cell cultures) and TRAP (PBMC cultures) activities.

2.2.4.9 Statistical analysis

Data were obtained from three separate experiments, each one performed in triplicate. Results are expressed as the mean ± standard deviation. Groups of data were evaluated with a two-way ANOVA, and no significant differences in the patterns of cell behaviour were found. A two-tailed Student's t test was performed to assess statistical differences between controls and experimental conditions P-values ≤ 0.05 were considered to be significant.

Chapter 3

Results

3.1 Osteoblastic Cell Response to Lanthanides

Osteoblasts are mesenchymal origin cells that lay down the extracellular matrix and regulate its mineralization (Neve, Corrado, & Cantatore, 2011). During their development, osteoblasts display various phenotypic markers, such as high ALP activity, and synthesize several proteins that are associated with the mineralized matrix in vivo, including OC and OPN (A. Yamaguchi, Komori, & Suda, 2000a). The effect of lanthanides on osteoblast precursor cell cultures was evaluated, at days 14 and 21, by the assessment of the DNA content, apoptosis, ALP activity, histochemical staining of ALP and collagen, and F-actin staining.

3.1.1 DNA Content

An increase in cell response was observed following zinc supplementation at 10^{-6} M (~ 44% higher than control), while an opposite behaviour occurred for higher concentrations, as shown in Figure 3.1A. At day 21, although a statistical significance was not always reached, the same response pattern was observed.

At day 14, magnesium concentrations did not affect significantly the DNA values of cell cultures, as observed in Figure 3.1B. At day 21, an increase in cell response was achieved in all tested condition (~ 6x higher than the control).

DNA content of osteoblast cell cultures was not significantly affected by cerium at day 14, with the exception of the concentration of 10^{-3} M, which elicited a decrease in the values (~ 29% lower than control). At day 21, only the concentrations of 10^{-7} and 10^{-4} M caused a significant effect in cell response, increasing it by the double, compared to the control (Figure 3.1C).

As shown in Figure 3.1D, at day 14 all lanthanum concentrations displayed an inhibitory role in cell response (~41% lower than control). On contrary, at day 21 all lanthanum concentrations elicited a marked increase of the DNA content (~ 5x higher than control).

At day 14, praseodymium had a significant positive effect on the DNA content, with the maximum response being achieved at concentration of 10^{-6} M (~ 3x higher than the control), as represented in Figure 3.1E. At day 21, a similar, although not so marked effect was observed.

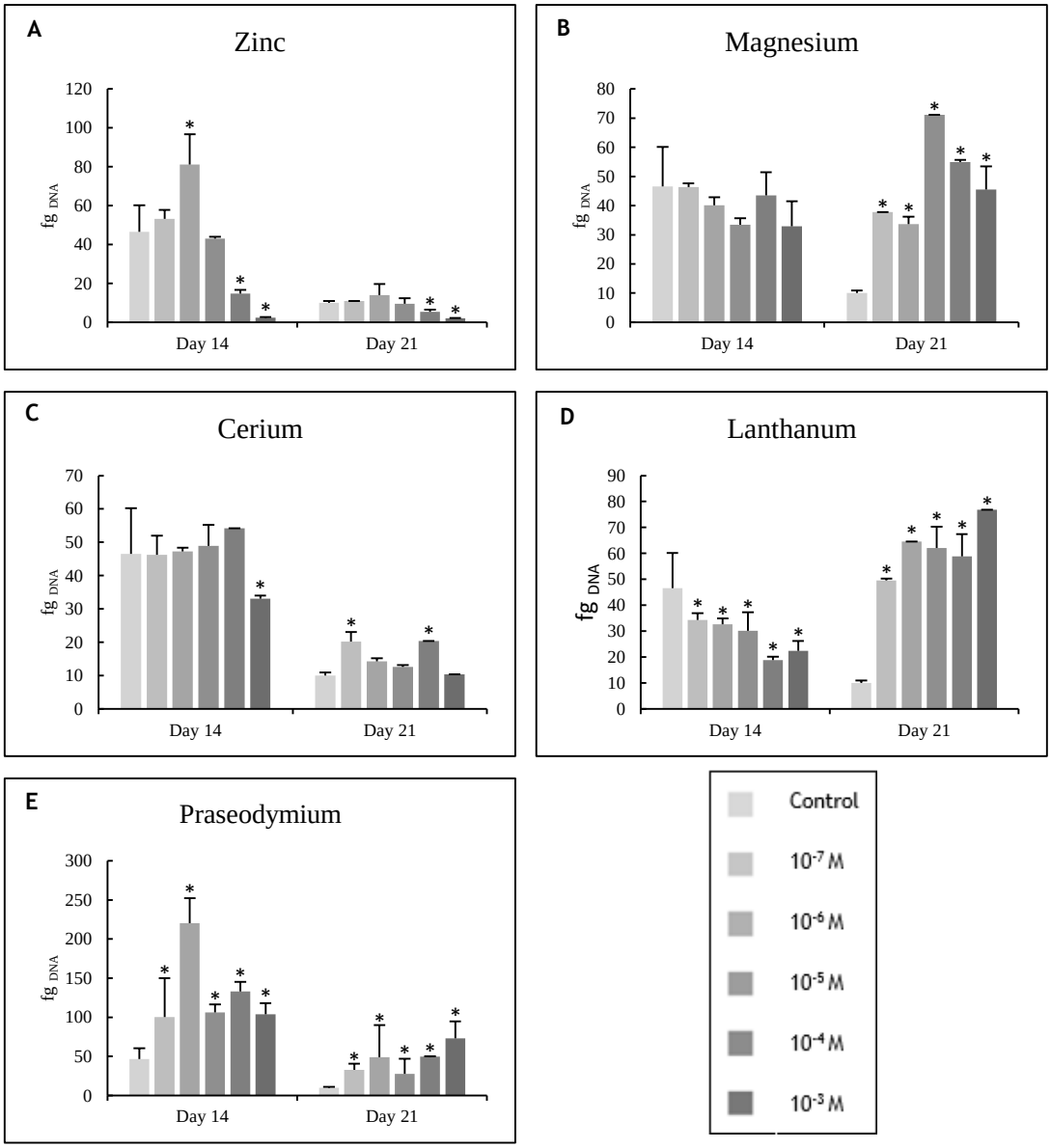


Figure 3.1 - DNA content of osteoblast precursor cells cultured in the presence of zinc, magnesium (A and B) and lanthanides (C to E) at days 14 and 21 of culture. * Significantly different from the control.

3.1.2 Apoptosis

At day 14 only zinc at concentration of 10^{-3} M had a significant effect on caspase-3 activity, by inducing its increase (~ 19% higher than the control), as observed in Figure 3.2A. This behaviour continued to be observed at day 21. Moreover, the lower concentrations started to affect significantly cultured cells, promoting a decrease of cell apoptosis. The maximum decrease was attained by zinc at 10^{-7} M (~ 47% lower than the control).

Magnesium did not significantly affect apoptosis at day 14. At day 21, magnesium at 10^{-5} M promoted a caspase-3 activity decrease (~ 22% lower than the control), as observed in Figure 3.2B. The further concentrations promoted the opposite effect, with magnesium at concentration of 10^{-3} M inducing the most significant rise (~ 30% higher than the control).

Cerium did not cause significant effects in caspase-3 activity at day 14, with exception of concentration 10^{-6} M, which presented an inhibitory role (~ 13% lower than the control), as observed in Figure 3.2C. At day 21, all the cerium tested concentrations elicited a decrease in

apoptosis (~22% lower than the control), with the one exception being 10^{-5} M, which cause an increase in cell response (~18% higher than the control).

As shown in Figure 3.2D, lanthanum at concentration of 10^{-6} and 10^{-5} M was responsible, at day 14, for an increase in apoptosis (~18% higher than the control). The other tested concentrations promoted an opposite effect (~20% lower than control). At day 21, only 10^{-5} M elicited an increase in caspase-3 activity (~16% higher than the control), while the remaining concentrations inhibited it (~20% lower than control).

Praseodymium at concentrations of 10^{-7}-10^{-4} M promoted a decrease of apoptosis (~ 29% lower than control), at day 14, as depicted in Figure 3.2E, while praseodymium at concentration of 10^{-3} M, induced the opposite influence (~ 29%). At day 21, praseodymium at 10^{-4} M inhibited caspase-3 activity (~27% lower than control), and the inverse effect was observed for 10^{-3} M (~10% higher than the control).

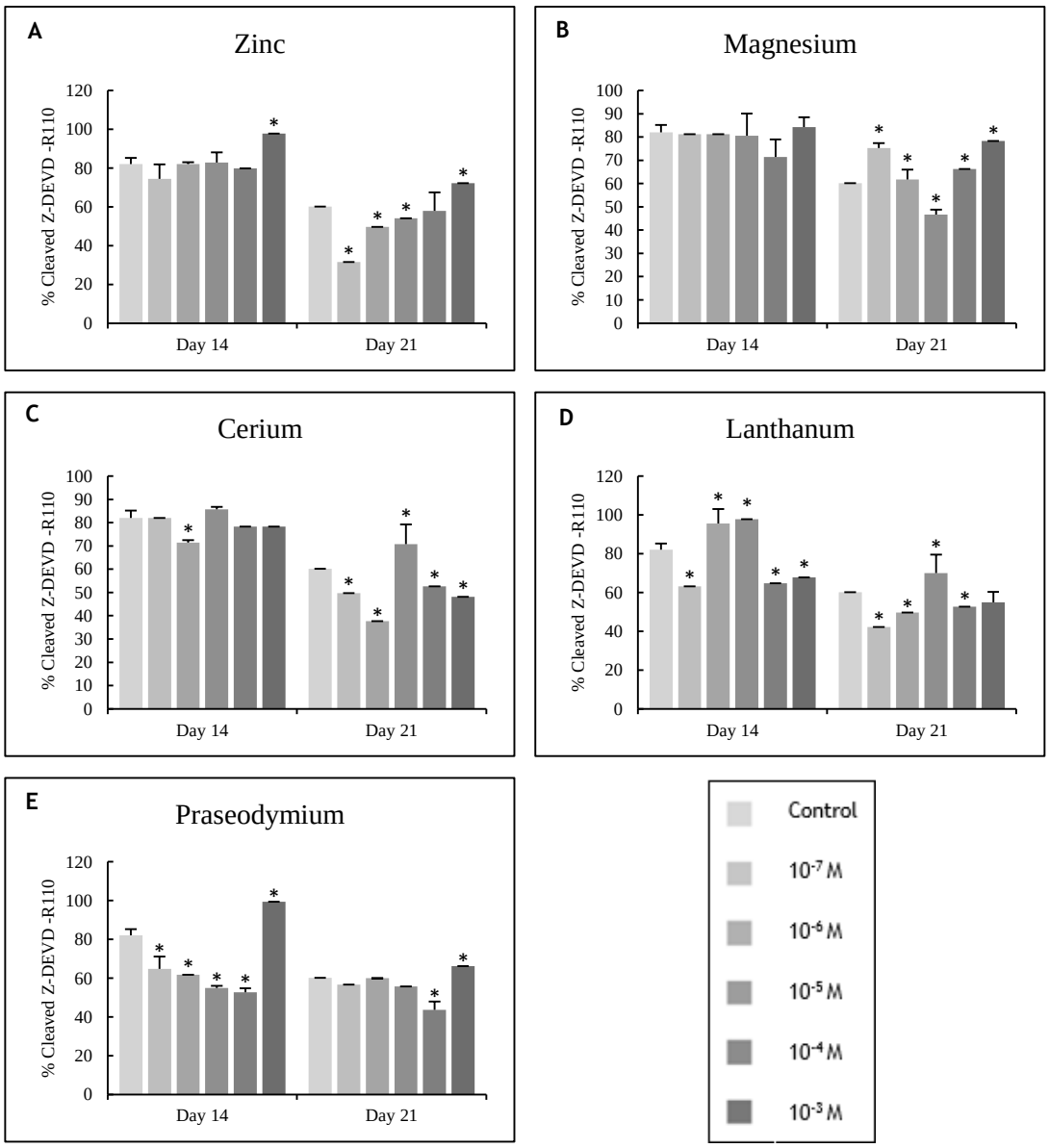


Figure 3.2 – Caspase-3 activity of osteoblast precursor cells cultured in the presence of zinc, magnesium (A and B) and lanthanides (C to E) at days 14 and 21 of culture. * Significantly different from the control.

3.1.3 ALP activity

At day 14, ALP activity of osteoblast precursor cells cultured in the presence of zinc was stimulated by all concentrations (~ 78% higher than the control), with exception of zinc at concentration of 10^{-7} M, which did not affect cell response significantly, as shown in Figure 3.3A. At day 21, zinc at concentration of 10^{-5} M induced the maximum increase of ALP activity (~ 84%), while the lowest concentrations tested elicited a decrease of osteoblastic marker activity (10^{-4} and 10^{-3} M; ~ 84%).

ALP activity was stimulated by magnesium at 10^{-6} -10^{-3} M at day 14 (~ 147% higher than control), whereas, only magnesium at concentration of 10^{-4} M improved the differentiation of osteoblast precursor cells at day 21 (~ 27%), as shown in Figure 3.3B. Magnesium at concentration of 10^{-3} M promoted a significant reduction of ALP activity (~ 97% lower than control), at day 21.

As observed in Figure 3.3C, at day 14 the majority of the cerium concentrations tested, modulated ALP activity positively (~ 166% higher than control). At day 21, cerium at concentration of 10^{-6} M induced the maximum response of osteoblast cells (~ 97% higher than control).

Osteoblast differentiation was affected by lanthanum in an opposite manner at both days, as shown in Figure 3.3D. ALP activity of osteoblast precursor cells was stimulated at day 14 (~ 4 times higher than the control) and inhibited at day 21 (~ 62% lower than control) by lanthanum.

As shown in Figure 3.3E, praseodymium at 10^{-5}-10^{-3} M promoted a significant increase of ALP activity (~ 108% higher than control) at day 14. At day 21, all concentrations tested, with exception of praseodymium at concentration of 10^{-6}, inhibited ALP activity.

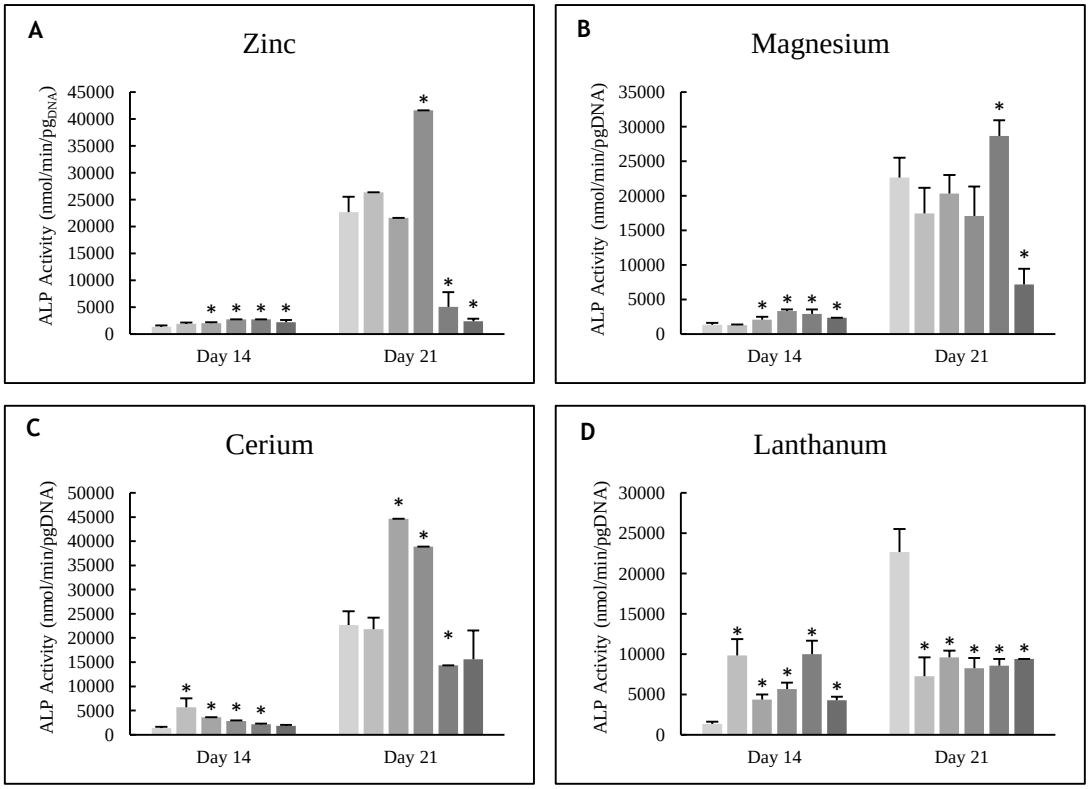


Figure 3.3 – ALP activity of osteoblast precursor cells cultured in the presence of zinc, magnesium (A and B) and lanthanides (C to E) at days 14 and 21 of culture. * Significantly different from the control.

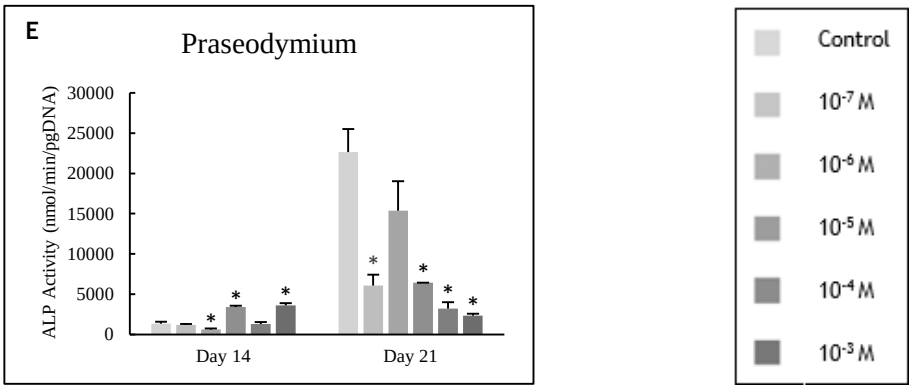


Figure 3.4 – ALP activity of osteoblast precursor cells cultured in the presence of zinc, magnesium (A and B) and lanthanides (C to E) at days 14 and 21 of culture. * Significantly different from the control.

3.1.4 Histochemical Staining of ALP and Collagen

Optical microscopy images from ALP activity at day 14, evaluated by histochemical staining, are presented in Figure 3.4. A dense monolayer organization can be observed in control (Figure 3.4A). Supplemented cultures (Figure 3.4B and 3.4C) showed a change in the culture morphology, with the formation of nodular structures in which grown cells converge to. A strong ALP staining was observed in the presented experimental conditions.

Optical microscopy images from collagen staining assessed by histochemical staining are presented in Figure 3.5. In control, a homogenous cell layer is observed at day 21, staining positive for total collagen (Figure 3.5A). Supplementation induced a nodular organization of the culture, with an increased stain at the nodular structures (Figure 3.5B and 3.5C).

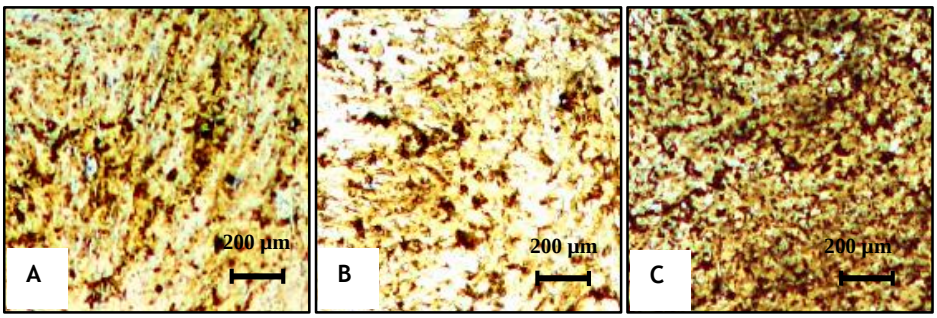


Figure 3.5 - Representative optical microscopy images of osteoblast precursor cell cultures at day 14, following ALP histochemical staining: (A) control, (B) magnesium at 10^{-4} M and (C) cerium at 10^{-6} M. Black bars represent 200 µm.

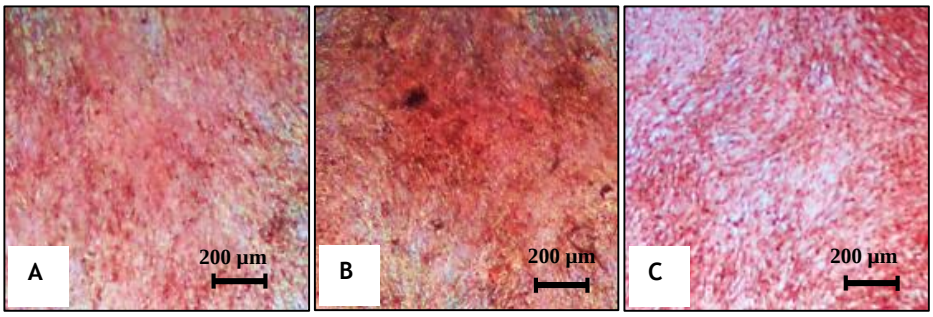


Figure 3.6 - Representative optical microscopy images of osteoblast precursor cell cultures at day 14, following collagen histochemical staining: (A) control, (B) zinc at 10^{-5} M and (C) cerium at 10^{-6} M. Black bars represent 200 µm.

3.1.5 F-actin Staining of Osteoblasts Precursor Cells

Figure 3.6 shows osteoblast precursor cell cultures at day 21, stained for F-actin visualized by CLSM. Cells were uniformly distributed in the wells, with visible cell-to-cell contacts, and displayed an expected spindle-shaped morphology. The amount of osteoblastic cells observed in the different conditions tested was somehow in compliance with the results obtained for ALP activity and staining.

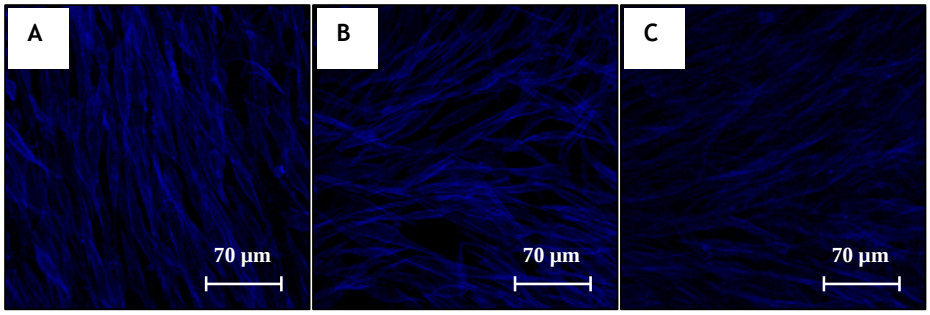


Figure 3.7 - CLSM visualisation of osteoblast precursor cell cultures at day 21, stained blue for F-actin: (A) control, (B) magnesium at 10^{-6} M and (C) praseodymium at 10^{-5} M. White bars represent 70 µm.

3.1.6 Intracellular Signalling Mechanisms

As shown in Figure 3.7A, all the tested pathways, with exception of PKC, were involved in the cell response in control cell cultures, as their inhibition promoted changes in ALP activity.

MEK pathway appeared to be involved in the cellular response developed in all conditions tested, with exception of magnesium and lanthanum (day 21) (Figure 3.7C and 3.7E). Moreover, the involvement of this pathway seemed to be more relevant in cultures performed with zinc and praseodymium, as observed in Figures 3.7B and 3.7F.

ALP production by osteoblast cells in all conditions tested seemed to be negatively modulated by both NFκB and JNK pathways. Moreover, the involvement of these pathways was identical in all conditions tested.

Although, PKC appeared to modulate the cellular response of all conditions tested, a more critical role of this pathway was detected in cells cultured in the presence of lanthanum, as its inhibition promoted an evident decrease of the enzymatic activity (Figure 3.7E).

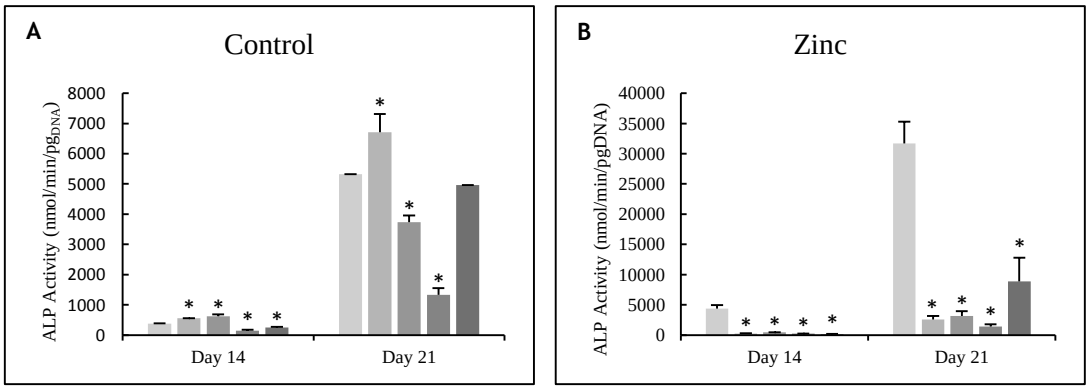


Figure 3.8 - Osteoblast precursor cells cultured with selective inhibitors of the MEK, NFκB, PKC and JNK signaling pathways and maintained in the absence (A) or in presence of the cations (B to F): zinc at 10^{-4} M, magnesium at 10^{-3} M, cerium, lanthanum and praseodymium at 10^{-5} M. Cell responses were evaluated for ALP activity. * Significantly different from the control.

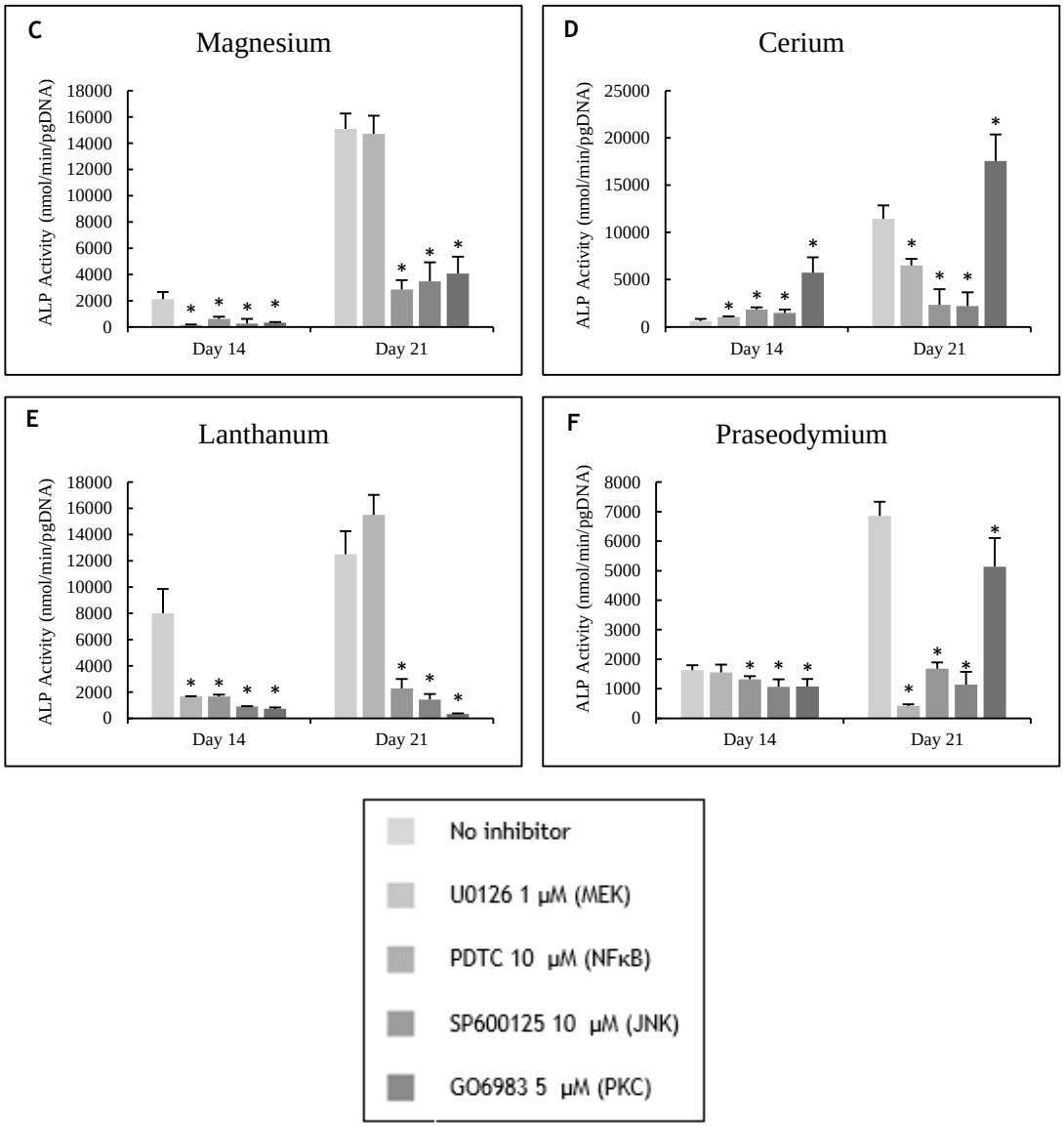


Figure 3.9 – Osteoblast precursor cells cultured with selective inhibitors of the MEK, NFκB, PKC and JNK signaling pathways and maintained in the absence (A) or in presence of the cations (B to F): zinc at 10^{-4} M, magnesium at 10^{-3} M, cerium, lanthanum and praseodymium at 10^{-5} M. Cell responses were evaluated for ALP activity. * Significantly different from the control.

3.2 Osteoclastic Cell Response to Lanthanides

Osteoclasts are ended-differentiated giant multinucleated cells of monocyte/macrophage lineage that are responsible for bone resorption. These bone-resorptive cells adhere and degrade bone matrix by secreting acid and lytic enzymes into the resorption compartment (Boyle *et al.*, 2003). Osteoclast precursors are mononucleated cells, that in contrast to early precursors which do not express TRAP, are TRAP positive. In the final step of osteoclast development, multinucleated osteoclasts are generated by the fusion of the mononucleated precursors (Martin, Wah Nga, & Sims, 2013). The main phenotypic markers of osteoclasts include, along with TRAP expression, CTR and VNR expression (Chambers, 2000).

3.2.1 DNA Content

Overall, regardless of the ion or concentration tested, it was observed a considerably decrease of the DNA content from day 14 to day 21 in all PBMC cultures.

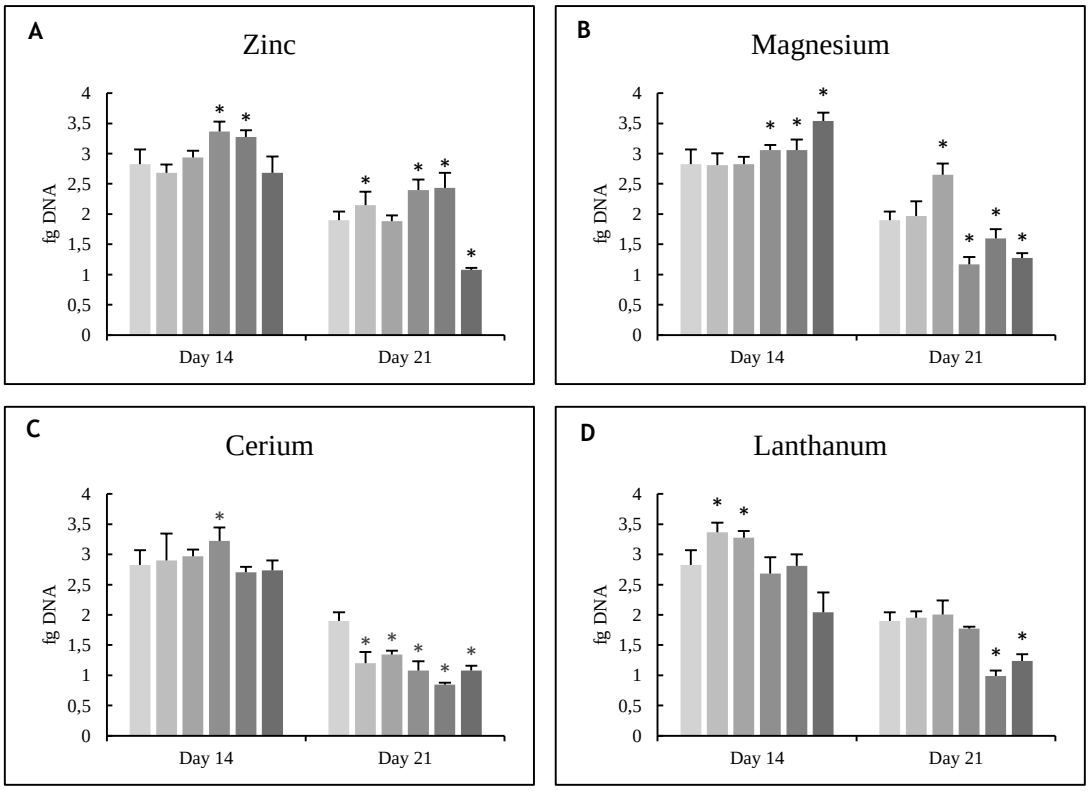
As shown in Figure 3.8A, zinc at concentrations of 10^{-5} and 10^{-4} M showed a stimulatory effect (~ 17% higher than the control) on the DNA content of PBMCs at day 14. At day 21, these concentrations continued to endorse a stimulatory effect (~ 27% higher than the control) and zinc at concentration of 10^{-7} M elicited an identical effect (~ 13% higher than the control). In contrast, zinc at 10^{-3} M induced an inhibitory effect (~ 43% lower than the control).

As observed in Figure 3.8B, magnesium at concentrations of 10^{-5} - 10^{-3} M increased the amount of DNA in the PBMC cultures (~ 14% higher than the control), at day 14. At day 21, all the tested concentrations of magnesium affect cell response, with exception of 10^{-7} M.

At day 14, only cerium at 10^{-5} M showed a significant effect on the DNA content of PBMC cultures (~ 14% higher than the control). At day 21, all cerium concentrations induced a decrease of DNA in the PBMC cultures, as observed in Figure 3.8C. The maximum decrease of DNA content was attained by the highest concentration tested (~ 49% lower than the control).

As depicted in Figure 3.8D, the lowest concentrations of lanthanum were shown to promote an increase (10^{-7} and 10^{-6} M; ~ 17% higher than the control) of DNA content at day 14, while the highest concentration tested originated the opposite effect (10^{-3} M; ~ 28% lower than the control). At day 21, only the highest concentrations tested had a significant effect in cell response, eliciting a decrease of DNA content (10^{-4} and 10^{-3} M; ~ 41% lower than the control).

At day 14, the DNA content of PBMC cultures was only significantly affected by praseodymium at concentration of 10^{-5} M (~ 35% lower than the control), as demonstrated in Figure 3.8E. At day 21, it was observed a decrease in cell behaviour caused by all tested conditions (~ 28% lower than the control)



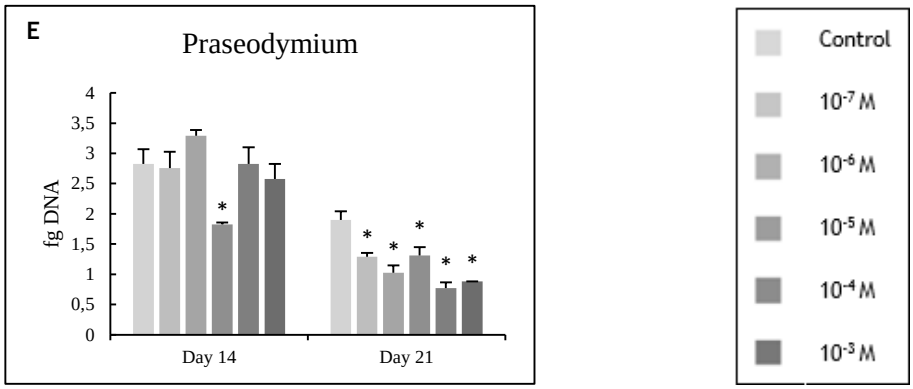


Figure 3.10 - DNA content of PBMCs cultured in the presence of zinc, magnesium (A and B) and lanthanides (C to E) at days 14 and 21 of culture. * Significantly different from the control.

3.2.2 Apoptosis

At day 14, all zinc concentrations promoted an identical increase (~ 39% higher than the control) of caspase-3 activity, as depicted in Figure 3.9A. In contrast, at day 21, the same concentrations induced a decrease in apoptosis (~ 21% higher than the control).

As shown in Figure 3.9B, cultures performed in the presence of magnesium showed an increased apoptotic response (~ 32% higher than the control). At day 21, magnesium induced a slight decrease of caspase-3 activity (~ 11% lower than the control)

Cerium at concentrations of 10^{-7} - 10^{-5} M promoted the increase of apoptosis (~ 48% higher than the control) at day 14, in contrast the lower concentrations induced a slight decrease of caspase-3 activity (~ 22% lower than the control), as shown in Figure 3.9C. At day 21, all cerium concentrations, with exception of cerium at 10^{-4} M which did not affect significantly the cellular response, promoted a reduction of apoptosis (~ 19% lower than the control).

At both days, lanthanum at 10^{-6} - 10^{-3} M inhibit the apoptotic response of PBMC cultures, as observed in Figure 3.9D. The lowest concentration tested did not significantly affect cell response on time points.

All praseodymium concentrations, with exception of 10^{-5} and 10^{-3} M, induced a reduction of apoptosis (~ 19% lower than the control), as represented in Figure 3.9E. At day 21, all concentrations tested inhibit the apoptotic response. The reduction was higher at the higher concentration (10^{-3} M; ~ 27% lower than the control) and identical at the lowest concentrations (10^{-7} M-10^{-5} M; ~ 14% lower than the control).

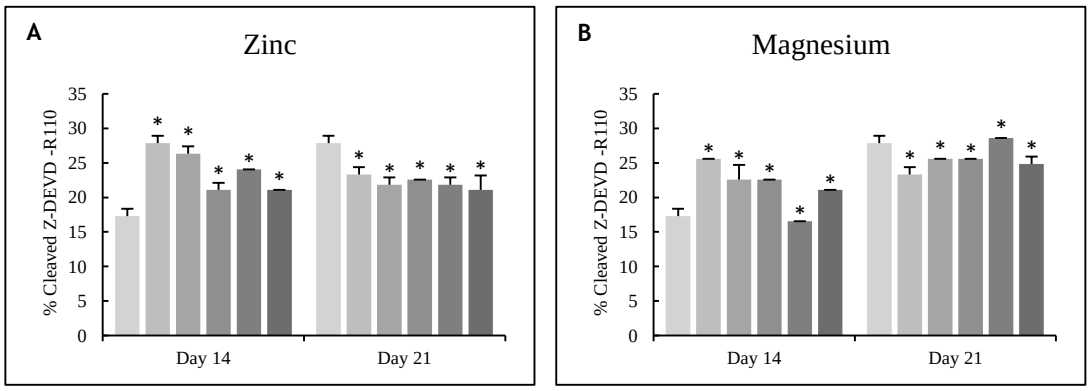


Figure 3.11 - Caspase-3 activity of PBMCs cultured in the presence of zinc, magnesium (A and B) and lanthanides (C to E) at days 14 and 21 of culture. * Significantly different from the control.

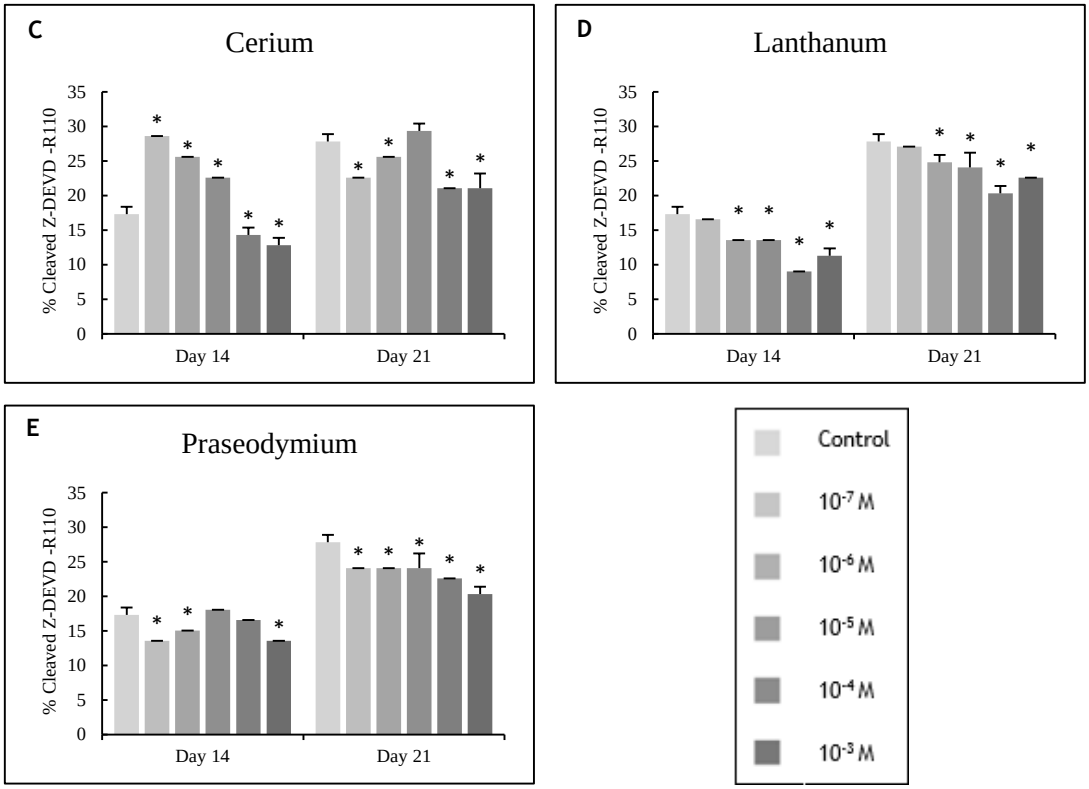


Figure 3.12 - Caspase-3 activity of PBMCs cultured in the presence of zinc, magnesium (A and B) and lanthanides (C to E) at days 14 and 21 of culture. * Significantly different from the control.

3.2.3 TRAP activity

As shown in Figure 3.10A, all zinc concentrations tested, with exception of 10^{-3} M, stimulated the differentiation of PBMCs amongst days 14 and 21 of culture, as demonstrated by the increase of TRAP activity comparatively to control. At day 14 the maximum response was observed at concentration of 10^{-5} M (~ 65% higher than the control), while a more pronounced response at day 21 was attained at concentrations of 10^{-6} - 10^{-4} M (~ 87% higher than the control). Compared to the control, the concentration of 10^{-3} M elicited a decrease in cell behaviour at day 21 (~ 20% lower than the control).

TRAP activity was increased on both days by magnesium at concentrations of 10^{-7} and 10^{-6} M (~ 58% and ~ 69% at days 14 and 21, respectively), as shown in Figure 3.10B. Magnesium at 10^{-3} M and 10^{-4} M inhibited the differentiation of PBMCs on day 14 (~ 68% lower than the control), but at day 21 they did not significantly affect cell response.

As shown in Figure 3.10C, all cerium concentrations tested increased TRAP activity on both days, as observed in Figure 2C (~ 61% and 125% at days 14 and 21, respectively).

As shown in Figure 3.10D, on both days lanthanum significantly stimulated the differentiation of PBMCs at the lowest concentrations tested (10^{-7} and 10^{-6} M; ~ 72% and ~ 63% at days 14 and 21, respectively), while the highest concentrations inhibited the differentiation (10^{-4} and 10^{-3} M; ~ 57% and ~ 63% at days 14 and 21, respectively). At day 21, the maximum response was achieved at a lanthanum concentration of 10^{-5} M (~ 97%).

At day 14, praseodymium at 10^{-7}-10^{-6} M promoted a significant increase of TRAP activity (~ 78%), whereas a decrease of the osteoclastic marker activity was elicited by the highest con-

centrations tested (10^{-4} and 10^{-3} M; ~ 30%), as shown in Figure 3.10E. This behaviour was maintained at day 21, though with more pronounced differences, when compared with the control (~ 140% increase for 10^{-7}-10^{-5} M; ~44% decrease for 10^{-4} and 10^{-3} M).

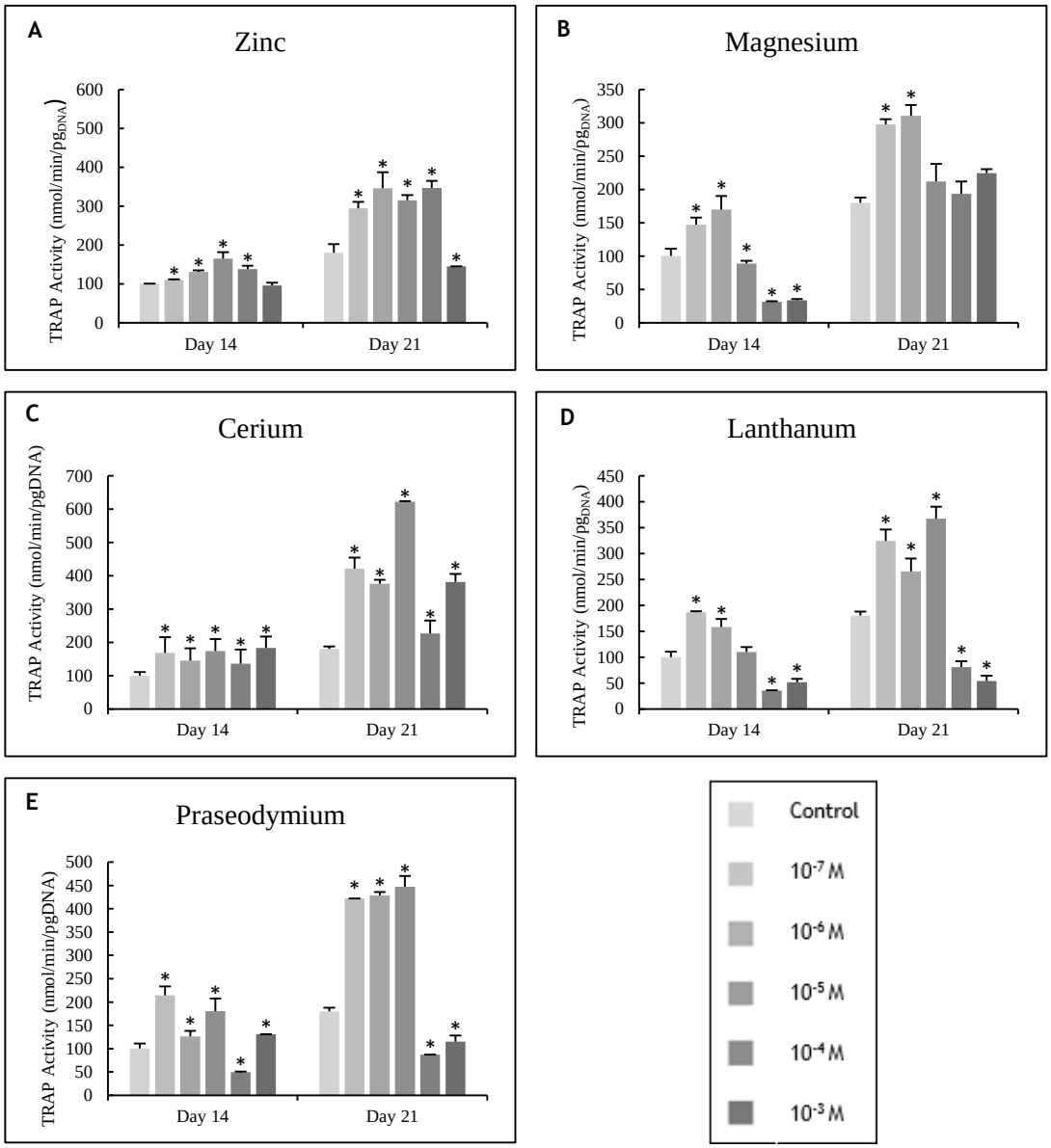


Figure 3.13 - TRAP activity of PBMCs cultured in the presence of zinc, magnesium (A and B) and lanthanides (C to E) at days 14 and 21 of culture. * Significantly different from the control.

3.2.4 Number of TRAP-Positive Multinucleated Cells

TRAP-positive multinucleated cells were formed in PBMCs cultures in the presence of zinc (Figure 3.11A). As observed for TRAP activity, at day 14 the maximum response was attained by zinc at concentration of 10^{-5} M (~ 97% higher than the control). At day 21 a more marked response was induced by zinc at 10^{-6} - 10^{-4} M (~ 58%).

In the presence of magnesium, TRAP-positive multinucleated cells formation was stimulated on both days by magnesium at concentrations of 10^{-7} - 10^{-5} M (~ 37% and ~ 21% at days 14 and 21, respectively), as observed in Figure 3.11B. Moreover, magnesium at concentrations of 10^{-4} and 10^{-3} M, inhibited the formation of TRAP-positive multinucleated cells on both days (~ 88%).

At day 14, cerium at 10^{-7} - 10^{-5} M prompted the formation of TRAP-positive multinucleated cells (~ 14% higher than the control), as represented in Figure 3.11C. At day 21, the majority of cerium concentrations promoted TRAP-positive multinucleated cells formation. The maximum response was induced by cerium at 10^{-5} M (~ 91% higher than the control).

As shown in Figure 3.11D, on both days lanthanum significantly stimulated the formation of TRAP-positive multinucleated cells at the lowest concentrations tested (10^{-7} and 10^{-6} M; ~ 43% and ~ 56% at days 14 and 21, respectively), while the highest concentrations inhibited the differentiation (10^{-4} and 10^{-3} M; ~ 89% and ~ 92% at days 14 and 21, respectively).

At day 14, praseodymium at concentrations of 10^{-7} and 10^{-6} M promoted a significant increase of TRAP-positive multinucleated cells formation (~ 53% higher than control), as shown in Figure 3.11E. As observed, at day 21, praseodymium at 10^{-4} and 10^{-3} M played an inhibitory role (~ 41% lower than control).

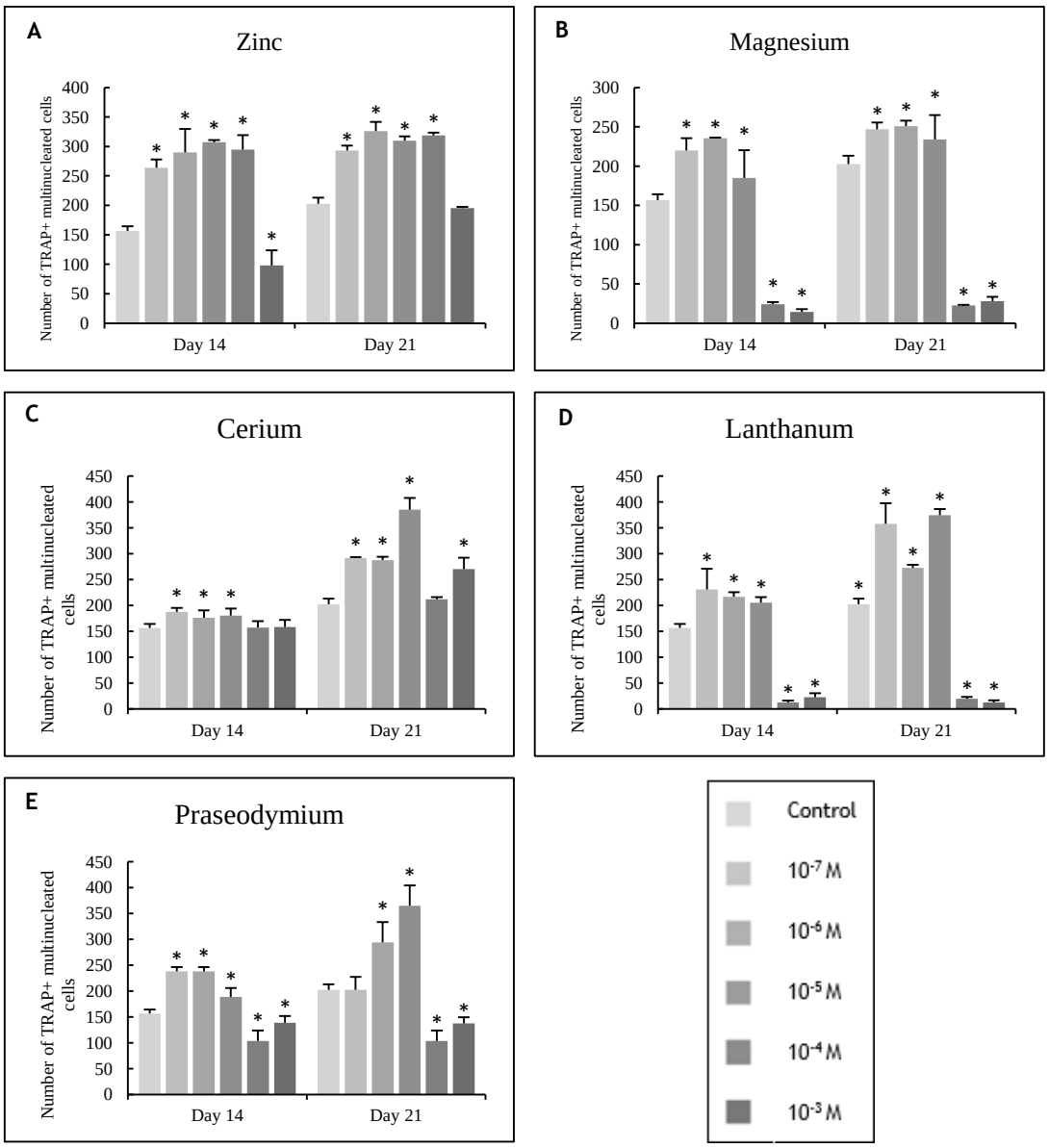


Figure 3.14 - Number of TRAP-positive multinucleated cells on PBMC cultures: in the presence of zinc, magnesium (A and B) and lanthanides (C to E) at days 14 and 21 of culture. * Significantly different from the control.

3.2.5 PBMCs Displaying F-actin Rings and Expressing VNR and CTR

Representative CLSM images of PBMC cultures, stained blue for F-actin and green for VNR and CTR, at day 21, are shown in Figure 3.12. Cells displayed well-defined F-actin rings and positive staining for VNR and CTR in all tested conditions. The amount of PBMCs in the different conditions was somehow correlated with the results observed for TRAP activity and staining, that means, the presence of zinc and lanthanum (10^{-6}M and 10^{-5}M, respectively) increased the amount of osteoclastic cells in culture.

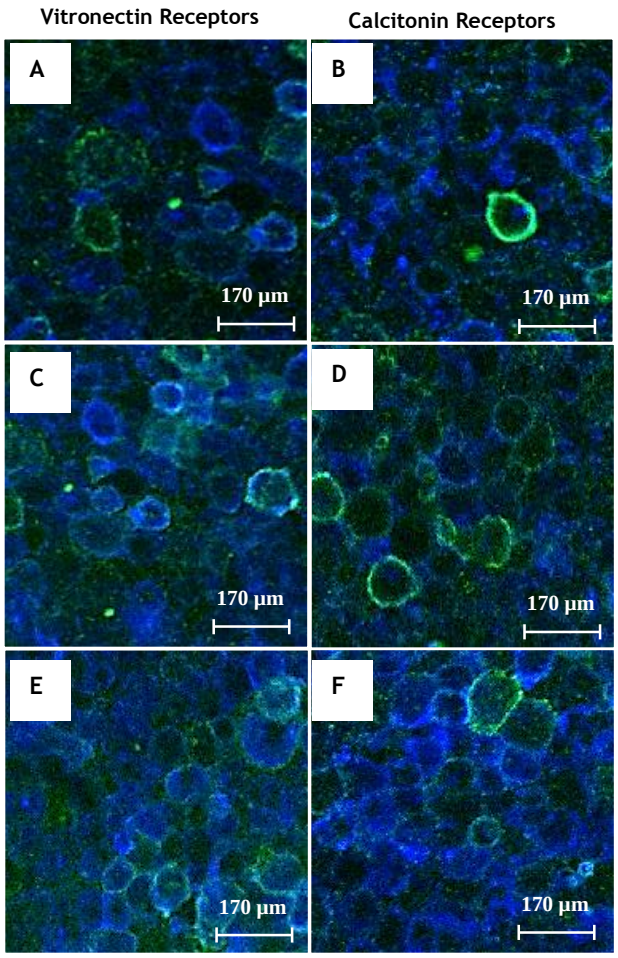


Figure 3.15 - CLSM visualisation of PBMC cultures at day 21, stained blue for F-actin and green for VNR and CTR: control (A and B), zinc at 10^{-6} M (C and D) and lanthanum at 10^{-5} M (E and F). White bars represent 170 µm.

3.2.6 Intracellular Signalling Mechanisms

In PBMC cultures maintained in control conditions (Figure 3.13A), all the tested pathways were involved in the cell response, but NFκB was essential, as its inhibition completely abolished TRAP synthesis.

The involvement of MEK pathway in PBMC development appeared not to modulate TRAP activity of cells cultured in the presence of lanthanum (day 21), while in the presence of the zinc, magnesium and the remaining lanthanides, it seemed to have a high relevance in the process.

NFκB seemed to play the most critical role in the cell response, in the different conditions tested (Figures 3.13B-3.13F).

JNK was not significantly involved in the response of cells cultured in the presence of zinc, magnesium (day 21) and cerium, as observed in Figures 13.3B, 13.3C and 13.3D. In the remaining conditions, JNK appeared to have a relevant role in osteoclastogenesis, as shown in Figures 13.3E and 13.3F.

PKC showed to be very important in all tested conditions, particularly in cell cultures performed in the presence of praseodymium, as demonstrated in Figure 13.3F.

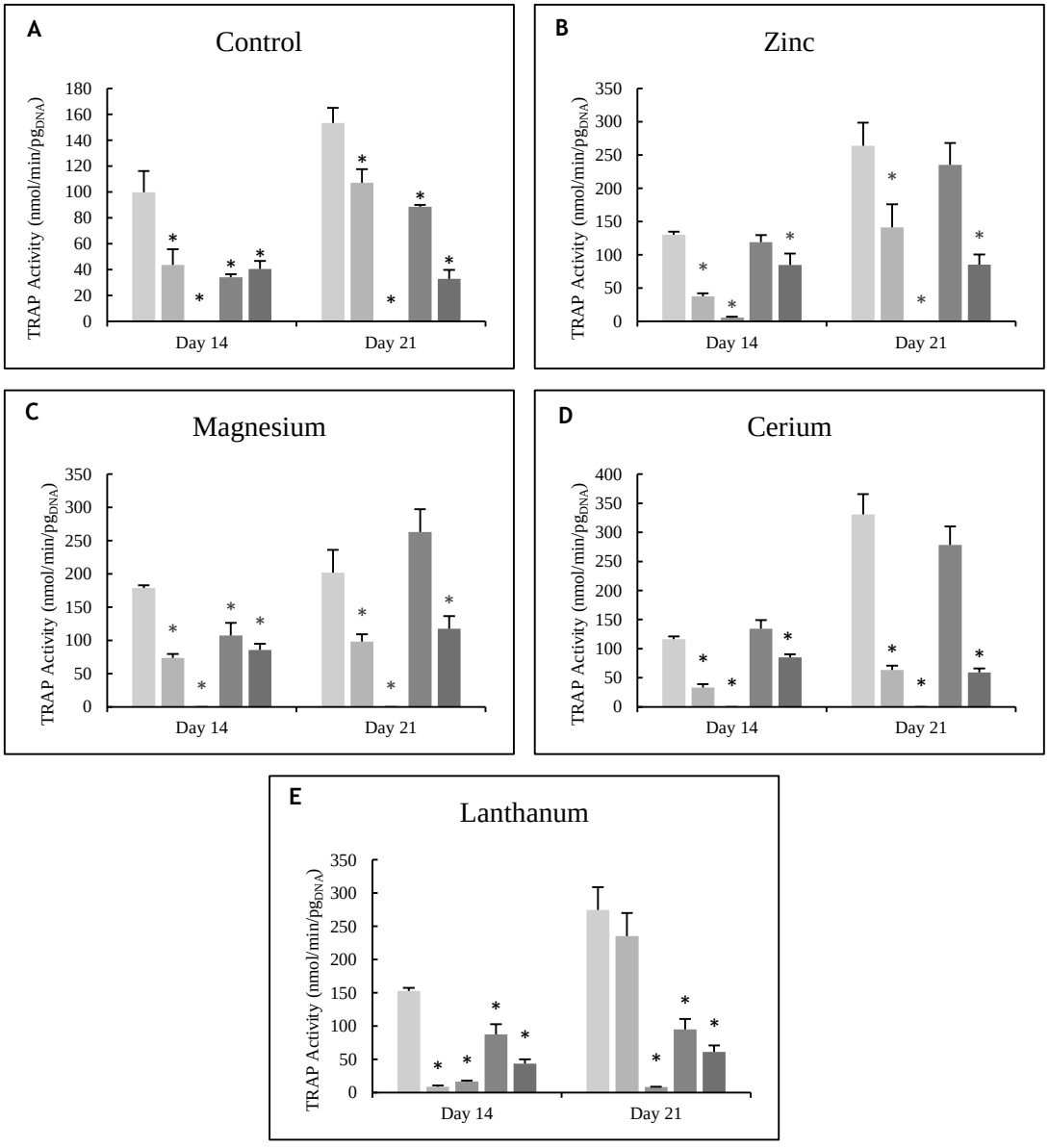


Figure 3.16 - PBMCs cultured with selective inhibitors of the MEK, NFκB, PKC and JNK signaling pathways and maintained in the absence (A) or in presence of the cations (B to F): zinc at 10^{-4} M, mag-nesium at 10^{-3} M, cerium, lanthanum and praseodymium at 10^{-5} M. Cell responses were evaluated for TRAP activity. * Significantly different from the control

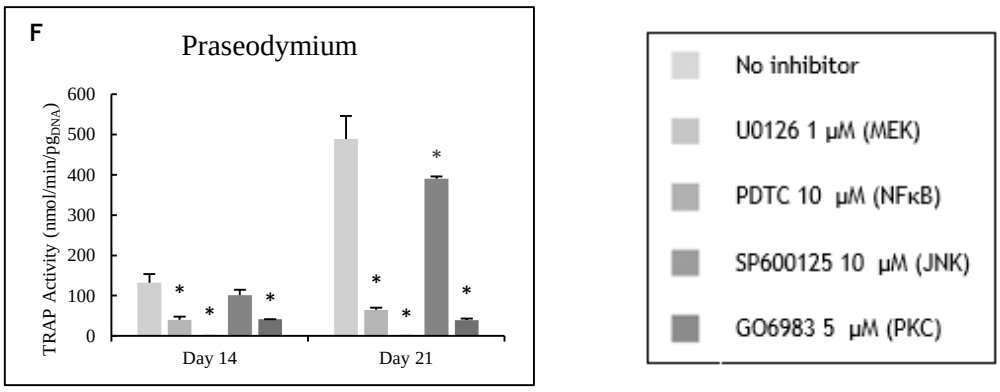


Figure 3.17 - PBMCs cultured with selective inhibitors of the MEK, NFκB, PKC and JNK signaling pathways and maintained in the absence (A) or in presence of the cations (B to F): zinc at 10^{-4} M, mag-nesium at 10^{-3} M, cerium, lanthanum and praseodymium at 10^{-5} M. Cell responses were evaluated for TRAP activity. * Significantly different from the control

Chapter 4

Discussion

4.1 Overview

Bone is a specialized and dynamic organ that undergoes a continuous remodeling throughout life (Clarke, 2008). Its structure and metabolic activity may become compromised by any imbalance between bone formation and resorption, which depend on osteoblasts and osteoclasts, respectively (Wróbel & Witkowska-Zimny, 2013).

Lanthanides are a group of metal ions with similar properties, which have been known for their diversity of biological effects and of great potential in medical applications *(K. Wang et al.*, 1999). Lanthanides have been shown to functionally mimic calcium, as they possess analogous chemical characteristics, namely, ionic radii, donor atom preferences and coordination numbers (Fricker, 2006; Sastri *et al.*, 2003). Due to these similarities, lanthanides can exchange with calcium in bone and, consequently, affect the remodeling cycle, by modulating osteoblast and osteoclast differentiation and function (Barta *et al.*, 2007; Pidcock & Moore, 2001; Webster, Massa-Schlueter, Smith, & Slamovich, 2004).

Due to these characteristics, lanthanides attracted much attention in recent years and became the focus of interest for the synthesis of bone related biomaterials (Aissa *et al.*, 2009). In this context, the activation and metabolic activity of osteoblast-like cell lines (HOS TE 85) on samarium oxide surfaces was reported. These cells displayed a normal morphology and growth, expressed ALP and osteocalcin and presented a slight increased proliferation comparatively to controls (Herath, Di Silvio, & Evans, 2010). In another work, Webster *et al.* doped HAp with various metal cations (such as, Mg^{2+}, Zn^{2+}, La^{3+}, Y^{3+}) in an attempt to enhance Hap properties, relevant for orthopaedic and dental applications. The results obtained indicated that human osteoblasts adhered and differentiated in response to HAp doped with trivalent cations at earlier time points than with HAp doped with divalent cations or undoped Hap (Webster *et al.*, 2004).

Apart from these studies, more classical approaches related to the effect of lanthanides on bone cells *in vitro* were also performed by several authors. Wang et al reported that ALP activity of primary mouse osteoblasts was enhanced by lanthanum (10^{-8} to 10^{-5} M) after two days in culture and inhibited by the same range of concentrations after seven days in culture (X. Wang *et al.*, 2008). Yttrium effect on primary mouse osteoblasts was also investigated. The results showed that yttrium inhibited the differentiation of osteoblasts at tested concentrations (10^{-9} to 10^{-4} M) at day 2 and that the lower concentrations (10^{-9} to 10^{-7} M) inhibited the differentiation at day 3. Moreover, tested concentrations showed to promote osteoblast proliferation at days 1, 2 and 3 (J. Zhang et al., 2010).

The influence of lanthanides on bone resorbing function of osteoclasts was also reported, although in a less extent than on osteoblasts. Zhang *et al.* investigated the effect of several

lanthanides by culturing Japanese white rabbit osteoclasts on bone slices. The number of osteoclasts and the surface areas of the lacunae indicated that lanthanum, samarium, erbium (10^{-7}, 10^{-6} and 10^{-5} M), neodymium, gadolinium and dysprosium (10^{-6} and 10^{-5} M) inhibited osteoclastic activity (J. Zhang *et al.*, 2003). Zhang *et al.* also analysed the effect of lanthanum (10^{-8} to 10^{-5} M) on the bone resorbing activity of rabbit mature osteoclasts co-cultured with osteoblasts. The results demonstrated that after 20 days in culture, osteoclasts started to spread and adhere different morphologies. Moreover, histochemical staining of TRAP revealed red deposits located in the cytoplasm. The authors suggested that in addition to osteoblast-osteoclast contact, lanthanum was also required to produce a change in the resorbing activity of osteoclasts (J. Zhang *et al.*, 2005).

Although the effect of lanthanides on mouse/rabbit bone cells had already been somehow described in several studies, to the best of our knowledge few reports focused on the effect of lanthanides on human bone cells. Therefore, in the present study, cultures of human osteoclastic and osteoblastic precursor cells were used as *in vitro* models to address the cellular and molecular effects of lanthanides (lanthanum, cerium and praseodymium) at different concentrations (10^{-7} to 10^{-3} M) on osteoblast and osteoclast development. Zinc and magnesium were used as reference physiological cations, since the effect of these elements on bone cells differentiation is already well-established.

Osteoblast and osteoclast differentiation are controlled by complex activities involving several signalling pathways and transcriptional regulators (Datta *et al.*, 2008). The intracellular signalling pathways involved in the cellular response induced by lanthanides has not been well-explored so far. Accordingly, to gain insight into the intracellular events that modulate osteoblast and osteoclast behaviour in the presence of lanthanides, several important signalling pathways involved in osteoblastogenesis and osteoclastogenesis were characterized in the present study.

4.2 Osteoblastic Cell Response to Lanthanides

During the differentiation of osteoblast precursor cells into osteoblasts, cells undergoes DNA synthesis and cell division, resulting in a rapid increase in cell number until confluence (Bellows, Aubin *et al.* 1991). The rate of DNA synthesis is known to vary in coordination with the rate of cell proliferation (Rubin, 2005). Accordingly, the effect of lanthanides on cell proliferation was evaluated by quantifying the total amount of double-stranded DNA through the culture time.

The results of the DNA content showed that, although a statistical significance was not always reached, the lowest concentrations of zinc increased DNA synthesis while the highest concentrations endorsed the opposite behaviour. The positive influence of zinc in bone formation was proposed to be in part related with bone protein synthesis, since stimulatory effect of zinc compound on DNA synthesis in osteoblastic cells is completely abolished when cells are cultured with inhibitors of protein synthesis (Hashizume & Yamaguchi, 1993). The effect of zinc on DNA synthesis was previously investigated in osteoblastic MC3T3-E1 cells. In one of the works, cells were cultured with a zinc compound, namely zinc sulfate, in a range of concentrations of 10^{-7} to10^{-5} M, for 72 hours. The results showed that zinc sulfate at concentration of 10^{-5} M produced a significant increase of cell number, DNA content, and protein concentration (M. Yamaguchi & Matsui, 1996). In another work, cells were cultured and treated with various con-

centrations of zinc (1, 3, 15, 25 uM) for 1, 5 and 10 days. The results showed that cell proliferation was stimulated at low zinc concentration (1-3 μM) at day 1. Furthermore, zinc-stimulated cell proliferation was more prominent at 15 and 25 uM at days 5 and 10 (Seo, Cho, Kim, Shin, & Kwun, 2010).

All concentrations of magnesium showed a positive effect on the DNA content of osteoblast precursor cells at day 21, although a less pronounced effect was denoted at the lowest concentrations. These results were not surprising, since magnesium is required for cell proliferation. Low magnesium concentrations is known to reduce protein synthesis, which is followed by a large reduction in the rate of DNA synthesis (Rubin, 2005). The effect of magnesium on DNA synthesis of osteoblast precursor cells was previously assessed on human osteoblast-like MG-63. In these study cells were incubated in the presence of different concentrations of both calcium (0.1 and 1 mM) and magnesium (0.01, 0.1, 0.4 and 0.8 mM) for 24 and 48 hours. DNA synthesis was significantly decreased at both time points under culture conditions of magnesium below 0.8 mM, at all concentrations of calcium (Abed & Moreau, 2007). Thus, it is observed a discrepancy between the results obtained in the present study and those obtained by Abed and Moreau. This difference could be explained by the different cells tested in both studies, and by the use of calcium in their experiment. Magnesium is commonly noted as a calcium antagonist, therefore, these two elements are often linked and can compete with each other for the same receptors (Jahnen-Dechent & Ketteler, 2012). Accordingly, the competition between the two elements could be responsible by the loss of magnesium effect. Moreover, the different time points used may also be responsible for the differences founded.

The DNA synthesis in osteoblast precursor cells was shown to be inhibited at day 14 by cerium at concentration of 10^{-3} M and promoted at day 21 by 10^{-7} and 10^{-4} M. The remaining concentrations tested did not elicited a statistical significant cell response, however, by analysing the qualitative behaviour of the cultures it was possible to verified that all conditions, with exception of 10^{-3} M at day 14, promoted an identical or slightly increased response compared to control at both days. Therefore, cerium apparently did not influence negatively the proliferation of osteoblast precursor cells, regardless of the concentration used. The effect of cerium (10^{-9} to 10^{-4} M) on the proliferation of primary mouse osteoblasts was already described by Zhang *et al*. The authors demonstrated that all concentrations tested promoted the proliferation of cells at days 1, 2 and 3. Moreover, the highest concentration of cerium tested (10^{-4} M) has been shown to promote the maximum effect at all the culture days.

Lanthanum-induced DNA synthesis presented an opposite response pattern at both time points analysed. At day 14 all concentrations tested reduced osteoblast precursor cells proliferation, whereas, an increased proliferation was attained by all concentrations at day 21. The present finding suggests a gradual effect of lanthanum on osteoblast proliferation. Apparently, cells need to adapt to lanthanum before the increase of DNA content becomes evident. This gradual response has already been described in osteoblasts, isolated from Sprague-Dawley fetal neonatal rats, cultured in the presence of lanthanum at a range of concentrations of 10^{-8} to10^{-4} M. After incubation of osteoblasts with lanthanum for 1 day, only lanthanum at 10^{-6} M showed a positive effect on the cell proliferation. However, all concentrations significantly increased cell number after two days (X. Wang, Huang, Zhang, & Wang, 2009).

All concentrations of praseodymium tested increased DNA content at both time points, though with a less pronounced response at day 21. These results may suggest that, although praseodymium affects positively cell proliferation, over time this effect is lost. As far as we

know, the influence of praseodymium on osteoblast proliferation has not been described heretofore.

Apoptosis is a highly organized process characterized by the progressive activation of precise pathways leading to specific biochemical and morphological alterations (Franco, DeHaven, Sifre, Bortner, & Cidlowski, 2008). Cell selection by apoptosis occurs during normal physiological functions as well as diseases (Corcoran *et al.*, 1994). Besides, it has been recognized that many of the toxic effects are mediated by regulation/induction of apoptosis (Franco, Sánchez-Olea, Reyes-Reyes, & Panayiotidis, 2009). Aside from promoting cell proliferation, lanthanides have been also shown to induce apoptosis (Shen *et al.*, 2010). Lanthanide induced-apoptosis has been described in several cell types, namely, rat skin fibroblasts, HeLa and PC12 cells (K. Wang *et al.*, 2003). Only a few reports described the effect of lanthanides on bone cell apoptosis.

Apoptosis results of cultures performed in the presence of zinc showed a concentration-dependent increase, at day 21. The maximum apoptotic response was attained, at both days, by zinc at concentration of 10^{-3} M, which suggests that this concentration may have a toxic effect on osteoblast precursor cells, a value significantly higher than the physiological levels of zinc in human serum (10.1 μM to 16.8 μM) The increased apoptotic response at the higher concentrations is in line with the observed decrease of the DNA content.

Osteoblast precursor cells culture in the presence of magnesium at concentration of 10^{-5} M showed a slightly lower caspase-3 activity. This result is in agreement with the results of the DNA content, since magnesium at 10^{-5} M was shown to induce a more pronounced DNA synthesis. The remaining conditions tested induced an increase in apoptosis.

Cultures performed in the presence of cerium and lanthanum presented an identical apoptotic profile. In both cases, an increased apoptotic response was induced by both lanthanides at 10^{-5} M (cerium at day 14 and lanthanum at days 7 and 14). Moreover, a decrease in caspase-3 activity induced by both the lowest and the highest concentrations was detected in the two conditions By comparing the results of the two lanthanides at concentration of 10^{-5} M was observed a slightly higher response when cells were cultured with cerium. This suggests that cerium presents a more toxic effect than lanthanum. This issue was already stated with male Sprague-Dawley rat pulmonary alveolar macrophages, exposed to cerium, lanthanum, and neodymium for 20 hours. The results showed that cerium was highly cytotoxic, followed by lanthanum, whereas neodymium showed only a slight reduction of viability (Palmer, Butenhoff, & Stevens, 1987).

Cells cultured in the presence of praseodymium showed an increased caspase-3 activity at both days at concentration of 10^{-3} M, nevertheless, the response promoted by this concentration was less pronounced at day 21. Moreover, it was verified that all concentrations tested (with exception of 10^{-3} M) inhibited apoptosis at day 14, while, a similar response to control was attained by these concentrations at day 21 (although a statistical significance was not reached). Praseodymium has been previously found to be toxic to human cells, such as, osteosarcoma cells, umbilical cord perivascular cells, causing their apoptosis (Polis *et al.*, 2013).

Osteoblast differentiation plays a critical role in bone formation and remodeling. At a molecular level, it is considered that an increase of ALP activity is related to the transition from an earlier to a more mature stage of osteoblast differentiation (Stein & Lian, 1993). Thus, as one of the most widely recognised biochemical markers of osteoblastic activity, ALP activity was used in the present work to assess the ability of lanthanides to modulate osteoblast precursor cells differentiation (Seo *et al.*, 2010).

ALP synthesis was highly stimulated by zinc at concentration of 10^{-5} M at day 21, while the highest concentrations tested appeared to prevent cell differentiation. The ability of zinc to stimulate ALP activity in a concentration-dependent manner was already reported in previous works, where mouse osteoblastic MC3T3-E1 showed an increased ALP activity pattern upon zinc treatment (1, 3, 15, 25 uM) (Seo *et al.*, 2010; M. Yamaguchi, 1998; M. Yamaguchi & Yamaguchi, 1986).

It was observed, at day 21, that while magnesium at 10^{-4} M induced a statistical significant increase of osteoblast differentiation, magnesium at 10^{-3} M elicited the opposite effect. In line with this, it was previously reported that high concentrations of magnesium (5 mM) caused an inhibition of ALP activity in human osteoblasts (Leidi, Dellera, Mariotti, & Maier, 2011).

Cerium promoted the differentiation of osteoblasts precursor cells at most concentrations tested, except at higher concentrations, where it displayed an opposite effect. These results are accordant with previous reports, where cerium at concentrations of 10^{-9} to 10^{-6} M promoted mouse osteoblasts differentiation and at higher concentrations (10^{-5} and 10^{-4} M) inhibited the differentiation (J. Zhang *et al.*, 2010).

Lanthanum showed a concentration- and time-dependent effect on ALP activity of osteoblasts precursor cells. At day 14 all lanthanum concentration enhanced osteoblast precursor cells differentiation, while an inhibitory effect was observed in the whole range of concentrations at day 21. This behaviour pattern is in agreement with previously data described by Wang and his co-workers with osteoblasts derived from rats that were cultured in the presence of lanthanum (10^{-8} M to 10^{-4} M) for 24, 48 and 72 hours) (X. Wang *et al.*, 2008). The results of the DNA content and ALP activity of cells cultured revealed an inverse relationship between cell proliferation and differentiation, as expected as a general cell behaviour.

ALP activity of the cultures performed in the presence of praseodymium revealed a small ability to induce osteoblast precursor cells differentiation at day 14. This behaviour continued to be observed at day 21, as all concentrations tested inhibited ALP synthesis. These results are somehow related to the results obtained previously. Zhang *et al.* investigated short-term effects of $PrCl_3$ on the differentiation of primary mouse osteoblasts. In this work, $PrCl_3$ (10^{-8} M to 10^{-4} M) was shown to inhibit osteoblast differentiation for 36 hours. Similarly to lanthanum, it was observed that praseodymium had a negative effect on cell differentiation but induced cell proliferation.

Collagen synthesis as an also well-recognized biochemical marker of osteoblastic differentiation, was assessed by histochemical staining (Seo *et al.*, 2010). As mentioned previously, the presence of mature osteoblasts is indicated by the production of ALP which in turn interacts with collagen during matrix mineralization (Linder, Enander, & Magnusson, 2014). Although, these results are qualitative it was observed a close relation between ALP activity and collagen staining. In fact, it was observed that the conditions that induced a more pronounced ALP synthesis, presented a more marked staining for collagen, suggesting a higher degree of osteoid production. Moreover, these conditions induced a nodular organization of the culture, with an increased stain at the nodular structures.

As mentioned above, osteoblast precursor cell cultures were also tested for the involvement of several signalling pathways (MEK, NFkB, JNK and PKC). Some significant alterations on the contribution of the tested pathways on the response of osteoblastic cells were observed. MEK pathway appeared not to be involved in the cellular response performed in the presence of magnesium and lanthanum. Although, this pathway seemed to the involved in the remaining

conditions, a more relevant role was observed in cultures performed with zinc and praseodymium. This result contrasts with a previous study that showed that lanthanum enhances the *in vitro* differentiation of osteoblast derived from Sprague-Dawley rats via ERK signalling pathway (Wang, Yuan, Huang, Zhang, & Wang, 2008). NFκB and JNK pathways seemed to be involved in all tested conditions which suggests that these pathways could be important targets of lanthanides on osteoblast precursor cells. Lanthanum chloride has been previously demonstrated to enhance osteoblastic differentiation and apoptosis by supressing the activation of JNK (Li *et al.*, 2009). PKC appeared to modulate the cellular response in all conditions tested, although a more critical role of this pathway was detected in cells cultured in the presence of lanthanum, as its inhibition promoted an evident decrease of the enzymatic activity.

4.3 Osteoclastic Cell Response to Lanthanides

Next, the effect of lanthanides on PBMC cultures was evaluated. As mentioned previously, even though the effect of lanthanides on osteoclasts formation, differentiation and activity has already been addressed, the number of reports is rather low compared to osteoblasts.

Osteoclasts are large multinucleated cells formed by the fusion of mononuclear progenitors of the monocyte–macrophage lineage (Hoebertz & Arnett, 2003). A number of studies have shown that RANKL and M-CSF are necessary and sufficient to promote osteoclastogenesis (Boyle *et al.*, 2003). Osteoclast differentiation is characterized by acquisition of mature phenotypic markers, such as the CTR, VNR, TRAP, integrin αvβ3, morphological conversion into large multinucleated cells and the capacity to form resorption lacunae on bone (Lacey *et al.*, 1998). While physiological osteoclastic bone resorption is essential for the maintenance of human skeleton, enhanced osteoclast formation and/or function result in excessive bone resorption, leading to either localized osteolysis or systemic bone loss *(Ying et al.*, 2015).

To evaluate the effect of the different ions and concentrations tested on the density of PBMCs, DNA content was measured after 14 and 21 days of culture. Overall, regardless of the ion or concentration tested, it was observed a reduction in the DNA content from day 14 to day 21, which was expected, since the first osteoclastogenic steps involve fusion of the precursor cells.

The DNA content of PBMCs cultured in the presence of zinc appeared to be decreased at the highest concentration tested. A similar finding was already described in mouse bone marrow-derived monocyte cells cultured in the presence of $ZnSO_4$ (1, 10, 30, 60 and 100 μM) for 4 days. In this work, it was identified a dose-dependent inhibition of osteoclast formation upon zinc treatment (Park *et al.*, 2013).

With regard to PBMCs cultured in the presence of magnesium, it was observed that the amount of DNA at day 21 was up-regulated by the lowest concentrations and down-regulated by the highest concentrations tested, though the influence observed was not particularly high. The role of magnesium at different concentrations (0.08, 0.4 and 0.8 mM), was previously evaluated using osteoclast precursor cells isolated from long bones and jaws from mice. Notwithstanding, the results did not show any differences in the DNA amount, between day 3 and 6 (Belluci *et al.*, 2013).

All concentrations of cerium and praseodymium induced a decrease of the DNA synthesis by PBMC cultures at day 21, while at day 14 almost no difference was observed comparatively to

control. This may suggest that both lanthanides are able to modulate PBMCs density in a concentration independent-manner, however, the effect is only observed over time.

Lanthanum at the lowest concentrations tested showed a stimulatory effect on the DNA content at day 14, however, the effect seemed to be lost over time. It was also observed that although the highest concentrations tested had induce a slight decrease of the DNA content at day 14, at day 21 the effect had become more evident and statistical significant. These outcomes may suggest a time- and concentration- influence of lanthanum on cell density.

Osteoclast apoptosis is an important point of control of bone resorption, since after performing their resorption activity, osteoclasts undergo apoptosis. Decreased osteoclast apoptosis is in general associated to an increased bone loss (Xing & Boyce, 2005). Hence, the regulation of the numbers of osteoclast precursors and mature osteoclasts via the induction of cell death may be a mechanism by which the number of active osteoclasts at any site is controlled, thus, allowing to control bone resorption (Roux & Brown, 2009). In order to assess the ability of the lanthanides to induce PBMCs apoptosis, caspase-3 activity was quantified at day 14 and 21.

It was observed an overall increase of apoptosis between day 14 and 21, regardless of the ion or concentration tested. These outcome could be also related to the reduction of the DNA content observed between the two time points. Apoptosis is defined by morphological changes of cells and nuclei, such as cell shrinkage, condensation and fragmentation of nuclei, and blebbing of the plasma membranes. Moreover, this event is often accompanied by degradation of chromosomal DNA, which could explain the observed phenomenon (Nagata, Nagase, Kawane, Mukae, & Fukuyama, 2003).

All zinc concentrations tested induced a higher apoptotic response at day 14. At day 21, the effect was opposite to the observed at day 14, however, the difference relatively to control (although statistical significant) was low. These results may indicate an ability of zinc to up-regulate osteoclast apoptosis, although the effect may be attenuate or lost over time. Likewise, the ability of zinc to induce apoptosis was previously described in osteoclast precursor from mice, where zinc at 10^{-5} M in the presence or absence of M-CSF and RANKL for 24 or 72 hours was shown to cause a significant increase in caspase-3 mRNA expression in osteoclastic cells (A. Yamaguchi & Uchiyama, 2008).

At day 14 cultures performed in the presence of magnesium showed an increased apoptotic response. On the other hand, at day 21, magnesium induced a very slight decrease of caspase-3 activity. These results may indicate that after cells being adapted to the presence of the magnesium ions, the apoptotic response is lowered to a level similar to control. Moreover, a study on the effect of magnesium on mature osteoclasts, showed that magnesium ions in the culture medium did not increase osteoclast apoptosis even at 100 ppm (Li, Senda, Ito, Sogo, & Yamazaki, 2008).

Cerium induced apoptosis at the lowest concentrations tested and inhibited this response at the highest concentrations, at day 14. The response induced by the highest concentrations was maintained at day 21, whereas the lowest concentrations started to present an inhibitory effect. This results may express the ability of cerium to act as a negative-modulator of osteoclast apoptosis.

Lanthanum and praseodymium presented an inhibitory effect on the apoptotic response at both days. These findings may suggest that both ions modulate apoptosis negatively, in a concentration- and time-independent manner.

TRAP is an enzyme highly expressed in both immature and mature osteoclasts (Fuchs *et al.*, 2009). Therefore, in order to evaluate the ability of lanthanides to induce PBMCs differentiation, TRAP activity and the number of TRAP positive multinucleated cells were assessed.

All zinc concentrations, with exception of 10^{-3} M, presented a stimulatory effect on TRAP activity. Zinc has been associated to the reduction of bone loss by supressing osteoclast differentiation. However, it was also reported that this ability is concentration-dependent. As matter of fact, TRAP activity of primary cultured mouse BMMs was shown to be inhibited by all $ZnSO_4$ concentrations tested (1, 10, 30, 60, 100 μM), yet, a more pronounced inhibition was observed at 100 μM (Park *et al.*, 2013). These observations could indicate that although zinc is able to reduce the differentiation of PBMCs, this ability is only observed at high concentrations. The results concerning the number of TRAP positive multinucleated cells also showed a decrease of fused multinucleated osteoclasts by zinc at 10^{-3} M. Since it was observed that this concentration did not induced an apoptotic response (at day 21), this could indicate that the inhibitory effect of zinc on PBMCs differentiation was not caused by zinc toxicity.

Magnesium at the lowest concentrations tested increased PBMCs differentiation on both time points. The highest concentrations tested presented an inhibitory effect at day 14. The inhibitory role was lost at day 21, even so, these concentrations were not able to induce a significant increase of TRAP activity. These results may suggest a positive effect of all magnesium concentrations tested at long term. A former work seemed to support this outcome, namely, Wu *et al.* indicated that $MgCl_2$ first enhanced and then opposed PBMCs differentiation in a concentration-dependent manner (peaking between 10 and 15 mM) at day 28 (Wu, Luthringer, Feyerabend, Schilling, & Willumeit, 2014). In accordance with these elements, the magnesium concentrations tested in the present work seemed to be too low to observe a decrease of PBMCs differentiation caused by the concentration.

Considering the results regarding TRAP activity and the number of TRAP positive multinucleated cells, it appeared that all cerium concentrations stimulate PBMCs differentiation. These results are somehow related to the low apoptotic response (day 21) observed.

Lanthanum induced a similar response at both days, with the lowest concentrations presenting a stimulatory effect on PBMCs differentiation and the highest concentrations presenting the opposite influence. The same effect was already described in the DNA content whereas apoptosis appeared not to be influenced by lanthanum concentration. Therefore, the ability of lanthanum to reduce PBMCs differentiation at the highest concentrations was not related to lanthanum toxicity.

The effect of praseodymium on PBMCs differentiation was similar to lanthanum, the lowest concentrations induced PBMCs differentiation while the highest concentrations inhibited it. Moreover, by analysing the apoptosis results it was also observed that the inhibitory effect caused by the highest concentrations was not related to praseodymium toxicity.

Confocal images of PBMCs were in line with the previous results, that is, the conditions that elicited PBMCs proliferation and differentiation presented an increased amount of osteoclastic cells in culture displaying osteoclast phenotypic features (VNR and CTR).

The involvement of the signalling pathways (MEK, NFkB, JNK and PKC) on PBMCs response was also evaluated. NFkB pathway seemed to be crucial in all tested conditions, which strongly suggests that, in our culture conditions, this can be a major osteoclastogenic intracellular mechanism. JNK pathway did not contribute to the cellular response of PBMC cultured in the presence of zinc, magnesium and cerium, whereas in the presence of lanthanum appeared to have a relevant role. PKC showed to be very important in all tested conditions, particularly in

cell cultures performed in the presence of praseodymium. MEK pathway appeared not to modulate TRAP activity of cells cultured in the presence of lanthanum.

4.4 General Discussion

Everything considered, lanthanides seemed to be able to affect human osteoclastic and osteoblastic proliferation and/or differentiation. Moreover, it was observed that the concentration and culture time were critical factors for switching the biological effects of lanthanides from toxicity to activity or from down-regulation to up-regulation, an outcome already stated previously (K. Wang *et al.*, 2003).

As already stated in previous works, it was observed that zinc contributes to the enhancement of osteoblast proliferation. This influence was only annulled at the highest concentrations tested, probably due to a toxic effect generated by this range of concentrations. Furthermore, ALP synthesis was also down-regulated by the highest concentrations. Osteoclast density and differentiation was affected by zinc in a way similar to osteoblast.

Magnesium was also shown to promote osteoblast proliferation. All the concentration range produced an up-regulation of proliferation, while, only magnesium at concentration of 10^{-4} M induced an increased differentiation. Osteoclast density was increased by the lowest concentrations of magnesium and inhibited by the highest at day 21. However, at day 21, all the concentrations tested exhibited an identical ability to induce cell differentiation.

Overall, cerium in the majority of the concentrations tested appeared not to affect osteoblast significantly proliferation. Additionally, it was observed that cerium at the lowest concentrations was able to induce osteoblast differentiation and that this effect was lost at the highest concentrations. In contrast, cerium was able to modulate osteoclast density at least at day 21. Additionally, it was observed that osteoclast differentiation was increased by cerium at both days, with suggests a strong ability of these lanthanide to promote this response.

Lanthanum inhibited osteoblast proliferation in all concentration range at day 14, whereas, the opposite effect was displayed at day 21. Moreover, lanthanum has shown to be unable to induce osteoblast differentiation at day 21. Osteoclast density appeared to be up-regulated by lanthanum at the lowest concentrations, still, this effect seemed to be lost over time. Even thought, the highest concentrations exhibited the opposite influence, this effect only became evident at day 21. Osteoclast differentiation was inhibited by the highest concentrations of lanthanum at both days.

Praseodymium was shown, at both time points, to up-regulate DNA synthesis at all concentration tested, which suggest that this cation is able to modulate positively osteoblast proliferation regardless of the concentration tested. On contrary, it showed to be a negative regulator of osteoblast differentiation. Osteoclast density was down-regulated by praseodymium in all concentrations tested, even though the effect was only detected at day 21. Praseodymium was shown to inhibit osteoclast differentiation at the highest concentrations. Therefore, praseodymium appeared to act as negative regulator of osteoclast density, however, only down-regulates their differentiation at the highest concentrations.

To sum up, it was observed that both lanthanum and praseodymium were able to modulate osteoblast proliferation, nevertheless, only praseodymium presented a stimulatory effect on both days. Osteoblast differentiation was modulated by all the lanthanides tested, even thought, only cerium was able to enhance differentiation at day 21. The results concerning the osteoclast

cultures showed a reduction in the DNA content, regardless of the ions or concentration tested. Moreover, it was observed that while lanthanum induced this influence only at the highest concentrations, cerium and praseodymium were able to induce this response in all the concentration range. Osteoclast differentiation was also shown to be regulated by cerium, lanthanum and praseodymium. Moreover, it was noted that, while cerium acted as positive regulator of osteoclast differentiation in a concentration-independent manner, lanthanum and praseodymium acted as positive or negative-regulators of osteoclast differentiation depending on their concentration.

Although the effect of lanthanides on osteoblast and osteoclast proliferation and differentiation was closely related to the concentration and culture time, the different tested ions elicited different patterns of cell response, as already stated in previous works *(J. Zhang et al.*, 2003). Cerium, for instance, was shown to induce osteoclast differentiation, whereas, praseodymium and lanthanum inhibited their differentiation, at least at the highest concentrations tested.

In some cases, the results obtained also highlighted an opposite effect of the same cation depending on the cell type. This contrasting effect was observed, for example, in the cultures performed in the presence of praseodymium. This lanthanide was shown to regulate positively osteoblast density, while the opposite effect was observed on osteoclast cultures.

The duality of the biological effects of lanthanides, observed in the present study, is considered one of the most common features of these elements (K. Wang *et al.*, 2003). This dual behaviour may result, in part, from lanthanide ability to interfere with the intracellular calcium ionic concentration in a bidirectional way (Riccardi, Finney, Wilkinson, & Kemp, 2009). Moreover, the different physicochemical characteristics, such as, ionic radius and charge density, may be also responsible for this twofold effect (K. Wang *et al.*, 1999). Lastly, the wide range of experimental models, culture conditions and evaluated parameters may also explain the contradictory results often found on literature reports.

The broad biological activity spectrum and the two-sidedness effect identified in the present study, reveal that lanthanides may have a potential to intervene in pathologic events characterized by an imbalance of the bone remodeling process, such as osteoporosis, Paget's disease, and inflammatory conditions such as rheumatoid arthritis (Vidaud *et al.*, 2012). In addition, lanthanides might be also relevant when considering the potential approaches for the development of biomaterials targeting bone tissue repair or regeneration (J. Coelho *et al.*, 2012). However, the clinical success of those applications will depend on the ability to find a balance between the differential effects that lanthanides have in different cell types. To accomplish this goal, further studies are required for the evaluation of lanthanides on bone cells activity, namely, the effect of lanthanides on the mineralized matrix formation, level of intracellular calcium, calcium phosphate-resorbing ability and gene expression. Besides, further experiments on the mechanisms of lanthanides affecting bone cell proliferation, apoptosis and differentiation should also be addressed in the future.

Chapter 5

Conclusion and Future Directions

5.1 Conclusion

The biological properties of the lanthanides, primary based on their similarity to the calcium, have been the basis for research into potential therapeutic applications of lanthanides since the early part of the twentieth century. This ability is of outermost importance, since calcium signals have been found to play a role in a wide range of cellular processes such as exocytosis, contraction, cell proliferation, differentiation and apoptosis.

More recently, lanthanides have been shown to be able to modulate the behaviour of bone-relevant cellular populations. Indeed, lanthanides ions exchange with calcium ions in bone, and modulate the remodeling cycle by acting on bone cells. This observation emphasised the prospective use of lanthanides on therapeutic approaches designed for the treatment of pathologic events characterized by an unbalanced bone turnover and for the development of biomaterials targeting bone tissue repair or regeneration.

Several works have already assessed the effect of these elements on bone cells activity, nonetheless, only a few reports evaluated these effect on human osteoblasts and osteoclasts. Moreover, the signalling pathways involved on the cellular response induced by lanthanides has not been elucidated so far. Therefore this study was set out to explore the cellular and molecular effects of different lanthanides at different concentrations on human osteoblasts and osteoclast precursor cells cultured *in vitro*. Moreover, the involvement of some osteoblastogenesis- and osteoclastogenesis-related signalling pathways on cellular response were addressed.

The results obtained confirmed the previous stated ability of lanthanides to modulate bone cells activity. In agreement with previous reports, it was often observed a relationship between the concentration/culture time and the biological effects attained by the lanthanides tested.

The experiments conducted with human osteoblast precursor cells provided some insights about the influence of cerium, lanthanum and praseodymium in this cell type. Cerium did not affect osteoblast precursor cells proliferation, in opposition, enhanced cell differentiation at the lowest concentrations. Lanthanum exhibited an opposite effect on osteoblast precursor cells proliferation and differentiation at the two time points investigated. Praseodymium stimulated osteoblast precursor cells proliferation at all the concentrations tested, yet, the same effect on differentiation was only observed at the lowest concentrations.

The experiments carried out on osteoclast precursor cell cultures showed, in some cases, contrasting effects to the observed on osteoblast precursor cells cultures. Cerium, for instance was shown to be able to modulate the density of osteoclast precursor cells, an effect that was not observed on osteoblast precursor cell cultures. Moreover, this cation increased osteoclast precursor cells density regardless of the concentration tested. Lanthanum was shown to modulate osteoclast precursor cells density, yet, this effect was only observed over time, highlight-

ing the impact of culture time. The effect of lanthanum on osteoclast precursor cells differentiation was also concentration-dependent. Praseodymium was shown to be able to down-regulated both osteoclast density and differentiation.

Due to the connexion between apoptosis and the cell cycle, the activity of caspase-3 was also assessed. Some interesting findings were identified. The apoptotic profile of osteoblast precursor cells culture in the presence of cerium and lanthanum was identical, however, the apoptotic response was shown to be pronounced when cells were cultured in the presence of cerium. Caspase-3 activity of osteoclast precursor cells was shown to be considerably inhibited by lanthanum and praseodymium.

Regarding the involvement of some osteoblastogenesis- and osteoclastogenesis-related signalling pathways in the cellular response promoted by the lanthanides some important findings were made. NFkB pathway, for instance, was shown to be involved in the response of both osteoblast and osteoclast precursor cells cultured in the presence of lanthanides. Furthermore, JNK pathway was shown to be as important as NFkB pathway in the response of osteoblast precursor cells, nevertheless, the same significance was not identified on osteoclast precursor cells.

All in all, this work provided new insights on the ability of lanthanides to modulate the development human bone cells and clarified the involvement of some signalling pathways on the cellular response induced by these elements. Lastly, this work underlined the dual behaviour of lanthanides and the importance of manage it in order to achieve different results in bone metabolism/regeneration. Although the results were promising, further experiments are required to understand the general influence of lanthanides on human bone cells activity and their mechanisms of action.

5.2 Future Directions

In the future, it would be important to determine the cytotoxic effect of lanthanides. In the present work, the assumptions regarding the potential toxicity of the elements tested, was supposed by means of the DNA content and the apoptotic response. Nevertheless, a more specific assay, such as, the lactate dehydrogenase (LDH) assay should be performed. In previous works, the cytotoxic effect of lanthanides on osteoblast was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, however, since this assay is based on mitochondrial activity of cells, is not the best option to evaluate the effect on osteoclast cultures.

Moreover, the duration of the study may be expanded, so that we can evaluate the effect of lanthanides at long-term. In this study it was often observed an opposite behaviour profile between day 14 and 21, thus, it would be interesting to observe the evolution of this profile. Likewise, based on the obtained results, the concentration range could be narrowed using the one that induced the intended response in order to establish a more specific interval.

Currently, it is well-stablished that osteoblasts not only play a central role in bone formation, but also regulate osteoclasts. Moreover, osteoclast-osteoblast interactions have been shown to perform an important role coupling bone resorption and formation. Therefore, it would be interesting to assess the effect of lanthanides on co-cultures of osteoblasts and osteoclasts, and understand if the communication between this two cell types, enhances or not the

effects observed in the present study. Zhang *et al*. have already evaluated the effect of lanthanum on the bone resorbing activity of rabbit osteoclasts co-cultured with osteoblasts, and observed that direct cell-cell contact between osteoclasts and osteoblasts regulate the response of osteoclasts to lanthanum(J. Zhang et al., 2005).

Additionally, it would be also relevant to evaluate the combined effect of the different lanthanides, to understand if the response attained by them is reinforced or not.

This study was developed on monolayer cell cultures, which do not reproduce the complex and dynamic three-dimensional environment experienced by bone cells *in vivo*. This type of structures allow to recapitulate and control the main biophysical, biochemical, and biomechanical cues that define the *in vivo* bone environment(Bouet, Marchat, Cruel, Malaval, & Vico, 2015). That being said, it would be interesting to analyse the behaviour of bone cells cultured in these structures in the presence of lanthanides.

As mentioned along this work, lanthanides have attracted much attention in the treatment of bone density disorders. Thus, in order to have a more accurate perspective of the effects of lanthanides on these disorders, it would be important to conduct *in vivo* experiments in animal models.

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