Escola Superior de Tecnologia da Saúde do Porto

Instituto Politécnico do Porto

Sara Raquel Carneiro da Rocha

Characterization of the effects of antiepileptic drugs on bone metabolism: *In vitro* studies with human osteoblastic and osteoclastic cells

MSc in Biochemical Technology in Health

Dissertation submitted to Escola Superior de Tecnologia da Saúde do Porto to fulfill the requirements needed to obtain the Masters level in Biochemistry Technology in Health, developed under the scientific orientation of Professor João Rodrigues, Laboratory for Bone Metabolism and Regeneration, Faculdade de Medicina Dentária da Universidade do Porto, institutional orientation of Professor Cristina Prudêncio, Escola Superior de Tecnologia da Saúde do Porto, Instituto Politécnico do Porto and under scientific co-orientation of Professor Ricardo Ferraz, Escola Superior de Tecnologia da Saúde do Porto, Instituto Politécnico do Porto.

September 2013

This dissertation is dedicated to my mom.
I love you, wherever you are.

"Love is watching someone die."

Benjamin Gibbard and Nicholas Harmer.
Death Cab for Cutie, 2005

Acknowledgments

In the first place, I would like to sincerely say thank you to **Professor João Rodrigues** for orienting and helping me throughout this growing and learning process.

To **Professor Maria Helena Fernandes** for receiving me in the Laboratory for Bone Metabolism and Regeneration – Faculdade de Medicina Dentária da Universidade do Porto, and for her wise words.

To **Professor Cristina Prudêncio** and **Professor Ricardo Ferraz**, for the co-orientation and permanent help throughout the Masters degree.

To **Professor Rúben Fernandes** and **Professor Mónica Vieira**, for all the help and good mood.

To my friends, especially **Tiago**, for supporting me in the most desperate moments, for always being there and not letting me discourage. Without you it wouldn't have been possible.

To my colleagues from the Master's degree of the Escola Superior de Tecnologia da Saúde do Porto, for all the companionship, joyful spirit and encouraging words.

To all those who, in a more or less permanent way, have shown their support, a big thank you.

At last, the biggest and most sincere thank you to **my dad** and **sister** for helping me accomplish another important step in my life, and to **my mom** for having been an inspiration and continuing to look after me.

THANK YOU!

Abstract

Bone is constantly being molded and shaped by the action of osteoclasts and osteoblasts. A proper equilibrium between both cell types metabolic activities is required to ensure an adequate skeletal tissue structure, and it involves resorption of old bone and formation of new bone tissue. It is reported that treatment with antiepileptic drugs (AEDs) can elicit alterations in skeletal structure, in particular in bone mineral density. Nevertheless, the knowledge regarding the effects of AEDs on bone cells are still scarce. In this context, the aim of this study was to investigate the effects of five different AEDs on human osteoclastic, osteoblastic and co-cultured cells.

Osteoclastic cell cultures were established from precursor cells isolated from human peripheral blood and were characterized for tartrate-resistant acid phosphatase (TRAP) activity, number of TRAP+ multinucleated cells, presence of cells with actin rings and expressing vitronectin and calcitonin receptors and apoptosis rate. Also, the involvement of several signaling pathways on the cellular response was addressed.

Osteoblastic cell cultures were obtained from femur heads of patients (25-45 years old) undergoing orthopaedic surgery procedures and were then studied for cellular proliferation/viability, ALP activity, histochemical staining of ALP and apoptosis rate. Also the expression of osteoblast-related genes and the involvement of some osteoblastogenesis-related signalling pathways on cellular response were addressed.

For co-cultured cells, osteoblastic cells were firstly seeded and cultured. After that, PBMC were added to the osteoblastic cells and co-cultures were evaluated using the same osteoclast and osteoblast parameters mentioned above for the corresponding isolated cell.

Cell-cultures were maintained in the absence (control) or in the presence of different AEDs (carbamazepine, gabapentin, lamotrigine, topiramate and valproic acid).

All the tested drugs were able to affect osteoclastic and osteoblastic cells development, although with different profiles on their osteoclastogenic and osteoblastogenic modulation properties. Globally, the tendency was to inhibit the process. Furthermore, the signaling pathways involved in the process also seemed to be differently affected by the AEDs, suggesting that the different drugs may affect osteoclastogenesis and/or osteoblastogenesis through different mechanisms.

In conclusion, the present study showed that the different AEDs had the ability to directly and indirectly modulate bone cells differentiation, shedding new light towards a better understanding of how these drugs can affect bone tissue.

Keywords: Bone remodeling, osteoclastic cells, osteoblastic cells, osteoclastogenesis, osteoblastogenesis, antiepileptic drugs, epilepsy**.**

Resumo

O tecido ósseo sofre remodelação constante por ação dos osteoclastos e osteoblastos. Um equilíbrio adequado entre as atividades metabólicas de ambas as células torna-se essencial para garantir uma estrutura apropriada do tecido esquelético, e envolve a reabsorção de osso velho e consequente formação de novo tecido ósseo. Alterações na estrutura esquelética, em particular na densidade mineral óssea, por parte de fármacos antiepilépticos, foram já documentadas. No entanto, o conhecimento acerca dos efeitos destes fármacos nas células ósseas é ainda escasso. Posto isto, o principal objetivo deste estudo foi investigar o efeito de cinco antiepilépticos diferentes em células ósseas humanas (osteoclastos, osteoblastos e culturas de ambas as células).

As culturas celulares de osteoclastos foram instituídas a partir de células percursoras isoladas de sangue periférico humano e caracterizadas para a atividade da TRAP (fosfatase ácida resistente ao tartarato), número de células multinucleadas TRAP positivas, presença de células com anéis de actina e que expressam recetores de vitronectina e calcitonina e taxa de apoptose. Para além disto, também o envolvimento de vias de sinalização na resposta celular foi testado.

As culturas celulares de osteoblastos foram obtidas a partir de cabeças de fémur de pacientes (25-45 anos) submetidos a cirurgia ortopédica e foram também caracterizadas para proliferação/viabilidade celular, atividade da ALP (fosfatase alcalina), e taxa de apoptose. Para além disto, também a expressão de genes relacionados com osteoblastos e o envolvimento de vias de sinalização na resposta celular foram estudadas.

Relativamente às co-culturas, em primeiro lugar as células osteoblásticas foram semeadas e cultivadas. De seguida, as células mononucleadas do sangue periférico (PBMC) foram adicionadas às células osteoblásticas e as co-culturas foram avaliadas para os mesmos parâmetros mencionados para as células osteoclásticas e osteoblásticas.

As culturas celulares estudadas foram mantidas na ausência (controlo) ou na presença de cinco antiepiléticos diferentes (carbamazepina, gabapentina, lamotrigina, topiramato e ácido valpróico).

Todos os fármacos testados foram capazes de afetar o desenvolvimento das células osteoclásticas e osteoblásticas, no entanto, mostraram modular de forma diferente estes processos.

De um modo geral, a tendência foi para inibir o processo de desenvolvimento de ambas as células. Adicionalmente, as vias de sinalização envolvidas também parecem ter sido afetadas pelos diferentes fármacos, sugerindo que estes podem afetar a osteoclastogénese e a osteoblastogénese por diferentes mecanismos.

Em jeito de conclusão, o presente estudo mostrou que os diferentes fármacos antiepilépticos possuem a capacidade de modular direta e indiretamente a diferenciação das células ósseas, fornecendo novas luzes para uma melhor compreensão de como estes fármacos podem afetar o tecido ósseo.

Palavras-chave: Remodelação óssea, osteoclastos, osteoblastos, osteoclastogénese, osteoblastogénese, antiepilépticos, epilepsia.

Page Index

List of tables

List of figures

Abbreviations and acronyms

AEDs Antiepileptic Drugs

ALP Alkaline Phosphatase

BMD Bone Mineral Density

BMPs Bone Morphogenetic Proteins (BMPs)

BMU Basic Multicellular Units

BSP Bone sialoprotein

BSUs Bone Structural Units

CATK Cathepsin K

CLSM Confocal Laser Scanning Microscopy

COLI – Collagen Type I

CTR Calcitonin Receptors

Dlx5 Distal-less homeobox-5

DMP-1 Dentin Matrix Protein-1

EDTA Ethylenediaminetetraacetic acid

ELISA Enzyme-Linked Immunosorbent Assay

GAPDH Glycerol - 3- phosphate dehydrogenase

JNK c-Jun N-terminal Kinase

MEK Methyl Ethyl Ketone

MEPE Matrix Extracellular Phosphoglycoprotein

MITF Micropthalmia-associated Transcription Factor

Msx2 Msh homeobox homologue-2

MTT 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

M-CSF Macrophage Colony Stimulating Factor

NFAT-c1 Nuclear factor of Activated T cells, Calcineurin dependent 1

NFk-B Nuclear Factor Kappa-B ´

OC osteocalcin

OPG osteoprotegerin

Osx Osterix

PBMC Peripheral Blood Mononuclear Cells

PBS Phosphate-Buffered Saline

PKC Protein Kinase C

***p*NPP** *para*-nitrophenilphospate

PTH/PTHrP Parathyroid Hormone/Parathyroid Hormone-related Peptide

RANK Receptor Activator of Nuclear Factor κB

RANKL RANK Ligand

RT-PCR Reverse Transcriptase – Polymerase Chain Reaction

Runx2 Runt-related transcription factor-2

TGF-β Transforming Growth Factor-β

TRAP Tartarate-Resistant Acid Phosphatase

VNR Vitronectin Receptors

α-MEM α-Minimal Essential Medium

CHAPTER 1 - General introduction

1.1 Bone

Contrary to the commonly held misconception, bone is a hard connective tissue and a dynamic organ that undergoes significant turnover as compared to other organs in the body (1). This organ has well recognized functions including structural support for the muscles, protection of vital organs and hematopoietic marrow, and storage and release of vital ions, such as calcium and of growth factors stored in the matrix (2). In addition, Guntur A., *et al* (3), also described the bone as an endocrine organ able to secrete its own factors to modulate metabolic functions. Bone is a highly vascularized tissue with a unique capacity to heal and remodel without scaring (4, 5). It is renewed continuously in the adult skeleton in response to a variety of stimuli by the process of bone remodeling (2). This remodeling is made possible by a group of specialized cells - osteoclasts, osteoblasts and osteocytes. A disruption or imbalance in this process can lead to either an increase or decrease in bone mineral density that may be detrimental to skeletal strength (3). So that bone strength is maintained, the process of bone turnover must be carefully regulated (1).

1.2 Bone: anatomy

The human body contains more than 200 bones, which can be classified as either long, short, flat or irregular (6, 7). Long bones are longer rather than wider. Most bones of the upper and lower limbs are long bones. Short bones are about as broad as they are long. They are almost cube-shaped or round. The wrist and ankle bones (carpals) and (tarsals) are short bones (4). Flat bones have a relatively thin, flattened shape and are frequently curved. Skull bones, the breastbone (sternum), ribs and shoulder blades (scapulae) are examples of flat bones. Irregular bones don’t fit easily into the other three categories (1, 6).

The normal structure of bones and their shape depend on two extraosseous influences: their interaction with the muscles and the hormonal regulation of calcium and phosphate metabolism. The proper balance between bone formation and bone resorption is crucial to the normal structure of bones (7).

1.2.1 Structure of the long bone

The growing long bone has three major components: the diaphysis, epiphysis, and epiphyseal plate. The diaphysis, also called shaft, is composed mostly of compact bone, which is fundamentally bone matrix and cells arranged into elementary units called *osteons* (6, 7). Compact bone is practically solid, being only 10% porous (4, 5). The epiphysis, or bone end, is composed primarily of spongy (or cancellous) bone, which is mostly cavities surrounded by bone matrix (6). Spongy bone has a higher porosity (50-90%) (4). The surface of the epiphysis is a layer of compact bone. The epiphyseal or growth plate is covered by hyaline cartilage, which contains large amounts of both collagen fibers and proteoglycans, located between the epiphysis and diaphysis (6). The growth plate and the adjacent terminal diaphysis are the most metabolically active segment of long bones (7). Together with the small spaces within spongy bone and compact bone, the diaphysis of a long bone can have a large space called the medullary cavity. This cavity and the cavities of spongy bone are filled with marrow (6).

1.2.2 Structure of the short, flat and irregular bone

Short and irregular bones have a similar composition to the epiphysis of long bones. They have compact bone surfaces that surround a spongy bone center with small spaces that usually are filled with marrow. Also, they have no diaphysis (because they are not elongated). Flat bones usually have no diaphysis or epiphysis and they contain an internal framework of spongy bone between two layers of compact bone (6).

1.3 Bone: major functions

Bone is involved in a serious of processes which are found to be essential in the human body (4). It provides important functions (Table 1), such as support, protection, movement, storage and the production of blood cells (6).

Table 1 - Functions of the skeletal system

Function	Description
Support	Bones, which are rigid and strong, are the major supporting tissue of the body. Cartilage provides a firm yet flexible support and ligaments (strong bands of fibrous connective tissue) attach to bone and hold them together.
Protection	Bone is hard and gives protection to the organ it surrounds.
Movement	The contraction of the skeletal muscles causes the bones to move, producing body movements. These muscles are attached to the bone by tendons (strong bands of connective tissue).
Storage	Some minerals in the blood are taken by the bone and stored. If blood levels of these minerals become lower, the minerals are released from bone into the blood. The main minerals stored are calcium and phosphorus. Adipose tissue is also stored in bone cavities.
Blood cell production	Many bones contain cavities filled with bone marrow that gives rise to blood cells and platelets.

1.4 Bone: histology and physiology

Bones are composed by cells and extracellular matrix (osteoid). The inside surface of the compact bone and the spongy are covered with bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts) (7). The composition of the bone matrix characterizes the bone. The bone cells produce the bone matrix, become enclosed within it, and break it down so that new matrix can replace the old matrix (6).

1.4.1 Bone matrix: organic and inorganic phase

Most of the outstanding properties of bone are related to its matrix composition (4). Bone is constituted by both mineral (inorganic) and organic phases (8). The organic material, which by weight is approximately 35% of the whole mature bone matrix, primarily is consisted of not only collagens and proteoglycans but also glycoproteins, sialoproteins and bone "gla" proteins (4). To better understand this, the components of bone organic phase are summarized in *Table 2*. The inorganic material (the remaining 65%) primarily consists of calcium phosphate crystals in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Both phases are responsible for the major functional characteristics of bone. Collagen lends flexible strength to the matrix, however the mineral components give the matrix compression strength (weight bearing). If the entire mineral is removed from a long bone, collagen becomes the main constituent, and the bone becomes overly flexible. On the other hand, if the collagen is removed from the bone, the mineral component becomes the primary constituent, and the bone becomes brittle (6).

1.4.2 Bone cells

Although bone tissue is populated by a variety of different cells, the synthesis, maintenance and resorption of this outstanding tissue results mainly from the interaction of three cell types: osteoclasts, osteoblasts and osteocytes (4, 5). All of them have defined tasks and are thus essential for the maintenance of a healthy bone tissue (4). Osteoblasts and osteoclasts have closely related activities although they originate from different cellular lineages and possess opposite functions within the bone remodeling cascade (9). The osteoblast produces the matrix which becomes mineralized in a well regulated fashion. This mineralized matrix can be removed by the activity of the

osteoclast when activated (8). Tissue yielded by osteoblastic formation is maintained by the osteocytes (10). Bone cell types, morphological characteristics and respective functions are summarized in *Table 3*.

Table 2 - Components of the bone organic phase. Adapted from Macromol. Biosci (2004), 4: 743-765.

Bone extracellular matrix components	Functions and properties
Collagen type I	Provides framework for skeletal structure.
Byglican and Decorin	Proteoglycans. Affect collagen fiber growth and diameter.
Osteonectin	Glycoprotein. Binds Ca^{2+} and collagen. Nucleates hydroxyapatite.
Thrombospondin	Glycoprotein. Binds calcium, hydroxyapatite, osteonectin and other cell surface proteins.
Fibronectin	Osteoblast attachment to surface.
Osteopontin	Sialoprotein. Involved in bone remodeling.
Bone Sialoprotein	Sialoprotein. Constituent of cement line.
Osteocalcin	Skeletal gla protein. Involved in bone remodeling.

1.4.2.1 Osteoclasts

The osteoclast is a tissue-specific macrophage polykaryon created by the differentiation of monocyte/macrophage precursors cells at or near the bone surface (11). Osteoclasts are large cells with several nuclei and are responsible for the resorption, or breakdown, of bone. Where the plasma membrane of osteoclasts contacts bone matrix, it creates many projections named a ruffled border. Hydrogen ions are

pumped across the ruffled border and produce an acid environment that causes decalcification of the bone matrix. Alongside this, the osteoclasts also release enzymes that digest the protein components of the matrix. Osteoblasts assist in the resorption of bone by osteoclasts by producing enzymes that break down the thin layer of unmineralized organic matrix that covers the bone (6).

1.4.2.2 Osteoblasts

Osteoblasts come from mesenchymal precursors that undergo a well-defined program of gene expression as they progress through osteoblastic commitment, proliferation, and terminal differentiation (12). They have an extensive endoplasmic reticulum and numerous ribosomes, produce collagen and proteoglycans. Osteoblasts also form vesicles that accumulate calcium ions (Ca^{2+}), phosphate ions (PO_4^{2-}), and various enzymes. The contents of these vesicles are released from the cell by exocytosis and are used to create hydroxyapatite crystals (6). Mature osteoblasts produce a particular extracellular collagen matrix that subsequently becomes mineralized by deposition of these hydroxyapatite crystals (12).

Ossification or osteogenesis is the formation of bone by osteoblasts. Elongated cell processes from osteoblasts link to cell processes of other osteoblasts through gap junctions. The osteoblasts then form an extracellular bone matrix that envelops the cells and their processes (6).

Osteoblast-lineage cells, in addition to regulating bone formation, also regulate bone resorption through an elegant signaling axis which controls osteoclasts generation and activity (10).

1.4.2.3 Osteocytes

Osteoblasts are encased by the matrix that they themselves synthesize and become osteocytes (1). Osteocytes are relatively inactive cells compared to most osteoblasts (6). In spite of that, osteocytes play a major part in the determination and maintenance of bone structure (1).

Osteocytes have a special function for triggering bone remodeling. These cells detect microcracks, mechanical strain, and the changes in the hormonal milieu of the bone. Osteocytes communicate with bone-lining cells, which initiate bone resorption and formation (9). The spaces occupied by the osteocyte cell bodies are called lacunae,

and the spaces occupied by the osteocyte cell processes are called canaliculi. In place of diffusing through the mineralized matrix, nutrients and gases can pass through the little amount of fluid surrounding the cells in the canaliculi and lacunae or pass from cell to cell through the gap junctions connecting the cell processes (6). Hence, osteocytes play a major part in coordinating bone remodeling (9).

Table 3 - Bone cell types, morphological characteristics and functions. Adapted from Macromol. Biosci (2004), 4: 743-765.

Bone cell type	Morphological characteristics	Functions
Osteoclasts	Polarized multinucleated cells. Hematopoietic origin.	Bone resorption.
Osteoblasts	Polarized and cuboidal cells. Located at the bone surface with their precursors (where they form a tight layer of cells). Mesenchymal origin.	Synthesis and regulation of bone extracellular matrix deposition and mineralization.
Osteocytes	Stellate shaped. Terminally differentiated osteoblasts.	Mechanosensor cells of the bone.

1.5 Molecular control of bone cell differentiation

1.5.1 Osteoclastogenesis

The osteoclast groups that quilt the cutting cone are derived from hematopoietic stem cells mainly present in the marrow and spleen. Osteoclastogenesis starts when a hematopoietic stem cell is stimulated to generate mononuclear cells, which then become committed pre-osteoclasts and enter the blood stream (10).

Two hematopoietic factors (expressed by osteoblasts and other cell types) are necessary and sufficient for osteoclastogenesis: the macrophage colony stimulating factor (M-CSF), and the receptor activator of nuclear factor κB ligand (RANKL) (11, 13-15). Other authors suggested that osteoclastogenesis is also dependent on the expression of several genes, including the AP-1 member c-fos, micropthalmia-associated transcription factor (MITF), and nuclear factor of activated T cells, calcineurin dependent 1 (NFAT-c1) (10, 15). Both, M-CSF and RANKL are required to induce expression of genes that typify the osteoclast lineage, including those encoding tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor (CTR) and β_3-integrin (or vitronectin receptor, VNR), leading to the development of mature osteoclasts (11, 16). M-CSF is believed to be critical for the proliferation of the osteoclast progenitors, while RANKL is membrane bound and directly controls the differentiation process by activating the receptor's activator of nuclear factor κB (RANK), which is expressed on the cell surface of mononuclear hematopoietic osteoclast precursors to trigger osteoclast formation (1, 13). Thus, the activation of RANK by its ligand leads to the expression of osteoclast-specific genes during differentiation, the activation of resorption by mature osteoclasts, and their survival and participation in new rounds of bone degradation at neighboring sites (11). The circulating precursors leave the peripheral circulation at or near the site to be resorbed, and fuse with one another to form a multinucleated immature osteoclast. The success in the production of the immature osteoclast is associated with the initiation of TRAP expression, which is used later in more mature cells to assist in bone resorption. Additional differentiation of the immature osteoclast happens only under the continued presence of RANKL and once the transition to mature osteoclast is reached, the bone-resorbing activity and survival of the mature osteoclast are regulated by RANKL (10). The mature, multinucleated osteoclast is activated by signals, which leads to the beginning of bone remodeling. The osteoclast cell body is polarized, and in response to activation of RANK by its ligand, it undergoes internal structural changes that prepare it to resorb bone, such as the rearrangements of the actin cytoskeleton and formation of a tight junction between the bone surface and basal membrane to form a sealed compartment (11). The mature osteoclast engages in bone resorption through peripheral attachment to the matrix, employing the β3 integrin, which creates a microcompartment between the ruffled basal border of the cell and the bone surface. H^+ ions are pumped

into the compartment by the osteoclast to solubilize the mineral component, followed by protease degradation of the organic matrix (10). Collagen fragments and solubilized calcium and phosphate (degradation products) are processed inside the osteoclast and released into the circulation (11).

The osteoblastic cells also produce a soluble protein that blocks osteoclast formation and bone resorption, the osteoprotegerin (OPG). It acts as decoy receptor by blocking RANKL binding to its cellular receptor RANK. OPG overexpression impairs osteoclast production, whereas OPG deletion results in enhanced remodeling of bone. Expression of RANKL and OPG is therefore coordinated to regulate bone resorption and density positively and negatively by controlling the activation state of RANK and osteoclasts (11, 13).

RANK signaling is mediated by cytoplasmic factors that activate downstream signaling pathways that control RANK's various functions. Some of the pathways are c-Jun N-terminal kinase (JNK) and Nuclear Factor Kappa-B (NFkB) (1, 11).

The osteoblast lineage plays an essential role in each step of this process (13).

1.5.2 Osteoblastogenesis

After resorption of a bone unit by the osteoclast, osteoblasts are then attracted to the surface of bone to begin refilling the pit that remains. As osteoclasts resorb bone matrix, they release factors that have been embedded in the bone matrix during bone formation. These factors may need some processing by the osteoclast to be activated before secretion, and may then stimulate bone formation by the osteoblast (13). Osteoblast development follows a different way, beginning with the local proliferation of mesenchymal stem cells residing in the marrow (10). During skeletal development osteoblast differentiation and deposition of bone matrix occurs, and involves spatiotemporal coordination of interaction among diverse endocrine, paracrine and autocrine factors. Osteoblast differentiation is stimulated by several factors at different stages of differentiation (1). Expression of the transcription factors runt-related transcription factor-2 (Runx2), distal-less homeobox-5 (Dlx5), and msh homeobox homologue-2 (Msx2) is required to push the precursor cells toward the osteoblast lineage and away from the adipocyte, myocyte, and chondrocyte lineages also yielded by the mesenchymal stem cell (10). Runx2 is frequently described as the master

regulator of osteoblastogenesis. It acts throughout the induction, proliferation and maturation of osteoblasts and regulates expression of many osteoblast genes. Numerous signaling pathways and transcription factors that influence osteoblastogenesis do so by influencing the production or activity of Runx2 (12). The committed pre-osteoblast expresses type I collagen and bone sialoprotein (Bsp). Further differentiation of the pre-osteoblast into a mature, bone-forming osteoblast phenotype requires the expression of Runx2, osterix (Osx), and several components of the Wnt signaling pathway (10). Osx is a zinc finger transcription factor expressed in osteoblasts and, like Runx2, ir required for bone formation (12). The mature osteoblast expresses the matrix proteins type I collagen (ColI) and osteocalcin (OC) and a key enzyme in the mineralization process, alkaline phosphatase (ALP). As a row of active osteoblasts secretes unmineralized matrix (osteoid) and advances away from the bone surface, a small number of cells fall behind and become incorporated into the matrix (10). These osteoblasts begin to generate long cytoplasmic processes to remain in communication with surrounding cells and upregulate expression of E11, an early osteocyte marker. At this point, the cells are considered immature osteocytes. As the matrix matures and mineralizes, and the osteoid seam moves further away, the osteocyte becomes entombed in a bony matrix and begins to mature and express a new set of genes, including dentin matrix protein-1 (DMP-1) and matrix extracellular phosphoglycoprotein (MEPE) (10).

Some authors suggested that Cbfa1 gene is essential for osteoblasts differentiation and bone formation (17). More recently, Zuo C., *et al* (9) affirmed, and Sims N., *et al* (13) supported, that osteoblasts are regulated by signals derived from autocrine, paracrine, various cell-cell interactions, and from the systemic level, including the endocrine and nervous systems. These signals include transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), Wnts, Notch, Eph-Ephrin interaction, parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP), and the leptin-serotonin-sympathetic nervous system pathway (9, 13, 14).

1.6 Bone formation and bone remodeling

Bone formation during fetal life and until the end of puberty occurs by two mechanisms: intramembranous and endochondral ossification. The terms describe the tissues in which bone formation takes place: intramembranous ossification in

connective tissue membranes and endochondral ossification in cartilage (6). The longitudinal growth of long bones is based on osseous transformation of the cartilage in the growth plate, called endochondral ossification. Most flat bones are formed through intramembranous ossification, whereby bone formation results from direct transformation of fibrous matrix into osteoid (extracellular matrix), followed by mineralization (deposition of calcium phosphate salts). Intramembranous ossification also accounts for the subperiosteal bone formation of long bones, which is the basis of the appositional growth and widening of long bones. Endochondral ossification ceases by the end of puberty and appears later only exceptionally and under pathologic conditions, for example during the healing of bone fractures. The intramembranous appositional growth of long bones continues, albeit at a slower rate, throughout the normal life span (7).

Bone is a dynamic tissue that is constantly formed and resorbed in response to changes in mechanical loading, altered serum calcium levels and in response to a wide range of paracrine and endocrine factors (13). Bone is capable of adapting its structure to mechanical stimuli and repairing structural damage through the process of remodeling, which removes and replaces discrete, measurable "packets" of bone. These replacement packets of bone, or bone structural units (BSUs), comprise secondary osteons. Bone is remodeled by groups of cells derived from different sources, which are collectively called the basic multicellular unit (BMU) (10). The BMU is a temporary anatomic structure that together with a canopy composed of bone-lining cells, and the associated capillary networks form a functional structure known as the bone remodeling compartment (9, 10). Within the BMU, cellular activity is matched, a principle that the amount of bone destroyed by osteoclasts in the BMU is equal to the amount of bone formed by osteoblasts. Matsuo K., *et al* (14) refer that bone remodeling had been described as a "bone remodeling cycle" consisting of activation, resorption, reversal, and formation phases. However, it is said that, in terms of osteoclast-osteoblast communication, it may be more convenient to describe bone remodeling as occurring in three phases: initiation, transition, and termination remodeling. (for an extensive review, see reference (14)). Thus, remodeling is a coordinated action between osteoclasts (cells that destroy bone) and osteoblasts (cells that form bone) as well as osteocytes within the bone matrix and osteoblast-derived lining cells that cover the surface of bone (13). In normal adults there is a balance between the amount of bone resorbed and formed.

During resorption, the osteoclasts release local factors from the bone, which might have two effects: inhibition of osteoclast function and stimulation of osteoblast activity. Furthermore, osteoclasts themselves produce and release factors that have a negative regulatory effect on their activity, and enhance osteoblast function (8).

For a better understanding of this process, see *Figure 1*.

1.7 Factors affecting bone growth

The bones in any skeleton usually reach a certain thickness, length, and shape. The potential shape and size of a bone and an individual's final adult height are determined genetically, but hormonal and exogenous factors can seriously modify the expression of those genetic factors (6, 18).

1.7.1 How do exogenous factors affect bone growth?

Exogenous factors that adversely affect bone growth include cigarette smoking, physical handicaps, certain medications and nutrition (18). Because bone growth requires osteoblast proliferation, any metabolic disorder that affects the rate of cell proliferation or the production of collagen and other matrix components affects bone growth, as well as the availability of calcium or other minerals needed in the mineralization process. Malnutrition during the time of bone growth can cause seriously problems (6). Certain vitamins are important in very specific ways to bone growth. Vitamin D is necessary for the normal absorption of calcium from the intestines. The body can either synthesize or ingest vitamin D. Vitamin C is necessary for collagen synthesis by osteoblasts. Normally, as old collagen breaks down, new collagen is synthesized to replace it (6).

1.7.2 How do hormones affect bone growth?

Hormones are paramount in bone growth. The growth hormone from the anterior pituitary increases general tissue, including overall bone growth, by stimulating interstitial cartilage growth and appositional bone growth. The thyroid hormone is also required for normal growth of all tissues, including cartilage; therefore, a decrease in this hormone can result in decreased size of the individual. Sex hormones also influence bone growth. Estrogen (a class of female sex hormones) and testosterone (a male sex hormone) initially stimulate bone growth, which accounts for the burst of growth at the

time of puberty, when the production of these hormones increases. Both hormones also stimulate ossification of epiphyseal plates, however, and thus the cessation of growth (6).

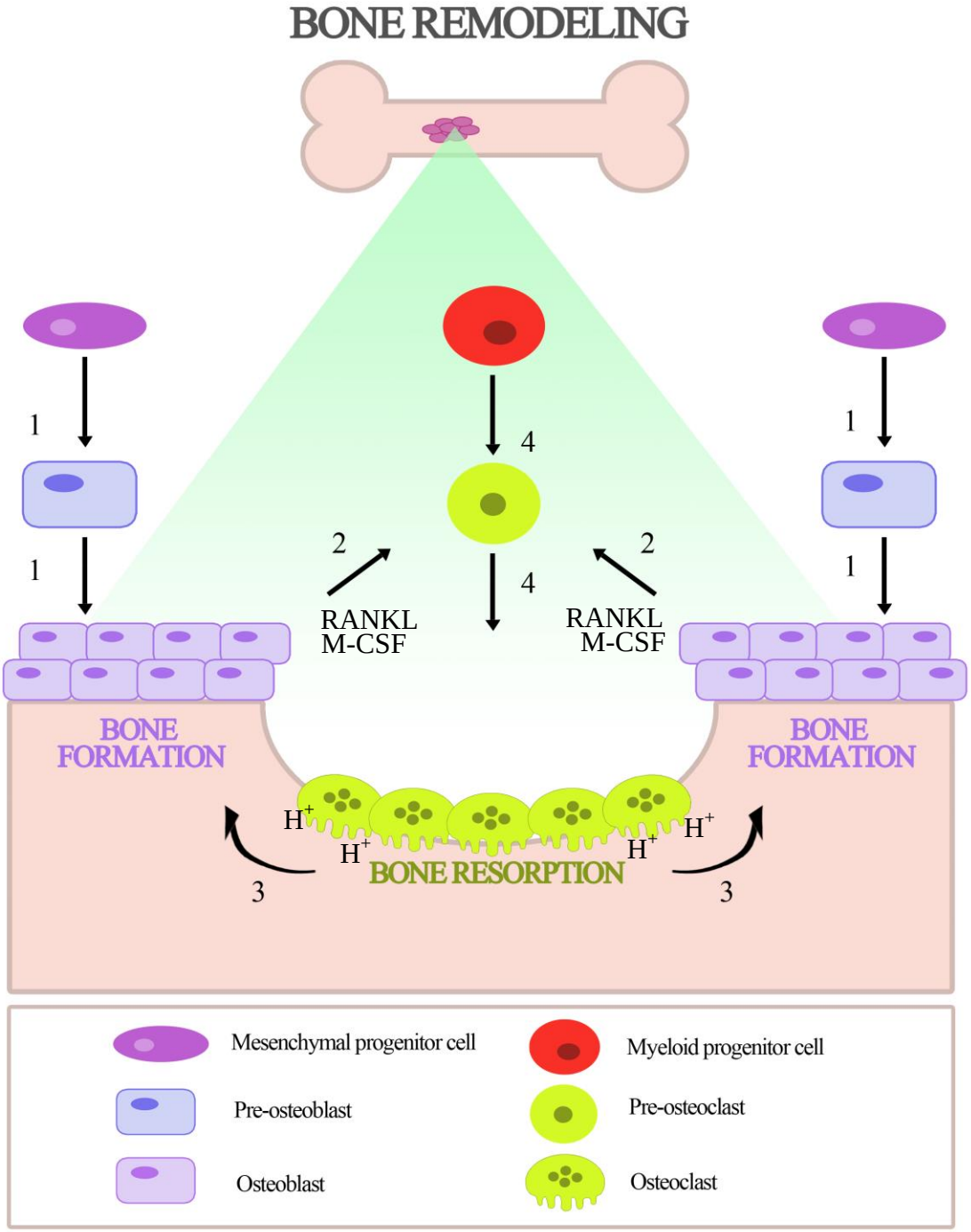

Figure 1 - The bone remodeling process - Osteoblasts arise from mesenchymal precursors [1] and are responsible for produce mineralized bone matrix [bone formation]. In addition to regulating bone formation, they also regulate bone resorption by generating and activating osteoclasts activity [2]. Osteoclasts originate by the differentiation of monocyte/macrophage precursor's cells [4]. As osteoclasts resorb bone matrix, they liberated matrix bound growth factors that then stimulate osteoprogenitor cells proliferation and bone formation by the osteoblast [3].

1.8 Epilepsy

Damjanov I. (7) characterized epilepsy in his book "Pathology for the Health Professions" as a group of diseases that lead to recurrent seizures. Seizures are typically characterized by convulsions, which are described as uncoordinated twitching of muscles and spastic contractions. Also, epileptic attacks may include abnormal motor activity, short periods of altered consciousness, altered sensory phenomena, or inappropriate behavior. All these manifestations of epilepsy are believed to result from abnormal synchronized electrical activity of the brain (7). Löscher W., *et al* (19) stated that epilepsy is one of the most common neurologic disorders, affecting about 4% of individuals over their lifetime.

1.8.1 Epilepsy and bone metabolism

Epilepsy is a chronic condition that may affect individuals for years (20). The evidence gathered over the past years show a strong link between long-term use of AEDs and disturbed bone metabolism, resulting in decreased bone mineral density (BMD) and an increased risk of fractures (21-24). Besides, AED therapy has been also associated with vitamin D deficiency and altered bone turnover (25). The propensity to fracture bones is increased in patients with epilepsy. Some are directly due to seizure or to falls, with or without associated seizures (26). Reduced bone accumulation in individuals with epilepsy and progressive bone loss caused by AEDs, may increase susceptibility to fractures (27). Risk of fractures after a few years of taking AEDs is two to six times greater in those who take it than in general population (28). Fractures can have catastrophic effects on the lives of patients with epilepsy (26).

1.8.2 Epilepsy and AEDs: should we be concern?

The mechanism of AED-related bone disease remains controversial (23, 29). Antiepileptic-induced bone disease has a heterogeneous spectrum of severity (30).

AEDs that induce the cytochrome P450 enzyme system are most commonly associated with abnormalities in bone (31-33). Carbamazepine is an inducer of the cytochrome P450 enzyme system. On the other hand, valproic acid is an inhibitor of the cytochrome P450 enzyme system (34). Enzyme-inducing AEDs alter vitamin D concentrations and may lead to the reduction of bone mass, although, nonenzyme

inducing AEDs may also affect bone by possibly altering osteoblastic function (18, 25, 27, 35). Notwithstanding, studies find conflicting results when evaluating its effect on bone and mineral metabolism (34, 36).

There are only a few studies of the effects of the other newer agents (gabapentin, lamotrigine and topiramate) on bone mineralization, so that, no conclusions can be drawn (18). However, Beerhorst K., *et al* (37) affirmed that not only the older AEDs but also the newer are associated with negative effects on bone metabolism. Gabapentin does not induce or inhibit hepatic enzymes, however, a few authors suggested that this drug can induce bone loss. The same scenario happens with lamotrigine (24, 38). Topiramate, for example, is a weak carbonic anhydrase inhibitor that may result in clinically significant metabolic acidosis and adverse effects on bone health. However, limited information is available (39).

At the present, the neurologists can choose from over 20 different AEDs, including older or first generation drugs such as carbamazepine, valproic acid, phenytoin and phenobarbital, and new or second generation drugs such as lamotrigine, topiramate, gabapentin, levetiracetam, vigabatrin and tiagabine (19, 40).

During recent years, a great number of new AEDs have been marketed worldwide, but the proportion of patients failing to respond to drug treatment has not been changed to any significant level. Furthermore, none of the old or new AEDs appears to represent a “cure” for epilepsy or an effective means for preventing epilepsy or its progression. Consequently, new concepts and original ideas for developing AEDs are urgently needed (19).

1.9 Outline and objectives of this thesis

Bone is the dynamic tissue comprising functionally distinct and unique cell populations that support the biochemical, mechanical and structural integrity of the skeleton. Bone undergoes remodeling, a continuing process of bone formation and resorption (41). The precise balance between bone formation and resorption is critical for the maintenance of bone mass density and systemic mineral homeostasis (9).

Epilepsy is one of the most common neurologic disorders, as well as a major public health issue. Current antiepileptic drugs are often accompanied by persistent side effects (19). Evidence shows that there is an association between long-term use of AEDs and disturbed bone metabolism. This is particularly problematic for people with epilepsy as their propensity to fractures is already elevated due to other drug side-effects, co-existing neurological deficits and seizure-related falls (21).

Taking into account all these factors, the main goal of this thesis was to perform an *in vitro* study that helps to characterize the effects of five different AEDs (Carbamazepine, Gabapentin, Lamotrigine, Topiramate and Valproic Acid) on bone metabolism. This goal was achieved through the following tasks:

1. Isolation of human osteoclastic and osteoblastic cells from healthy individuals;
2. Evaluation of the effect of five different AEDs in human osteoclastic and osteoblastic cell cultures;
3. Analysis of the influence of signaling pathways important for osteoclastogenesis and osteoblastogenesis in cellular responses of bone cells to AEDs.

Lastly, this thesis ends with Chapter 6 which describes the main conclusions drafted from this study, as well as possible future directions that should be addressed in order to answer some of the main questions that this work has raise.

CHAPTER 2 – Effects of AEDs on PBMC cultures

2.1 Introduction

Osteoclasts are specialized cells derived from the monocyte/macrophage hematopoietic lineage that develop and adhere to bone matrix, then secrete acid and lytic enzymes that degrade it in a specialized, extracellular compartment (11). So that resorption happens, osteoclasts attach to the bone surface by means of integrins, secrete acid hydrolyses, and resorb bone matrix. Because of the dynamic nature of bone, constant remodeling occurs (41). It was documented that AEDs induce disturbances on bone integrity (33). Hereupon, we performed an in vitro study with human osteoclasts in order to try to understand how AEDs these bone cells.

2.1.1 Aims

As discussed in Chapter 1, osteoclasts are responsible for bone resorption. This chapter describes the way we cultured osteoclastic cells, and the main aim was to investigate the effects of five different AEDs on the differentiation and function of human osteoclasts. We also tried to analyze the influence of signaling pathways important for osteoclastogenesis in cellular responses of bone cells treated with AEDs.

2.2 Material and methods

2.2.1 The culture of osteoclastic cells

Peripheral blood mononuclear cells (PBMC) were isolated from blood of 25-35 years old healthy male donors, after informed consent, as previously described (42). Shortly, after dilution with phosphate-buffered saline (PBS) (2:1), blood was applied on top of Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences). Samples were centrifuged at 400 g for 30 minutes and PBMC were collected at the interface between Ficoll-Paque and PBS. Cells were washed twice with PBS. On average, for each 90 mL of processed blood about $450x10^6$ PBMC were obtained. PBMC, seeded at $1.5x10^6$cells/cm^2, were maintained in α-minimal essential medium (α-MEM) supplemented with 30% human serum (from the same donor from which PBMC were collected), 100 IU/ml penicillin, 2.5 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 2 mM L-glutamine and the osteoclastogenic inducers M-CSF (25 ng/mL) and RANKL (40 ng/mL) (11, 43).

Cell cultures were performed in the absence (control) or presence of five different Antiepileptic Drugs (AEDs). The tested AEDs (Carbamazepine, Gabapentin, Lamotrigine, Topiramate and Valproic Acid) were used at five different concentrations within the range of 10^{-8}-10^{-4} M. Cell cultures were then characterized throughout a 21 day period, at days 7, 14 and 21, using the following parameters:

1. Protein quantification,
2. TRAP activity,
3. Number of TRAP-positive multinucleated cells,
4. Presence of cells with actin rings and expressing vitronectin and calcitonin receptors,
5. Apoptosis rate.

Cell cultures were incubated at 37ºC in a 5% CO_2 humidified atmosphere. Culture medium was replaced once a week and the AEDs were renewed at each medium change.

Next, PBMC were treated with the minimum concentration of each AED that elicited a significant effect on cellular response, and were further characterized for:

a. Calcium phosphate resorbing ability,
b. Involvement of some osteoclastogenesis-related signalling pathways on cellular response.

The AEDs tested were renewed at each medium change. Cell cultures were incubated at 37ºC in a 5% CO_2 humidified atmosphere.

2.2.2 Osteoclastic cultures characterization

Cell cultures were then characterized throughout a 21 day period, at days 7, 14 and 21, for the following parameters:

1. Protein quantification – Total protein content of cell cultures was quantified at days 7, 14 and 21 by Bradford's method (44). Firstly, PBMC cultures were washed

twice with PBS and then they were solubilized with 0.1 M NaOH. Coomassie® Protein Assay reagent (Fluka) was added to the cell cultures and the absorbance at 595nm was determined in an ELISA plate reader (Synergy HT, Biotek). Results were expressed as mg/mL.

2. TRAP activity – TRAP activity was determined by the *para*-nitrophenilphosphate (*p*NPP) hydrolysis assay, at days 7, 14 and 21, as previously described (45). Shortly, after being washed twice with PBS and solubilized with 0.1% (V/V) Triton X-100, samples were incubated with 22.5 mM *p*NPP prepared in 0.225 M sodium acetate, 0.3375 M KCl, 0,1% Tx-100, 22.5 mM sodium tartarate and 0.225 mM iron chloride (pH = 5.8) for 1 hour at 37ºC. The reaction was stopped with 5 M NaOH, and the absorbance of the samples at 400 nm was measured in an ELISA plate reader (Synergy HT, Biotek). Results were normalized to total protein content of cultures and expressed as nmol/min/$\mu g_{protein}$.

3. Number of TRAP-positive multinucleated cells - At days 14 and 21, PBMC cultures were washed twice with PBS, fixed with 3.7% formaldehyde for 15 min, rinsed with distilled water, and stained for TRAP with Acid Phosphatase, Leukocyte kit (Sigma), according to the manufacturer's instructions. Briefly, cells were incubated with 0.12 mg/ml naphtol AS-BI, in the presence of 6.76 mM tartarate and 0.14 mg/ml Fast Garnet GBC at 37ºC for 1 h in the dark. After incubation, cell layers were washed and stained with hematoxylin. After being washed with water, cells were visualized by light microscopy (Nikon TMS phase contrast microscope). Multinucleated (>2 nuclei) and TRAP-positive (purple/dark red) cells were counted.

4. Presence of cells with actin rings and expressing vitronectin and calcitonin receptors - PBMC cultures, at day 21, were washed twice with PBS and after being fixed with 3.7% *para*-formaldehyde for 15 minutes, cells were permeabilized with 0.1% (V/V) Triton X-100 for 5 minutes. Cells layers were stained for F-actin with 5 U/mL Alexa Fluor® 647-Phalloidin (Invitrogen), and for vitronectin receptors (VNR) and calcitonin receptors (CTR) with 50 µg/mL mouse IgGs anti-VNR and IgGs anti-CTR (R&D Systems), respectively. Anti-VNR and anti-CTR detection was performed with

2µg/mL Alexa Fluor® 488-Goat anti-mouse IgGs. Cultures were observed by Confocal Laser Scanning Microscopy (CLSM) (Leica TCP SP2 AOBS confocal microscope).

5. Apoptosis Rate - Apoptosis was quantified by measuring caspase-3 activity. For that, cell cultures, at days 14 and 21, were washed twice with PBS and assessed for caspase-3 activity with EnzCheck® Caspase-3 Assay Kit #2 (Molecular Probes), according to the manufacturer's instructions. Fluorescence was analysed at 496/520 nm (excitation/emission) in an ELISA plate reader (Synergy HT, Biotek). Results were presented as a % of activity (normalized with the corresponding total protein content value) compared to the control.

Next, PBMC were treated with the minimum concentration of each AED that elicited a significant effect on cellular response, and were further characterized for:

a. Calcium phosphate resorbing ability – PBMC cultures were performed on BD BioCoat™ Osteologic™ Bone Cell Culture Plates (BD Biosciences), for 21 days. After that culture period, cells were removed with 6% NaOCl and 5.2% NaCl, following manufacturer's protocol. The remaining calcium phosphate layers were visualized by phase contrast light microscopy (Nikon TMS phase contrast microscope). Resorption lacunae were identified and total resorbed area was quantified with ImageJ 1.41software.

b. Involvement of some osteoclastogenesis-related signalling pathways on cellular response - PBMC cultured for 7, 14 and 21 days were treated with inhibitors of signalling pathways involved in the osteoclastogenic response (11). U0126, a MEK (methyl ethyl ketone) signalling pathway inhibitor, was tested at 1 µM. PDTC, a NFkB (nuclear factor kappa-B) signalling pathway inhibitor, was used at 10 µM. GO6983, a PKC (protein kinase C) signaling pathway inhibitor, was tested at 5µM. Finally, SP600125, a JNK (c-Jun N-terminal kinase) signalling pathway inhibitor, was used at 10M. Cultures were assessed for total protein content and for TRAP activity.

2.2.3 Statistical analysis

Data presented in this work is the outcome of experiments performed with cells from two different blood donors. Three replicas of each condition were made for each experiment. Data was evaluated using a two-way analysis of variance (ANOVA) and no significant differences in the pattern of the cell behavior were observed. Statistical differences found between control and experimental conditions were determined by Bonferroni's method. For values of $P \leq 0.05$, differences were considered statistically significant. Data is expressed as the mean ± standard deviation.

2.3PBMC results

2.3.1 TRAP activity

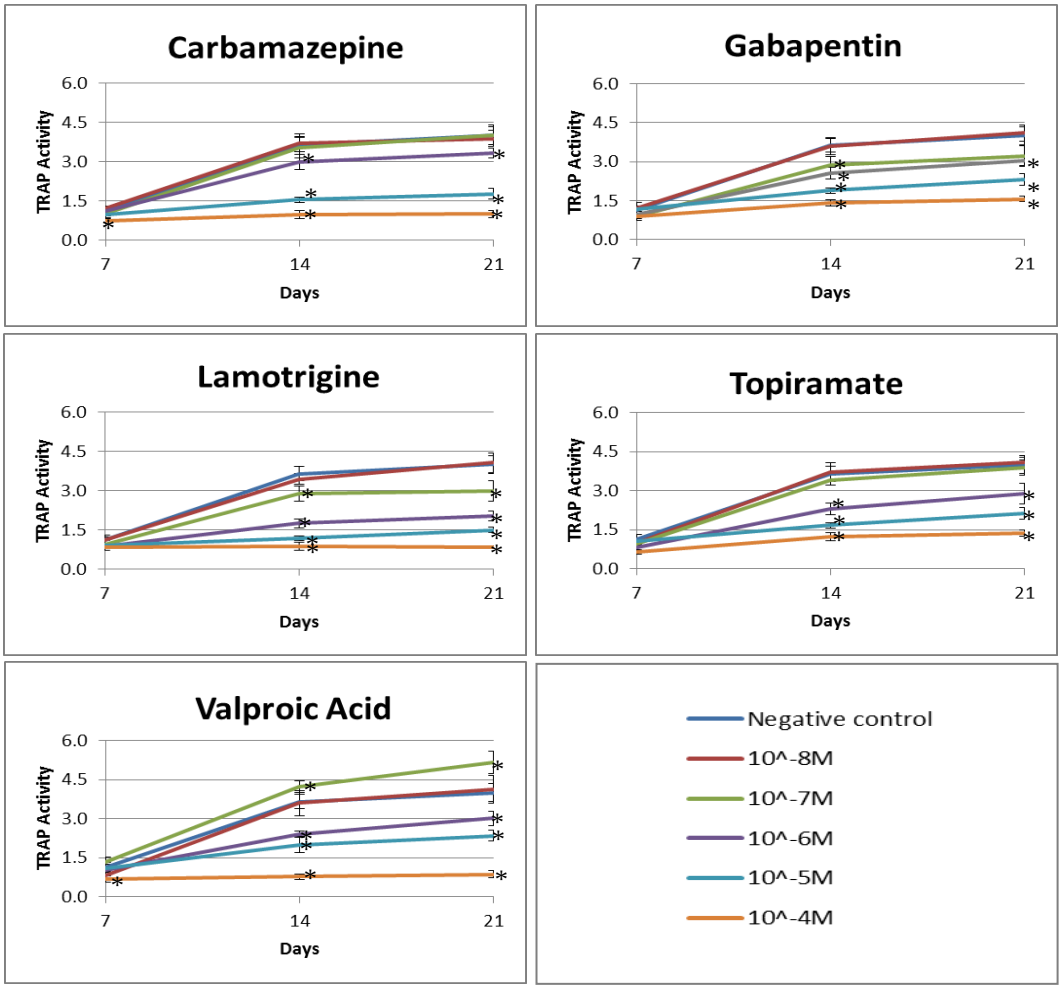

Figure 2 - TRAP activity of PBMC cultures maintained in the presence of recombinant M-CSF and RANKL, in the absence (negative control) or supplemented with different AEDs, cultured for 7, 14 and 21 days. * Significantly different from the control.

Figure 2 shows that, at control conditions, TRAP activity clearly increased until day 14. Between days 14 and 21, TRAP activity increased more slowly. The different AEDs had the ability to modulate the osteoclastogenic process. The majority of the tested AEDs negatively modulated it, leading to a dose-dependent decrease of TRAP activity. At lower doses (10^{-8}M) TRAP activity seems not to be affect by the presence of any AED. The decrease became statistically significant at 10^{-7}M gabapentin and lamotrigine (~19.95 and 24.92%, respectively) and 10^{-6}M carbamazepine and topiramate (~16.70% and 27.65%, respectively). Valproic acid appeared as an exception since at lower doses (10^{-7}M) caused an increase of TRAP activity (~29.02%). For concentrations higher than that, a dose-dependent decrease on cell response was observed.

2.3.2 Number of TRAP-positive multinucleated cells

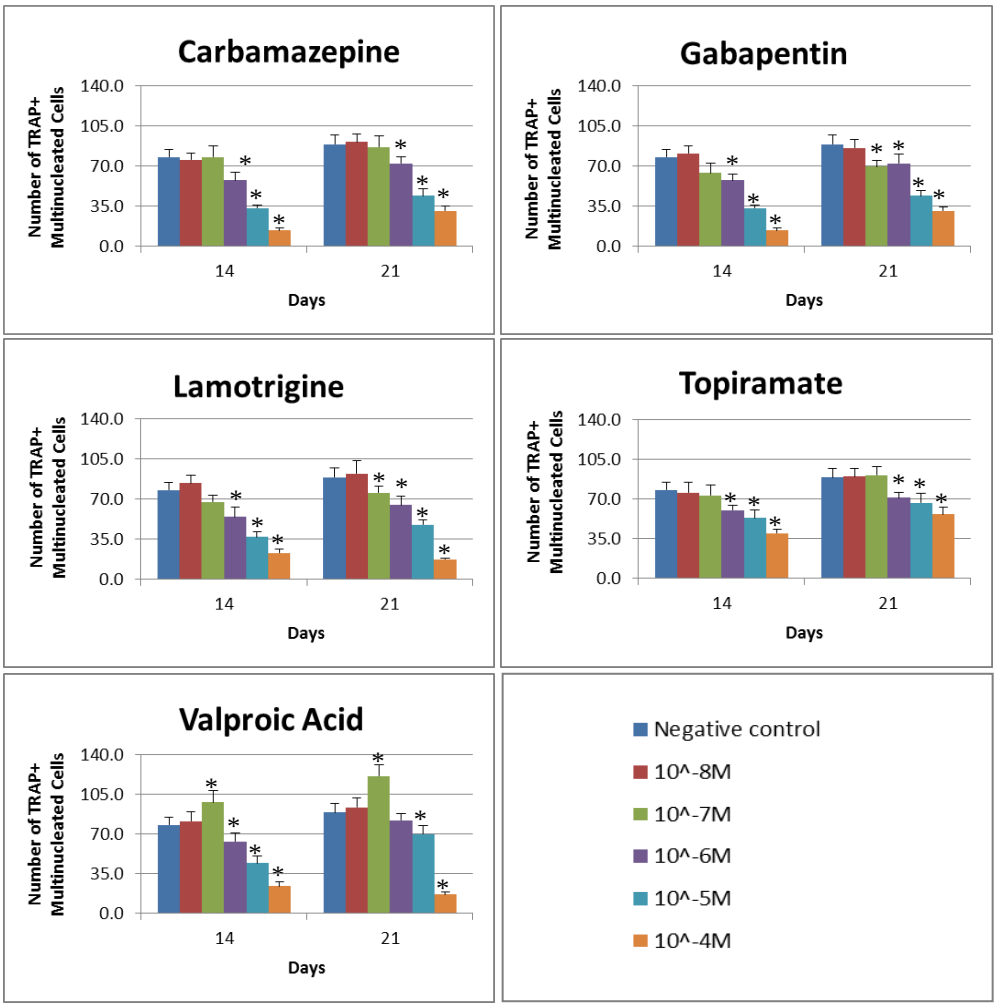

Figure 3 - Number of TRAP+ multinucleated cells on PBMC cultures maintained in the presence of recombinant M-CSF and RANKL, in the absence (negative control) or supplemented with different AEDs. * Significantly different from the control.

The results obtained for TRAP+ multinucleated cells (Figure 3), after 14 and 21 days of culture, followed the same profile observed for TRAP activity. Briefly, at lower doses (10^{-8}M) the number of TRAP+ multinucleated cells seems not to be affected by the presence of any AED. Also, supplementation with 10^{-7}M of Carbamazepine and Topiramate seems not to affect this number. The decrease became statistically significant at 10^{-7}M gabapentin and lamotrigine (~21.78% and 15.20%, respectively) and 10^{-6}M carbamazepine and topiramate (~18.93% and 20.18%, respectively). Valproic acid appeared as an exception since at lower doses (10^{-7}M) caused an increase of number of TRAP+ multinucleated cells (~35.94%), as noticed in TRAP activity results. For concentrations higher than that, a dose-dependent decrease on cell response was observed.

2.3.3 Presence of cells with actin rings and expressing vitronectin and calcitonin receptors

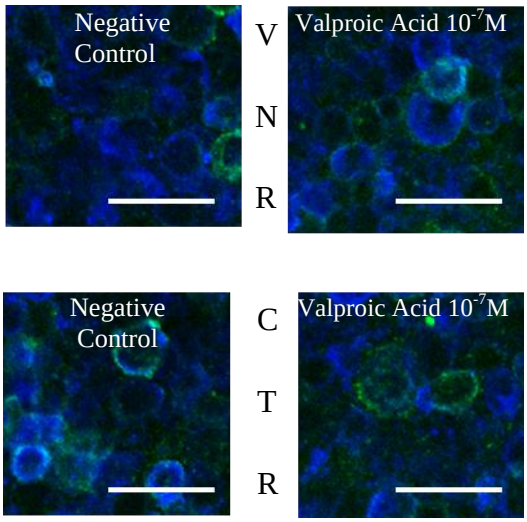

Figure 4 - Representative images of PBMC cell cultures visualized by confocal laser scanning microscopy (CLSM). Cells were stained blue for F-actin and green for Vitronectin Receptors (VNR) and Calcitonin Receptors (CTR). White bars represent 120 µm.

At day 21, PBMC cultures showed cells displaying osteoclastic features, that is, cells with actin rings and expressing vitronectin and calcitonin receptors. The amount of osteoclastic cells in the different conditions was somehow correlated with the qualitative results observed for TRAP activity. Figure 4 shows representative images of cultures maintained in control conditions or treated with Valproic acid.

2.3.4 Apoptosis rate

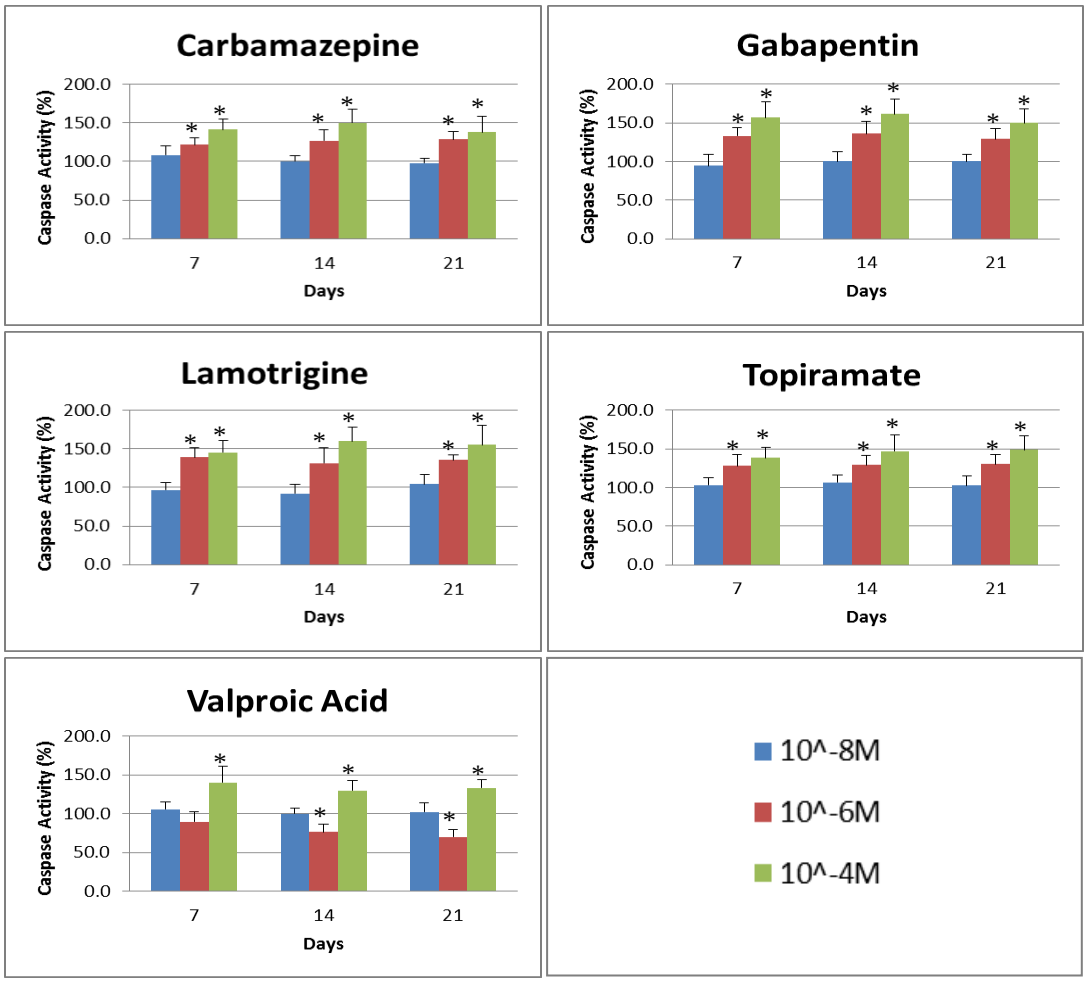

Figure 5 - Caspase-3 activity on PBMC cultures maintained in the presence of recombinant M-CSF and RANKL and treated with different AEDs. * Significantly different from the control.

Figure 5 shows that caspase-3 activity remained similar in the three culture periods tested, in all tested conditions. Supplementation with carbamazepine, gabapentin, lamotrigine and topiramate elicited a dose-dependent increase of caspase-3 activity. Supplementation with valproic acid caused a decrease of caspase-3 activity at 10^{-6}M concentration, while at 10^{-4}M an increase on cell response was observed.

2.3.5 Calcium phosphate resorbing ability

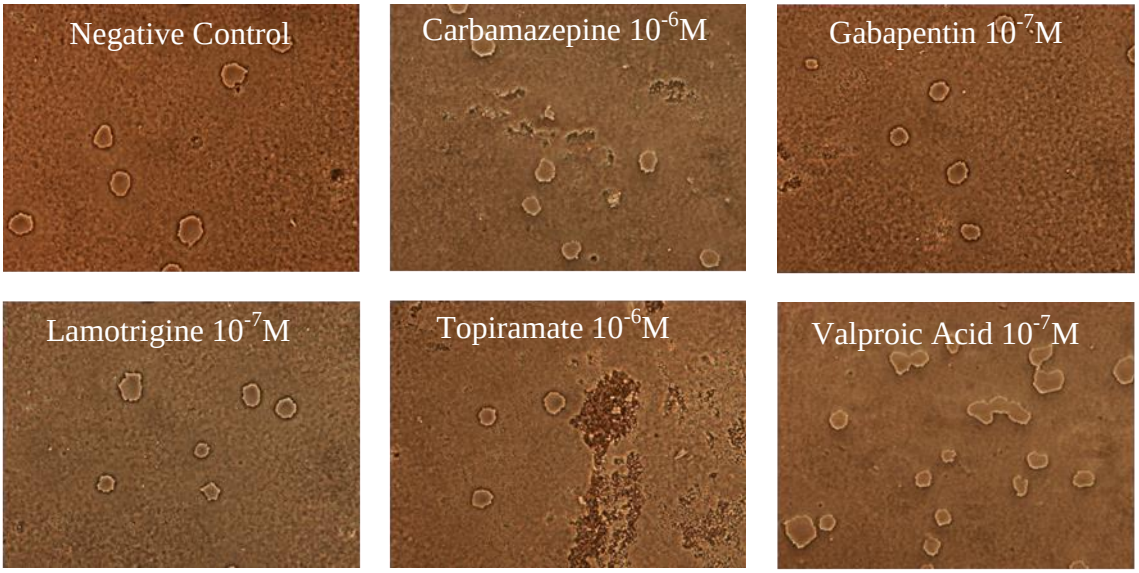

Figure 6 - Calcium phosphate resorbing ability of PBMC cultures maintained in the presence of recombinant M-CSF and RANKL, in the absence (negative control) or treated with different AEDs.

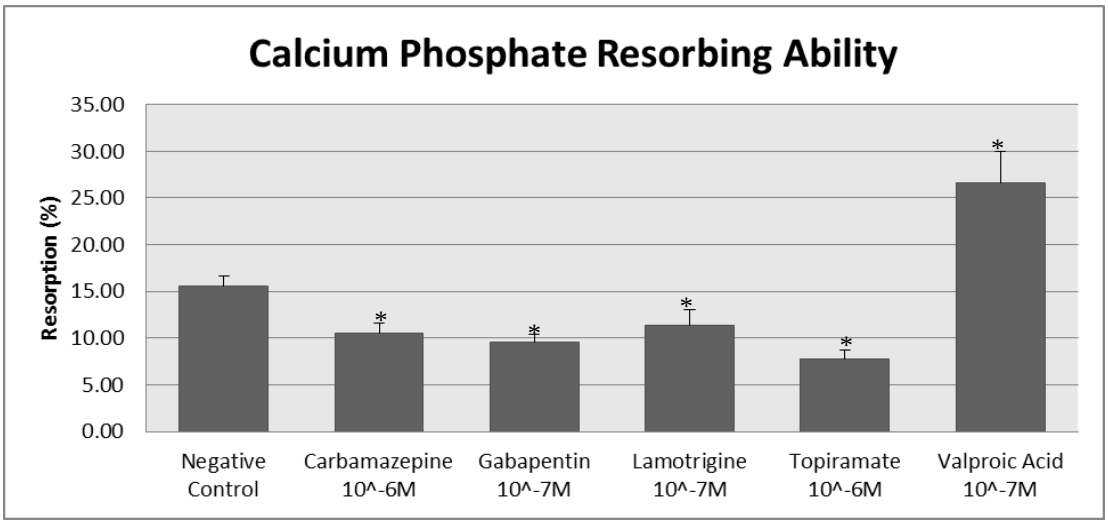

Figure 7 - Total resorbed area of PBMC cultures maintained in the presence of recombinant M-CSF and RANKL, in the absence (negative control) or treated with different AEDs. * Significantly different from the control.

Figures 6 and 7 show that the presence of Valproic acid induced an increase on the total resorbed area (~70.95%). However, the other drugs elicited a decrease on the resorbing ability. Topiramate was the one that most elicited this decrease (~50.10%), while the remaining molecules caused a similar response (~32.70% lower than in the control).

2.3.6 Involvement of some osteoclastogenesis-related signaling pathways on cellular response

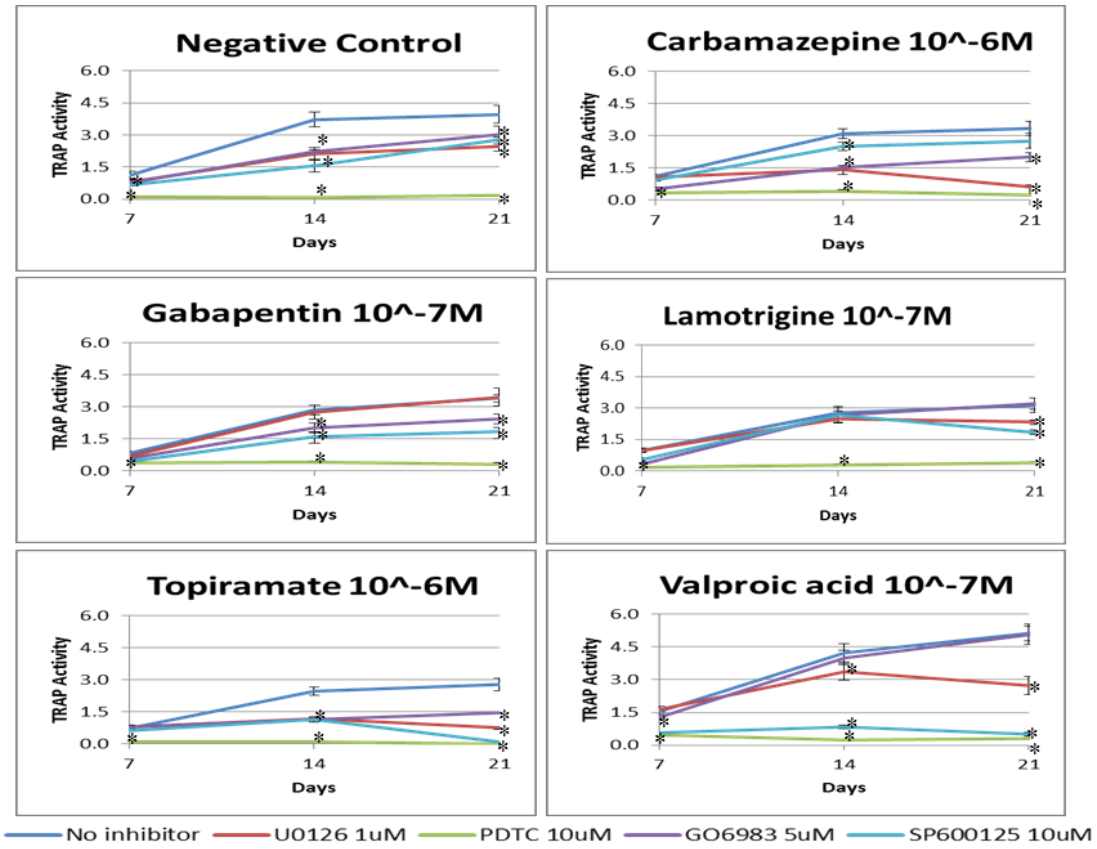

Figure 8 - TRAP Activity of PBMC cultures performed in the presence of different AEDs. Cell cultures were supplemented with different osteoclastogenic-related signaling pathways, namely U0126 (MEK inhibitor), PDTC (NFkB inhibitor), GO6983 (PKC inhibitor) and SP600125 (JNK inhibitor). * Significantly different from the control.

In the control, TRAP activity decreased in the presence of all tested signaling pathways inhibitors, especially in the case of PDTC that significantly abolished TRAP activity to values barely detectable. Comparatively, the presence of U0126 seemed not to affect the cellular response in cells cultures treated with gabapentin, while cultures supplemented with carbamazepine exhibited a higher inhibition. When supplemented with AEDs, the presence of PDTC also abolished TRAP activity to low values. GO6983 did not significantly affect the cellular response in cell cultures treated with lamotrigine and valproic acid, while in the presence of the remaining AEDs it elicited a decrease on TRAP activity slightly higher than the one observed in the control. Supplementation with SP600125 did not affect the behavior of cultures maintained in the presence of carbamazepine, while those treated with lamotrigine, topiramate and valproic acid exhibited a significantly higher inhibition than in the control.

CHAPTER 3 – Effects of AEDs on osteoblastic cell cultures

3.1 Introduction

The most important function of osteoblasts is to create mineralized bone tissues. These cells display various phenotypic markers such as high alkaline phosphatase (ALP) activity and synthesize collagenous and noncollagenous bone matrix proteins which include osteocalcin (46). The coordinated actions of osteoblasts and osteoclasts result in bone turnover or remodeling. Unfortunately, some AEDs can result in metabolic bone disease, affecting this balance between bone formation and resorption. Adverse effects of AEDs in osteoblast-like cells have been reported (41). In order to try to understand how AEDs affect bone cells, we performed an in vitro study with osteoblasts.

3.1.1 Aims

As discussed throughout Chapter 1, osteoblast-lineage cells, in addition to regulating bone formation, also regulates bone resorption via an elegant signaling axis that controls osteoclast differentiation and activity (10).

This chapter describes the way we cultured osteoblastic cells, and the main aim was to investigate the effects of five different AEDs on the differentiation and function of human osteoblasts. We also tried to analyze the influence of signaling pathways important for osteoblastogenesis in cellular responses of bone cells treated with AEDs.

3.2 Material and methods

3.2.1 The culture of osteoblastic cells

Osteoblasts were obtained from femur heads of patients (25-45 years old) undergoing orthopaedic surgery procedures, after informed consent, as previously described (47). Briefly, bone was broken in small pieces, which were maintained in 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 about 70-80% confluence, cells were enzymatically detached with 0.05% trypsin and 0.5 mM ethylenediamine tetraacetic acid (EDTA). Thereafter, they were seeded at 10^4cells/cm^2. Cell cultures, performed in the same culture medium mentioned above, were treated with 10 mM β-glycerophosphate (phosphate donor) and 10 nM dexamethasone

(osteogenic stimulator), and were maintained in the absence (control) or presence of five different AEDs, using five different concentrations within the range of 10^{-8}-10^{-4} M. Cell cultures were then characterized throughout a 21 day period, at days 7, 14 and 21, for the following parameters:

1. Cellular proliferation/viability,
2. ALP activity,
3. Protein quantification,
4. Histochemical staining of ALP,
5. Visualization of cellular morphology by CLSM,
6. Apoptosis rate.

Cultures were incubated in a 5% CO_2 humidified atmosphere at 37ºC. The culture medium was replaced once a week and the AEDs were renewed at each medium change.

Next, osteoblasts were treated with the minimum concentration of each AED that elicited a significant effect on those parameters, and were further characterized for:

a. Expression of osteoblast-related genes
b. Involvement of some osteoblastogenesis-related signaling pathways on cellular response

3.2.2 Osteoblastic cultures characterization

Cell cultures were then characterized throughout a 21 day period, at days 7, 14 and 21, for the following parameters:

1. Cellular proliferation/viability – The cellular proliferation/viability assessment was performed by MTT (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Cell cultures were incubated with 5mg/mL MTT, at 37ºC for 3 hours. After that, the culture medium was removed and dimethyl sulfoxide (DMSO) was added. The absorbance was quantified at 550 nm in an ELISA plate reader (Synergy HT, Biotek).

2. **ALP activity** – ALP activity was determined by the pNPP hydrolysis assay, at days 7, 14 and 21, as previously described (48). Shortly, after being washed twice with PBS and solubilized with 0.1% (V/V) Triton X-100, samples were incubated with 22.5 mM pNPP prepared in 0.15 M bicarbonate buffer (pH~10.3), for 1 hour at 37ºC. The reaction was stopped with 5 M NaOH, and the absorbance of the samples at 400 nm was measured in an ELISA plate reader (Synergy HT, Biotek). Results were normalized to total protein content of cultures and expressed as nmol/min/$\mu g_{protein}$.

3. **Protein quantification** - Total protein content of cell cultures was quantified at days 7, 14 and 21 by Bradford's method (44, 49), as described above.

4. **Histochemical staining of ALP** – The method used was based on the hydrolysis of α-naphtyl phosphate by ALP and the precipitation of phosphate liberated by reaction with a salt, giving rise to the formation of a coloured product, as previously described (48). Cell cultures were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer for 20 minutes and then washed with cacodylate buffer. After that, samples were incubated in the dark for 1 hour at 37ºC in 0.1 M Tris buffer (pH 10) containing 2 mg/ml Na-α-naphtyl phosphatase and 2 mg/ml of 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. Cells were visualized by phase contrast light microscopy (Nikon TMS phase contrast microscope).

5. **Visualization of cellular morphology by CLSM** - Cell cultures, at day 21, were washed twice with PBS and after being fixed with 3.7% para-formaldehyde for 15 minutes, cells 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) and for nuclei with 500 nM propidium iodide. They were then observed by Confocal Laser Scanning Microscopy (CLSM) (Leica TCP SP2 AOBS confocal microscope).

6. **Apoptosis rate** – Apoptosis was quantified as described above.

Next, osteoblasts were treated with the minimum concentration of each AED that elicited a significant effect on those parameters, and were further characterized for:

a. Expression of osteoblast-related genes - 21-day Cell cultures were assessed for the expression of the housekeeping gene glycerol-3-phosphate dehydrogenase (GAPDH) and the gene collagen type I (COL I) (1). RNA, 0.5 µg, was reverse transcribed and amplified (25 cycles) with the Titan One Tube RT-PCR System (Roche), with an annealing temperature of 55ºC. The primers used are listed on Table 2. PCR products were separated on an 1% (w/V) agarose gel, and the bands were analysed by densitometry with ImageJ 1.41 software. Values were normalized for the corresponding GAPDH value of each experimental condition.

Table 4 - Primers Used on RT-PCR Analysis of Osteoblastic Cell Cultures

Gene	5’ Primer	3’ Primer
GAPDH	5’-CAGGACCAGGTTCACCAACAAGT-3’	5’-GTGGCAGTGATGGCATGGACTGT-31’
COL I	5’-TCCGGCTCCTGCTCCTCTTA-3’	5’-ACCAGCAGGACCAGCATCTC-3’

b. Involvement of some osteoblastogenesis-related signaling pathways on cellular response – Osteoblastic cells cultured for 7, 14 and 21 days were treated with inhibitors of signalling pathways involved in the osteoblastogenic response (50). U0126, a MEK signalling pathway inhibitor, was tested at 1 µM. PDTC, a NFkB signalling pathway inhibitor, was used at 10 µM. GO6983, a PKC signaling pathway inhibitor, was tested at 5µM. Finally, SP600125, a JNK signalling pathway inhibitor, was used at 10µM. Cultures were assessed for total protein content and for ALP activity.

3.2.3 Statistical analysis

Data presented in this work is the result of separated experiments performed with cells from different blood donors. Three replicas of each condition were made for each experiment. Data was evaluated using a two-way analysis of variance (ANOVA) and no significant differences in the pattern of the cell behaviour were observed. Statistical differences found between control and experimental conditions were determined by Bonferroni’s method. For values of $P \leq 0.05$, differences were considered statistically significant. Data is expressed as the mean ± standard deviation.

3.3 Osteoblastic cells results

3.3.1 Cellular proliferation/viability

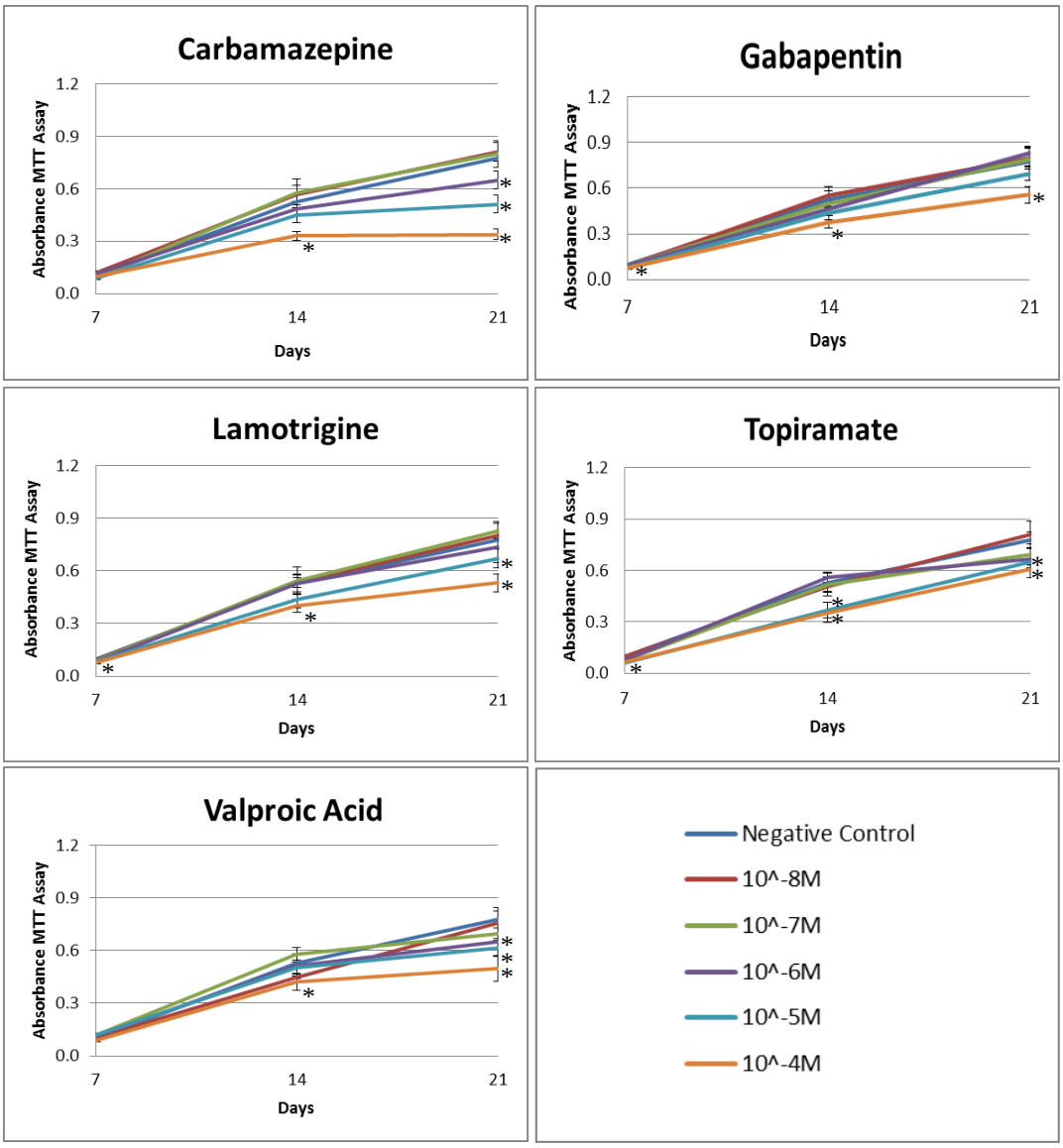

Figure 9 - Osteoblasts proliferation and viability performed by the MTT assay. Cell cultures were treated with different AEDs. * Significantly different from the control.

In general, cellular proliferation / viability increased through the 21 days of culture. When supplemented with low doses (10^{-8}M) of different AEDs, this behavior didn't significantly change. At high doses, all AEDs cause a dose-dependent decrease on cellular proliferation / viability, especially observed at 21 days of culture.

3.3.2 ALP activity

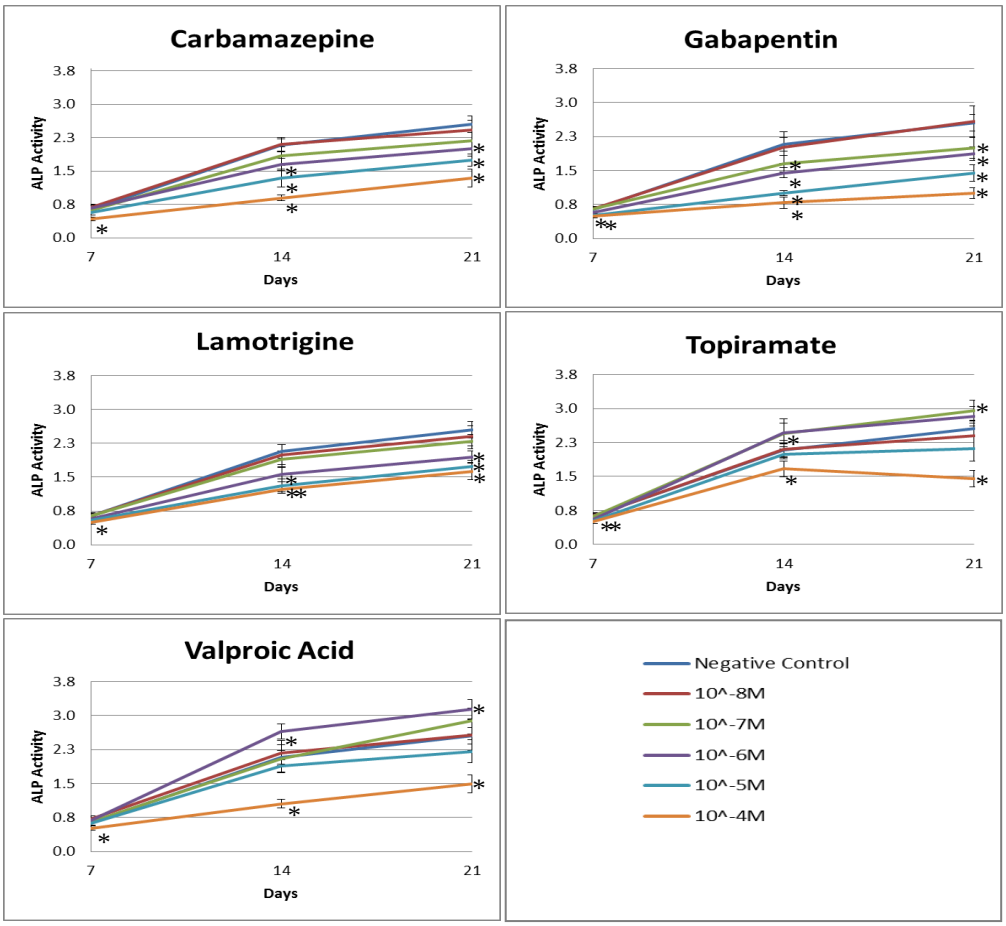

Figure 10 - ALP activity of osteoblastic cell cultures maintained in the absence (control) or presence of different AEDs, cultures for 7, 14 and 21 days. * Significantly different from the control.

Figure 10 shows that, without supplementation (negative control) with AEDs, ALP activity increased during the culture period (21 days). This increase was especially observed until day 14. Between days 14 and 21, ALP activity increased more slowly. The majority of the tested AEDs negatively modulated osteoblastogenesis, leading to a dose-dependent decrease of ALP activity. However, at lower doses (10^{-8}M) ALP activity seemed not to be affect by the presence of any AED. The decrease became statistically significant at 10^{-7}M gabapentin (~21.59%) and 10^{-6}M carbamazepine and lamotrigine (~21.59% and 23.98% respectively). Topiramate and valproic acid appeared as an exception since at lower doses (10^{-7}M and 10^{-6}M respectively) caused an increase of ALP Activity (~15.91% and 23.00% respectively). For concentrations higher than that, a dose-dependent decrease on cell response was observed.

3.3.3 Histochemical staining of ALP

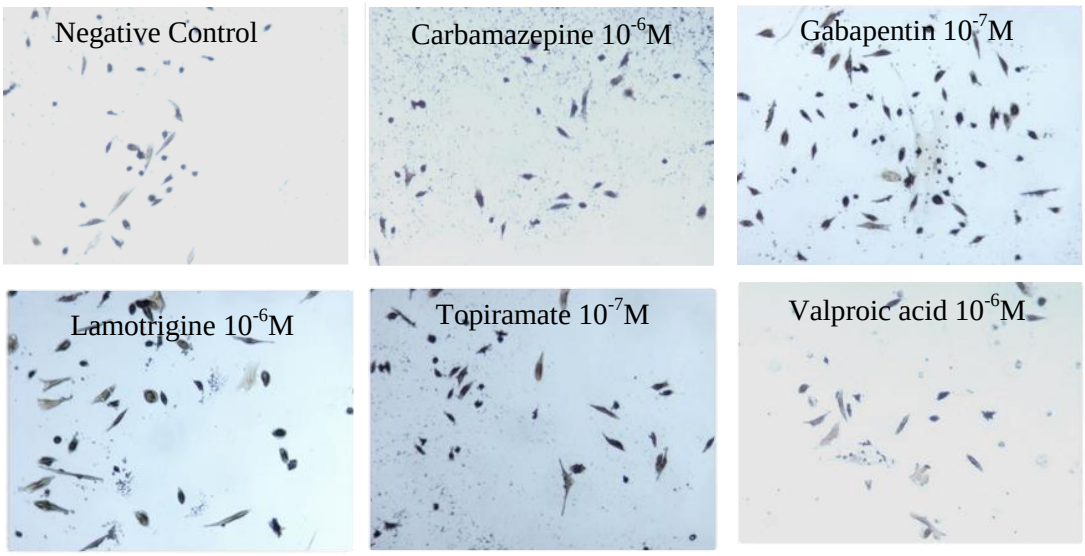

Figure 11 - Histochemical staining of ALP in osteoblastic cell cultures.

Figure 11 shows that, cells were positively stained for ALP in all tested conditions. No significant differences were observed in the intensity of the staining among the different tested AEDs. Cells were uniformly spread in the culture well and exhibited the characteristic elongated morphology of this lineage.

3.3.4 Visualization of cellular morphology by CLMS

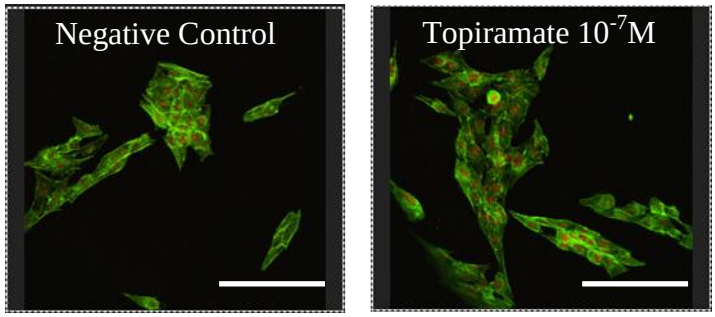

Figure 12 - Presence of multinucleated cells displaying actin rings and nuclei in osteoblastic cell cultures assessed by CSLM. Fluorescence images showing actin rings (green) and nuclei (red). White bars represent 120 µm.

Figure 12 shows osteoblastic cell cultures stained for actin and nuclei and visualized by CLSM. Cells were uniformly distributed in the wells, with visible cell-to-cell contacts, and displaying a proper elongated morphology.

3.3.5 Apoptosis rate

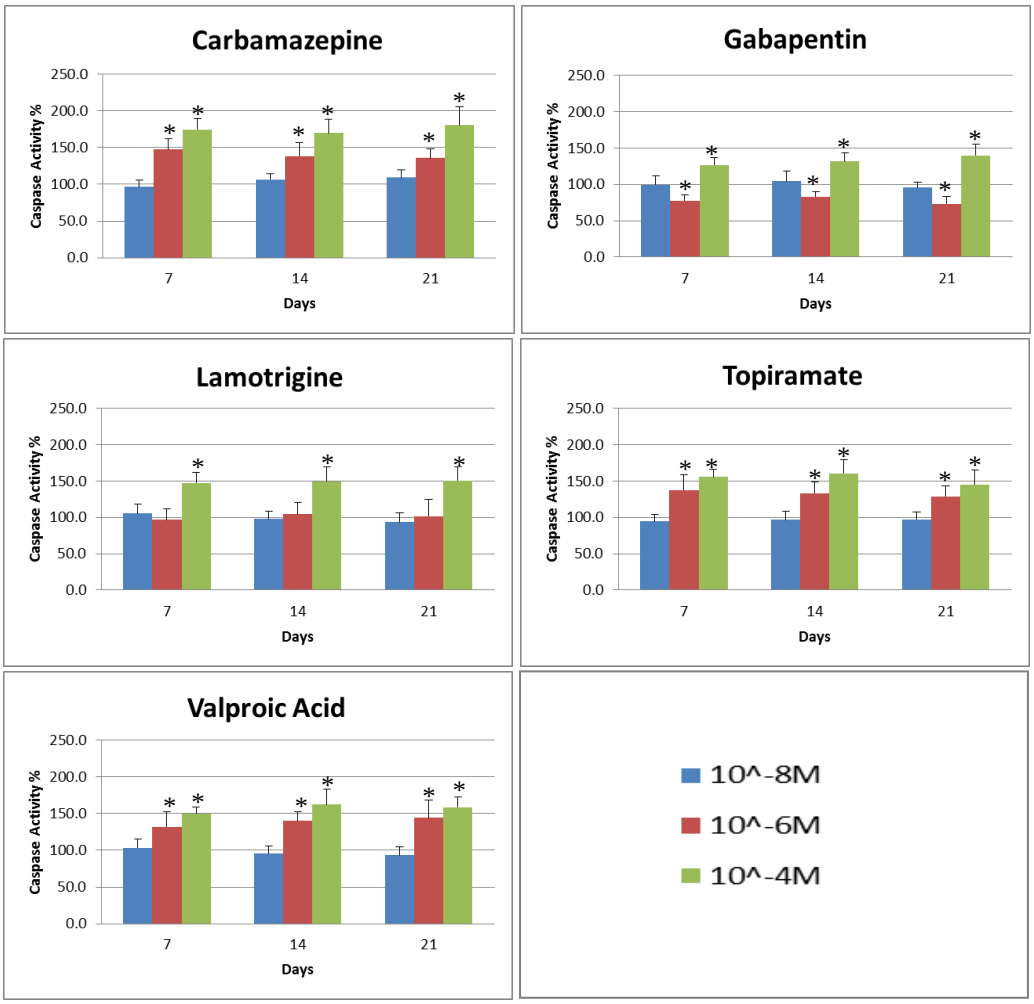

Figure 13 - Caspase-3 activity on osteoblastic cell cultures treated with different AEDs. * Significantly different from the control.

Figure 13 shows that caspase-3 activity remained similar in the three culture periods tested, in all tested conditions. Supplementation with carbamazepine, lamotrigine, topiramate and valproic acid elicited a dose-dependent increase of caspase-3 activity. Supplementation with gabapentin caused a decrease of caspase-3 activity at 10^{-6}M concentration, while at 10^{-4}M an increase on cell response was observed.

3.3.6 Expression of osteoblast-related genes

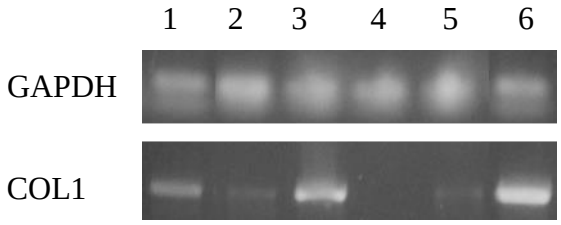

Figure 14 - Representative agarose gel of osteoblastic cell cultures. Densitometric analysis of RT-PCR products normalized by GAPDH. 1 – Negative control; 2 – Carbamazepine; 3 – Gabapentin; 4 – Lamotrigine; 5 –Topiramate; 6- Valproic acid.

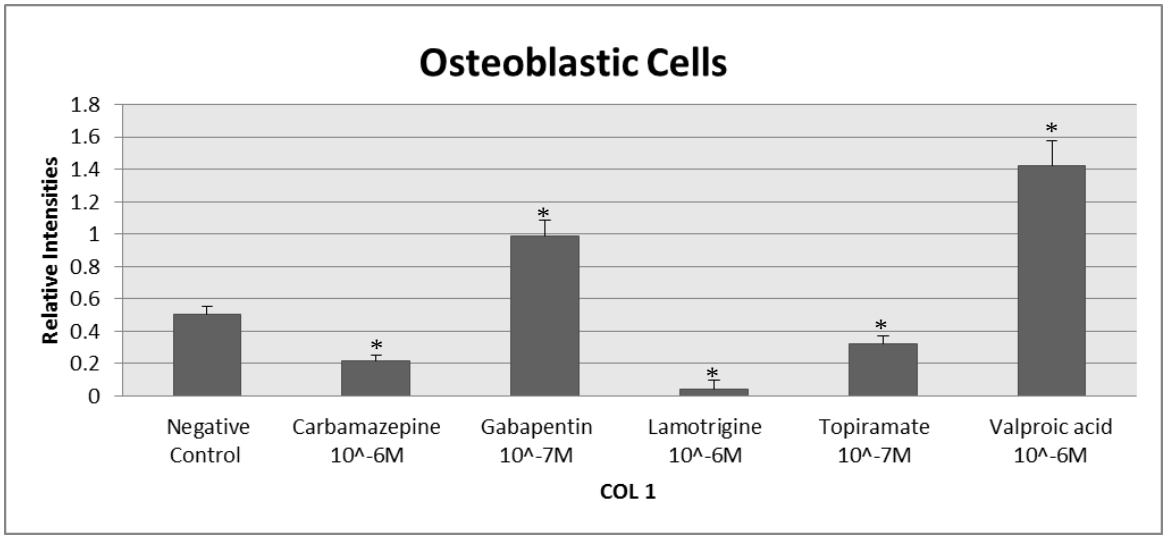

Figure 15 - Expression of osteoblastic-associated genes.

Cell layer was assessed by RT-PCR, in order to verify the expression of the housekeeping gene GAPDH and the osteoblastic gene COL1. Cell cultures revealed expression of both genes, in the presence of AEDs. In the control, the expression of COL1 was low. However, when cell cultures were treated with carbamazepine, lamotrigine and topiramate the expression levels significantly decreased (56.44%, 91.79% and 35.95% respectively). On the other hand, when cell cultures were treated with gabapentin and valproic acid, the expression levels significantly increased (96.15% and 183.30%, respectively).

3.3.7 Involvement of some osteoblastogenesis-related signaling pathway on cellular response

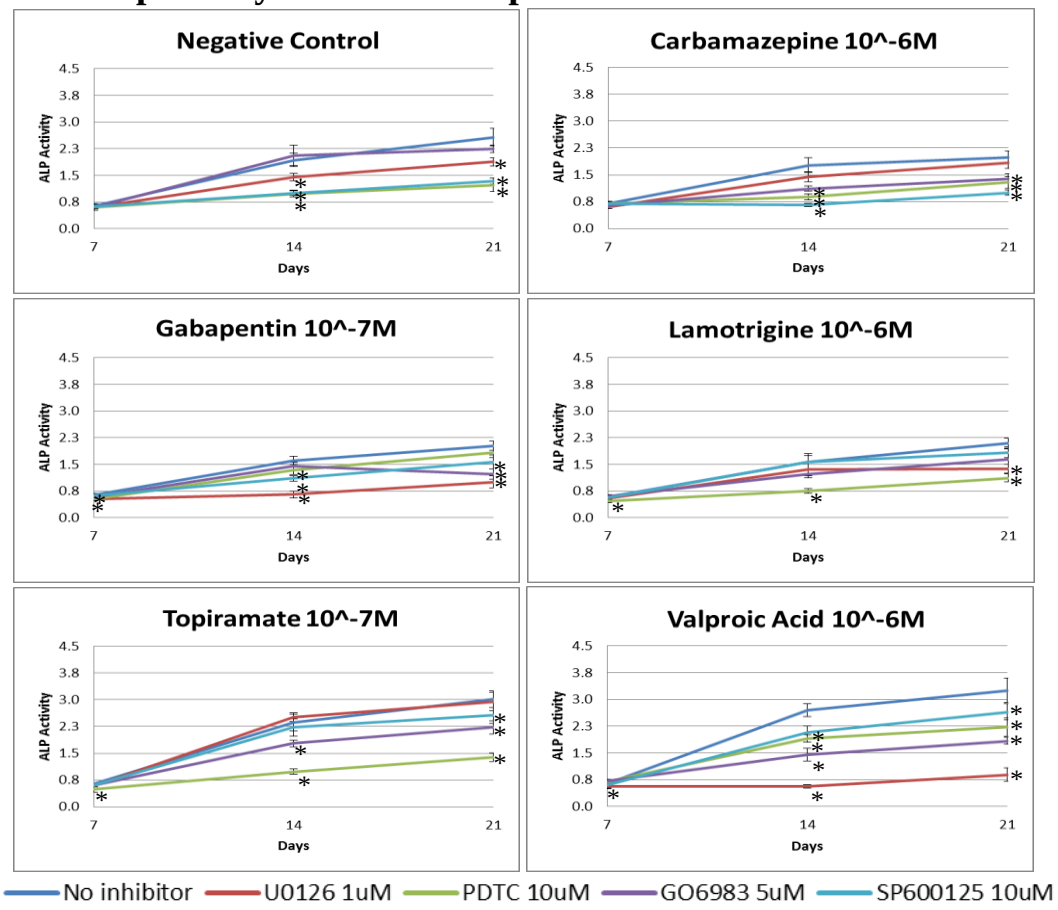

Figure 16 - ALP activity of osteoblastic cell cultures performed in the presence of different AEDs. Cell cultures were supplemented with different osteoblastogenic-related signaling pathways, namely U0126 (MEK inhibitor), PDTC (NFkB inhibitor), GO6983 (PKC inhibitor) and SP600125 (JNK inhibitor). * Significantly different from the control.

In the control, ALP activity decreased in the presence of all tested signaling pathways inhibitors. Comparatively, the presence of U0126 seemed not to affect the cellular response in cells cultures treated with carbamazepine and topiramate, while cultures supplemented with gabapentin and valproic acid exhibited a higher inhibition. When supplemented with AEDs, the presence of PDTC also abolished ALP activity to low values, especially with lamotrigine and topiramate. GO6983 elicited a decrease on ALP activity slightly higher than the one observed in the control. Supplementation with SP600125 did not affect the behavior of cultures maintained in the presence of lamotrigine, while those treated with carbamazepine exhibited a significantly higher inhibition than in the control.

CHAPTER 4 – Effects of AEDs on co-cultured cells

4.1 Introduction

Osteoclasts are cells specialized in bone resorption and osteoblasts are bone-forming cells (47). In addition to the well-established role of osteoblasts in osteoclastogenesis, recent studies have shown that osteoclasts are not simply bone resorbing cells, but that they also regulate osteoblast functions positively and negatively (2). Osteoclast-osteoblast interactions occur at various stages of differentiation, contributing to coupling of bone resorption and formation (14). Communication pathways exist between each of these cell types and the osteocytes, which are embedded in the bone matrix (13). Hereupon, osteoclastic and osteoblastic cells were cultured together.

4.2 Aims

As discussed throughout Chapter 1, both epilepsy itself and treatment with AEDs has been recognized to have an adverse effect on bone metabolism. A proper balance between bone formation and resorption is crucial for a healthy bone tissue.

It has been documented that in the normal bone microenvironment osteoclast-osteoblast crosstalks appear to have a direct influence in bone formation events (45).

This chapter describes the way we cultured both cell types together, and the main aim was the investigation of the effects of five AEDs, guarantying that all the information regarding osteoclast and osteoblast differentiation was related to the cellular crosstalks between both cell types, so that we can study how they influence each other and their functions.

4.3 Material and methods

4.3.1 The co-culture of osteoclastic and osteoblastic cells

Osteoblastic cells were seeded ($1x10^3$cells/cm^2) and cultured for 24 hours in the same conditions used for the culture of isolated osteoblastic cells. After that, PBMC were added ($1.5x10^6$cells/cm^2) to the osteoblastic cells and co-cultures were maintained in the same conditions described for the PBMC cultures but in the absence of any osteoclastogenic or osteogenic stimuli. Thus, it was expected that all the information regarding osteoclast and osteoblast differentiation was related to the cellular crosstalks between both cell types.

Cell cultures were maintained in the absence (control) or presence of the five tested AEDs (10^{-8}-10^{-4} M).

Cultures were maintained at 37ºC in a 5% CO_2 humidified atmosphere and were characterized throughout a 21 day period, at days 7, 14 and 21. Culture medium was replaced once a week and the AEDs were renewed at each medium change.

Cellular response was evaluated through the same osteoclast and osteoblast parameters mentioned and described above for the corresponding isolated cell cultures:

1. Protein quantification,
2. TRAP activity,
3. Number of TRAP + multinucleated cells,
4. Calcium phosphate resorbing ability,
5. ALP activity,
6. Histochemical staining of ALP,
7. Cellular morphology,
8. Expression of osteoclast and osteoblast-related genes,
9. Involvement of some osteoclast and osteoblastogenesis-related signaling pathways on cellular response.

4.4 Statistical analysis

Data presented in this work is the result of separated experiments performed with cells from different blood donors. Three replicas of each condition were made for each experiment. Data was evaluated using a two-way analysis of variance (ANOVA) and no significant differences in the pattern of the cell behaviour were observed. Statistical differences found between control and experimental conditions were determined by Bonferroni's method. For values of $P \leq 0.05$, differences were considered statistically significant. Data is expressed as the mean ± standard deviation.

4.5 Co-cultures results

4.5.1 TRAP activity

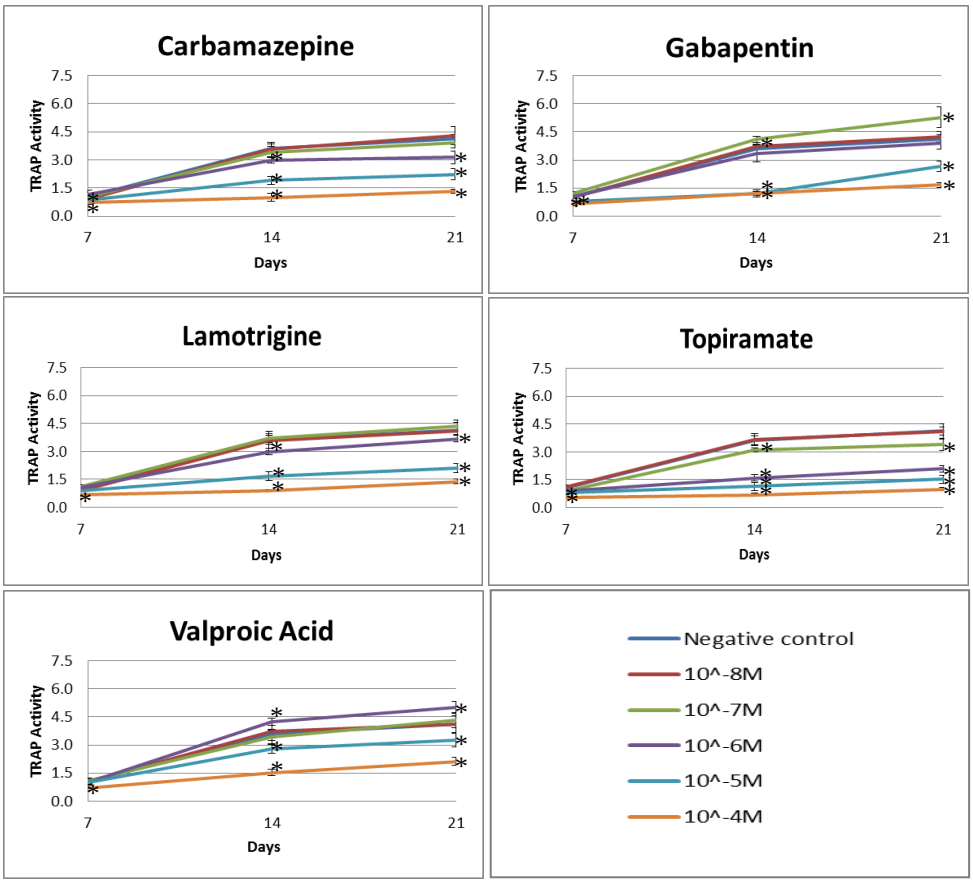

Figure 17 - TRAP activity of co-cultures maintained in the absence (negative control) or supplemented with different AEDs, cultured for 7, 14 and 21 days. * Significantly different from the control.

Figure 17 shows that, at control conditions, TRAP activity clearly increased until day 14. Between days 14 and 21, TRAP activity increased more slowly. The different AEDs had the ability to modulate the osteoclastogenesis process. The majority of the tested AEDs negatively modulated osteoclastogenesis, leading to a dose-dependent decrease of TRAP activity. At lower doses (10^{-8}M) TRAP activity seems not to be affect by the presence of any AED. The decrease became statistically significant at 10^{-7}M topiramate (~17.31%) and 10^{-6}M carbamazepine and lamotrigine (~24.12% and 10.98%, respectively). Gabapentin and valproic acid appeared as an exception since at lower doses (10^{-7}M and 10^{-6}M respectively) caused an increase of TRAP Activity (~27.84% and 21.37% respectively). For concentrations higher than that, a dose-dependent decrease on cell response was observed.

4.5.2 Number of TRAP-positive multinucleated cells

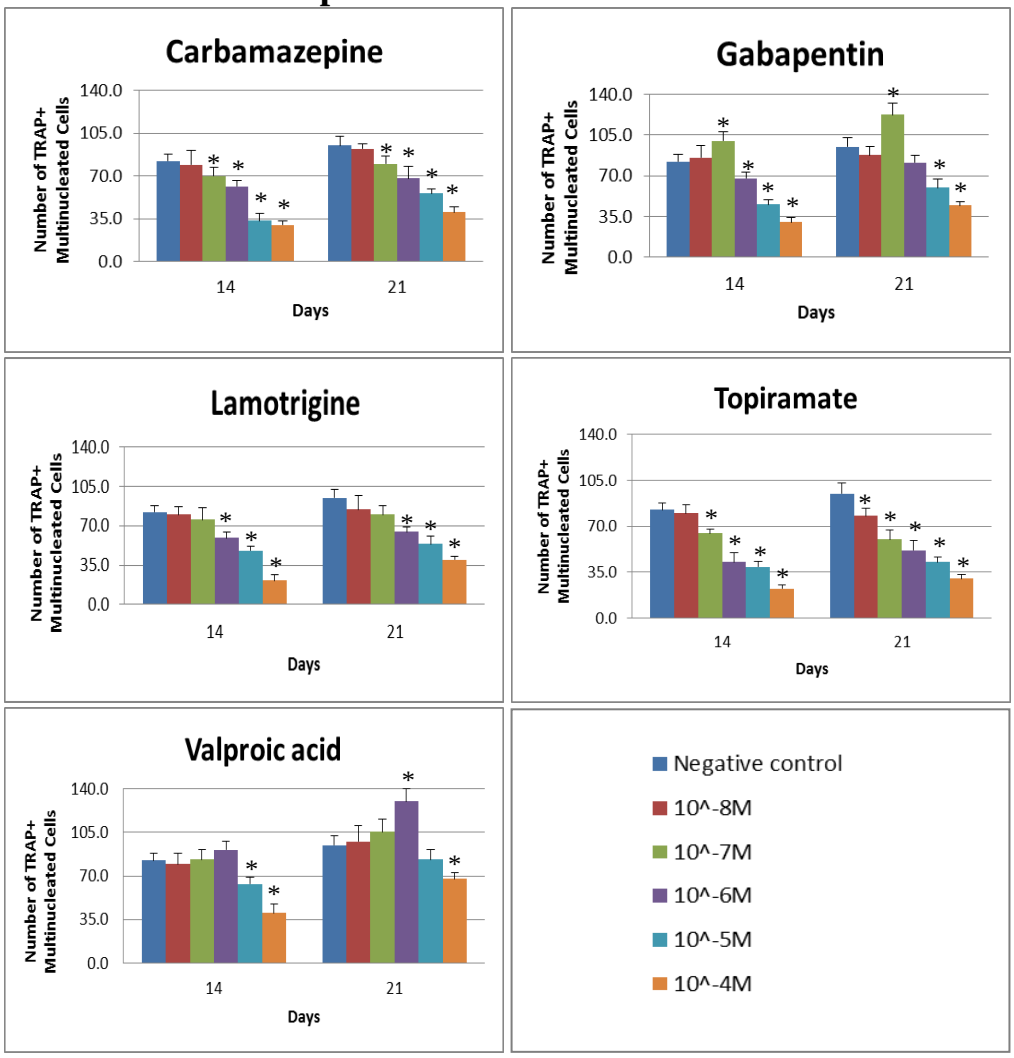

Figure 18 - Number of TRAP+ multinucleated cells on co-cultures maintained in the absence (negative control) or supplemented with different AEDs. * Significantly different from the control.

In general, the results obtained for TRAP+ multinucleated cells (Figure 18), after 14 and 21 days of culture, followed the same profile observed for TRAP activity. Briefly, at lower doses (10^{-8}M) the number of TRAP+ multinucleated cells seems not to be affected by the presence of any AED, except topiramate which caused a statistically significant decreased of number of TRAP+ multinucleated cells (~17.98%). The decrease became statistically significant at 10^{-7}M carbamazepine (~15.60%) and 10^{-6}M lamotrigine (~31.99%). Gabapentin and valproic acid appeared as an exception since at lower doses (10^{-7}M and 10^{-6}M respectively) caused an increase of number of TRAP+ multinucleated cells (~29.06% and 37.08%, respectively). For concentrations higher than that, a dose-dependent decrease on cell response was observed.

4.5.3 Calcium phosphate resorbing ability

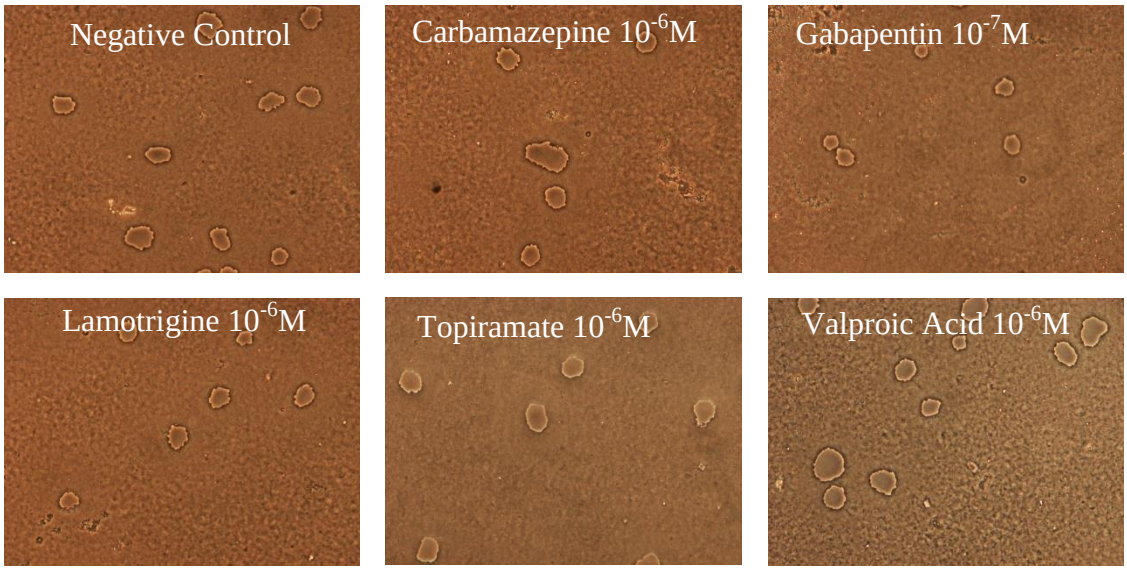

Figure 19 - Calcium phosphate resorbing ability of co-cultures maintained in the absence (negative control) or treated with different AEDs.

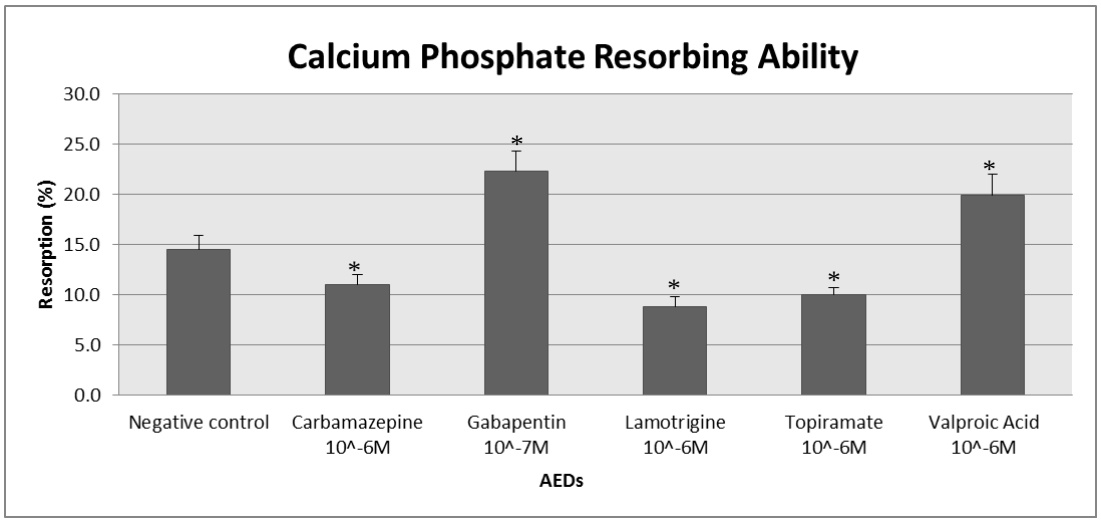

Figure 20 - Total resorbed area of co-cultures maintained in the absence (negative control) or treated with different AEDs. * Significantly different from the control.

Figures 19 and 20 show that the presence of gabapentin and valproic acid induced an increase on the total resorbed area (~54.14% and ~37.71% respectively). However, the other drugs elicited a decrease on the resorbing ability. Lamotrigine was the one that most elicited this decrease (~38.91%).

4.5.4 ALP activity

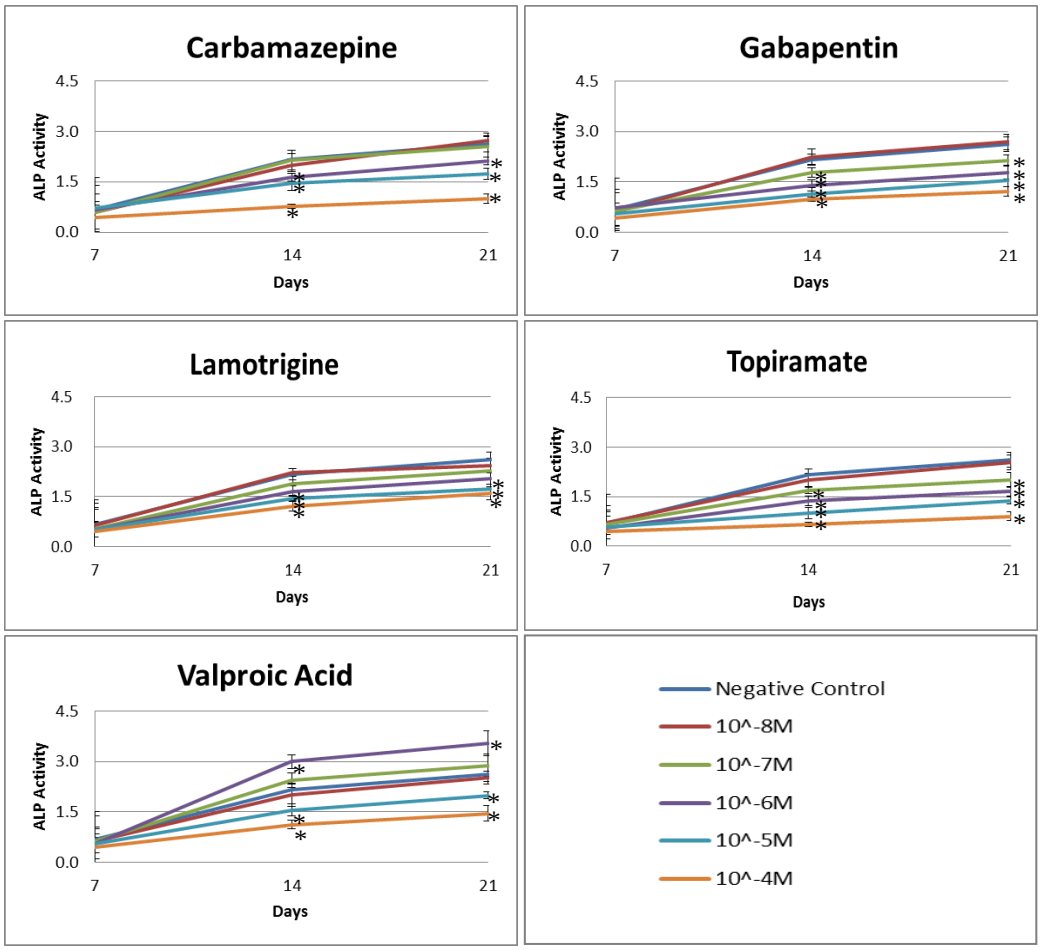

Figure 21 - ALP activity of co-culture cells maintained in the absence (control) or presence of different AEDs, cultures for 7, 14 and 21 days. * Significantly different from the control.

Figure 21 shows that, without supplementation (negative control) ALP activity increased during the culture period (21 days). This increase is especially observed until day 14. Between days 14 and 21, ALP activity increased more slowly. The majority of the tested AEDs negatively modulated osteoblastogenesis, leading to a dose-dependent decrease of ALP activity. At lower doses (10^{-8}M) ALP activity seems not to be affect by the presence of any AED. The decrease became statistically significant at 10^{-7}M gabapentin and topiramate (~18.79% and 23.58%, respectively). The decrease became statistically significant at 10^{-6}M carbamazepine and lamotrigine (~19.12% and 21.73%, respectively). Valproic acid appeared as an exception since at lower doses (10^{-6}M) caused an increase of ALP Activity (~34.98%). For concentrations higher than that, a dose-dependent decrease on cell response was observed.

4.5.5 Histochemical staining of ALP

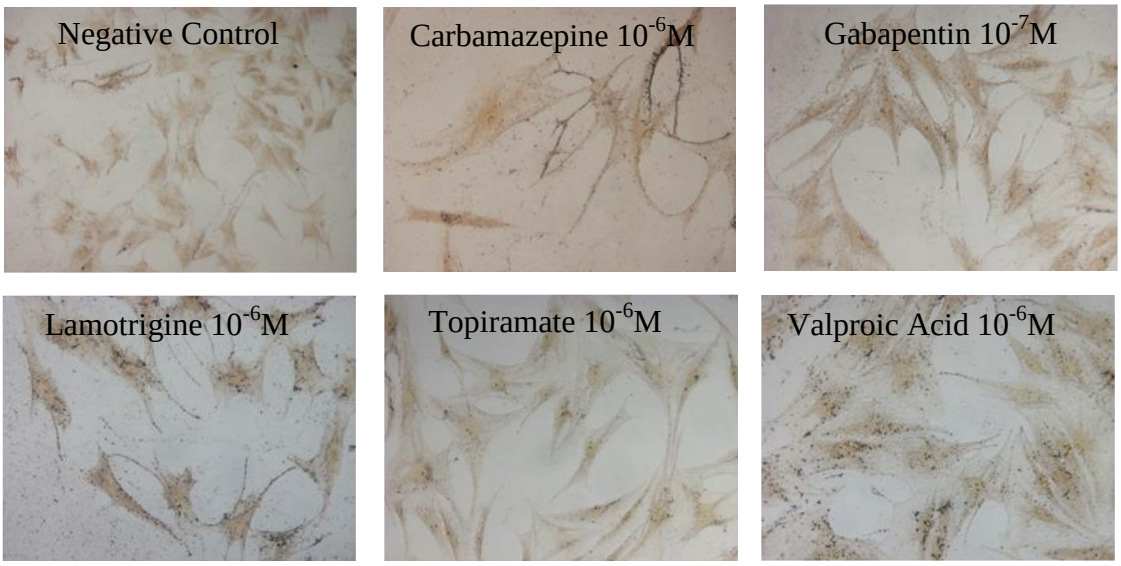

Figure 22 - Histochemical staining of ALP in co-cultures.

Figure 22 shows that cells were positively stained for ALP in all tested conditions. No significant differences were observed in the intensity of the staining among the different tested AEDs. Cells were uniformly spread in the culture well and exhibited the characteristic elongated morphology of this lineage.

4.5.6 Cellular morphology

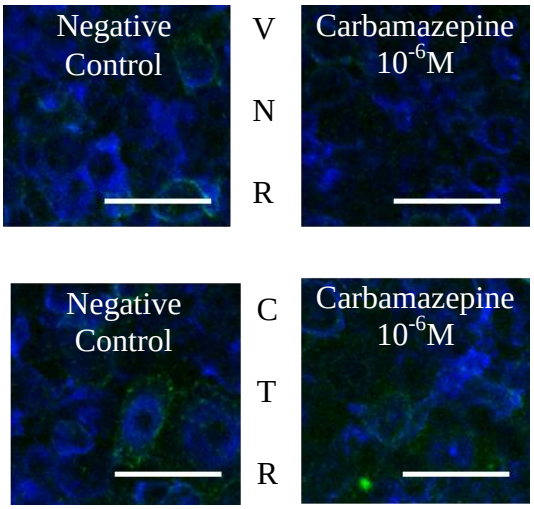

Figure 23 - Representative images of co-cultures visualized by confocal laser scanning microscopy (CLSM). Cells were stained blue for F-actin and green for Vitronectin Receptors (VNR) and Calcitonin Receptors (CTR). White bars represent 120 µm.

At day 21, co-cultures showed cells displaying osteoclastic features, that is, cells with actin rings and expressing vitronectin and calcitonin receptors, as well as few elongated cells only positive for actin staining. Figure 23 shows representative images of co-cultures maintained in the absence or presence of carbamazepine.

4.5.7 Expression of osteoclast and osteoblast-related genes

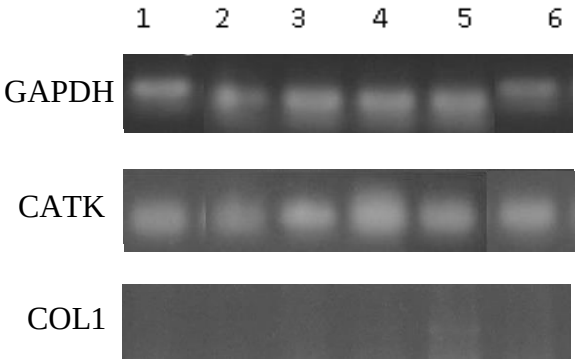

Figure 24 - Representative agarose gel of co-cultures. Densitometric analysis of RT-PCR products normalized by GAPDH. 1 – Negative control; 2 – Carbamazepine; 3 – Gabapentin; 4 – Lamotrigine; 5 –Topiramate; 6 – Valproic acid.

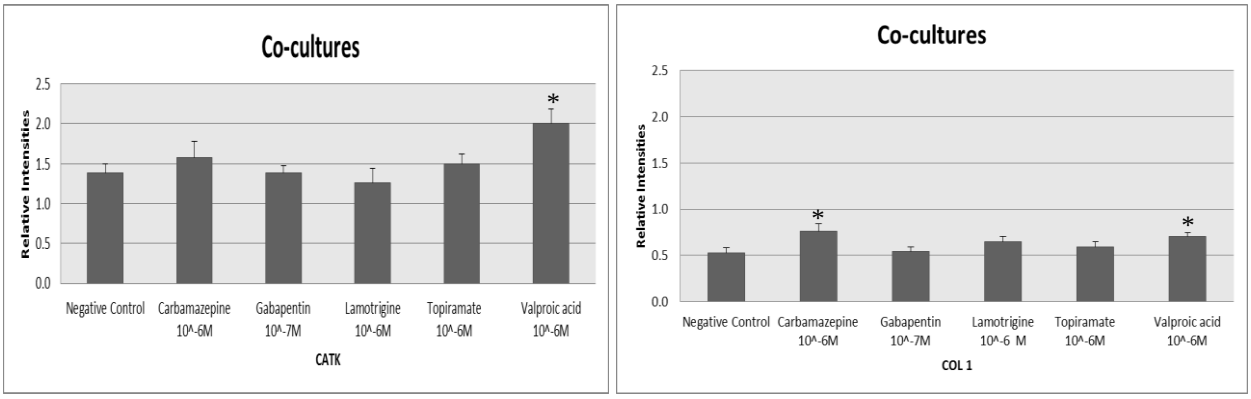

Figure 25 - Osteoclastic and osteoblastic-related genes

Cell layers were assessed by RT-PCR (Figure 24), in order to verify the expression of the housekeeping gene GAPDH and the genes CATK and COL1. The co-cultures revealed expression of both genes, either in the absence or presence of AEDs (Figure 25). When cell cultures were treated with valproic acid the expression levels of CATK significantly increased (~45.02%). On the other hand, in the control, the expression of COL1 was low, and this was not significantly affected by the presence of gabapentin, lamotrigine and topiramate. However, when cell cultures were treated with carbamazepine and valproic acid the expression levels significantly increased (~43.38% and 33.70%, respectively).

4.5.8 Involvement of some osteoclastogenesis-related signaling pathways on cellular response

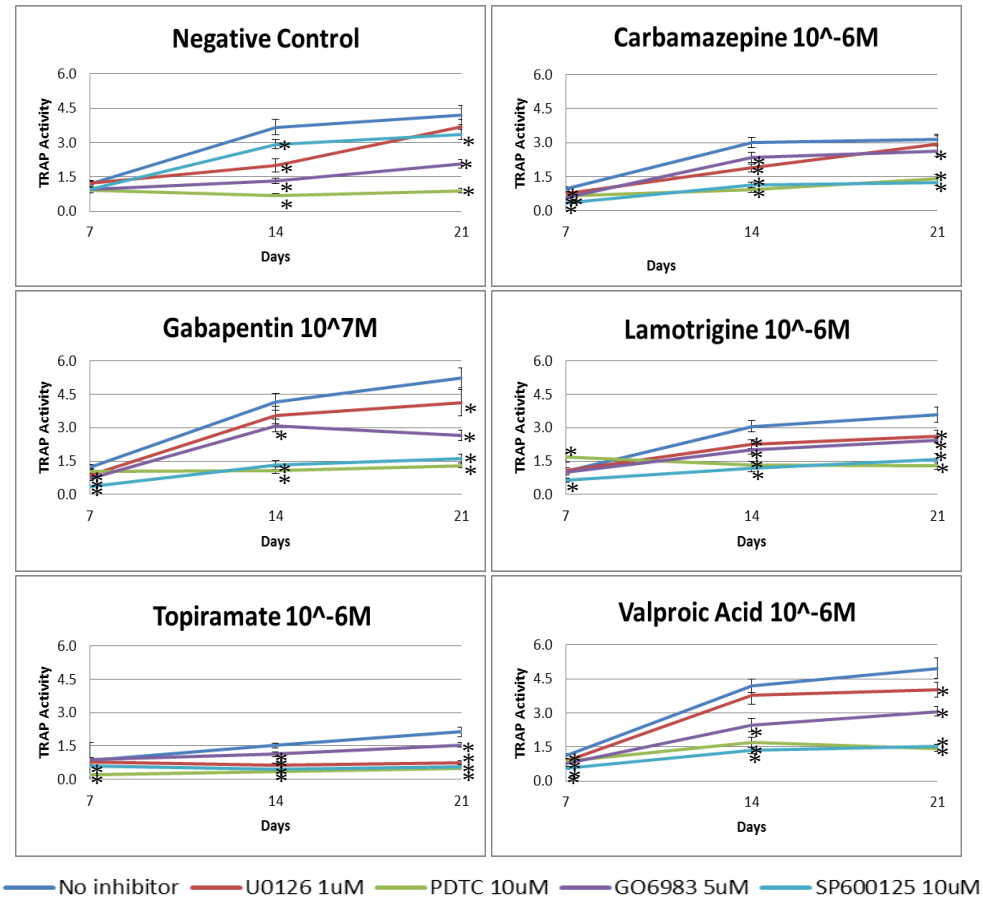

Figure 26 - TRAP Activity of co-cultures performed in the presence of different AEDs. Cell cultures were supplemented with different osteoclastogenic and osteoblastogenic-related signaling pathways, namely U0126 (MEK inhibitor), PDTC (NFkB inhibitor), GO6983 (PKC inhibitor) and SP600125 (JNK inhibitor). * Significantly different from the control.

In the control, TRAP activity decreased in the presence of all tested signaling pathways inhibitors, especially in the case of PDTC that sharply abolished TRAP activity. The one exception was U0126, which did not affect cellular response at day 21. Comparatively, the presence of U0126 seemed to affect the cellular response in cell cultures treated with all tested AEDs, except carbamazepine. When supplemented with AEDs, the presence of PDTC also abolished TRAP activity to low values. In the presence of the tested AEDs, GO6983 elicited a decrease on TRAP activity lower (carbamazepine and topiramate) or similar to the one observed in the control. Supplementation with SP600125 caused a higher inhibitory effect in the presence of all AEDs.

4.5.9 Involvement of some osteoblastogenesis-related signaling pathways on cellular response

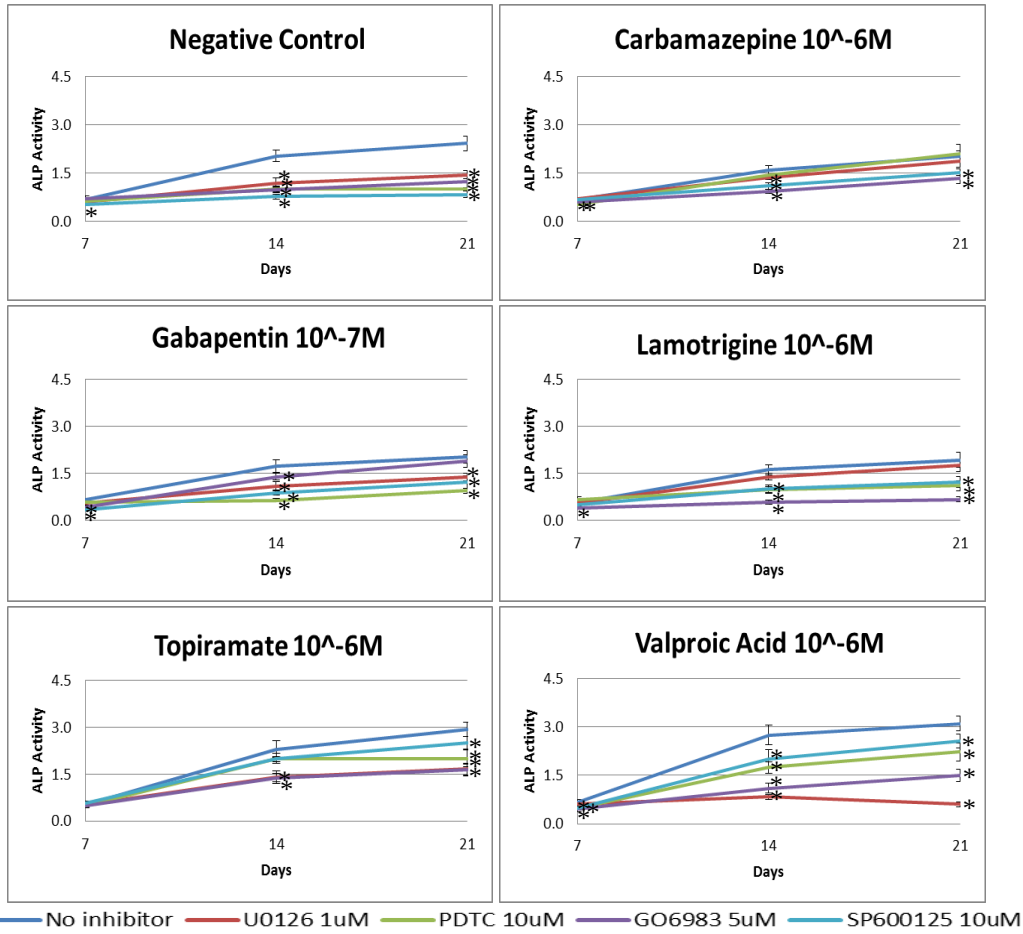

Figure 27 - ALP activity of co-cultures performed in the presence of different AEDs. Cell cultures were supplemented with different osteoclastogenic and osteoblastogenic-related signaling pathways, namely U0126 (MEK inhibitor), PDTC (NFkB inhibitor), GO6983 (PKC inhibitor) and SP600125 (JNK inhibitor). * Significantly different from the control.

In the control, ALP activity decreased in the presence of all tested signaling pathways inhibitors. Comparatively, the presence of U0126 did not affect cellular response in the presence of carbamazepine and lamotrigine, while cultures supplemented with topiramate and valproic acid exhibited a higher inhibition. No inhibition was also observed in the presence of PDTC in carbamazepine-treated cell cultures. GO6983 significantly affected the cellular response in cell cultures treated with the AEDs, except in the case of gabapentin. Supplementation with SP600125 affected the behavior of cultures maintained in the presence of all tested AEDs, although at a lower significance than the one observed in the absence of the drugs.

CHAPTER 5 – Discussion

5.1 Discussion

The precise balance between bone formation and bone resorption is critical for the maintenance of bone mass density and systemic mineral homeostasis. These events are responsible for the life-long bone remodeling in order to ensure a healthy tissue formation and maintenance. Any disturbance of this balance may lead to various bone diseases (9, 14). In this work, cultures of human osteoclastic, osteoblastic and co-cultures of both cells were used as an *in vitro* model to address the effects of AEDs on bone metabolism.

Antiepileptic drugs were first associated with bone disorders in both adults and children in the late 1960s (51). The most severe manifestations of these disorders are osteopenia/osteoporosis, osteomalacia and fractures (52).

There are conflicting reports on effects of AEDs on bone strength and metabolism. Furthermore, the effects of AEDs on the osteoclast and osteoblast development are scarcely reported in the literature, with only a few published studies about it. In addition, these studies are mostly performed *in vivo*. Thus, without a detailed characterization of the *in vitro* effects of AEDs on bone cells, the knowledge about the underlying mechanisms is still unknown.

*

PBMC cultures were performed in the presence of the osteoclastogenic recombinant growth factors M-CSF and RANKL. A number of studies have shown that both, M-CSF and RANKL, are required to induce the expression of genes that typify the osteoclast lineage, such as TRAP (an important cytochemical marker of osteoclasts, whose concentration in serum is used as a biochemical marker of osteoclast function and degree of bone resorption (53)), CTR, VNR (11).

The different AEDs had the ability to modulate the osteoclastogenic process. The majority of the tested AEDs (carbamazepine, gabapentin, lamotrigine and topiramate) negatively modulated it, leading to a decrease on osteoclastogenesis. This situation was observed by quantification of TRAP activity, number of TRAP+ multinucleated cells and calcium phosphate resorbing ability. All obtained results showed a dose-dependent decrease on the cellular response. Valproic acid appeared as an exception since, at lower doses (10^{-7} M), caused an increase on the process.

However, higher concentrations elicited a dose-dependent inhibition on osteoclast development.

Caspase-3 activity results were also in line with these findings, since supplementation with carbamazepine, gabapentin, lamotrigine and topiramate elicited a dose-dependent increase of caspase-3 activity. However, supplementation with valproic acid caused a decrease of caspase-3 activity at 10^{-6}M concentration, suggesting that this drug may stimulate osteoclastogenesis by increasing cell survival.

NFkB pathway seemed to be crucial in all tested conditions (control and presence of AEDs), which strongly suggests that, in our culture conditions, this can be a major osteoclastogenic intracellular mechanism. PKC signaling pathway did not contribute to the cellular response in PBMC cultures treated with lamotrigine and valproic acid, although in the presence of the remaining AEDs it elicited a decrease on TRAP activity slightly higher than the one observed in the control. JNK pathway was down regulated by carbamazepine, while supplementation with lamotrigine, topiramate and valproic acid exhibited a significantly higher involvement of that pathway on osteoclastic response.

The osteoblastic cells have a fundamental role in bone formation and regeneration producing extracellular matrix proteins and regulators of matrix mineralization (12). The proliferation/viability of osteoblasts was performed by the MTT assay. Cell proliferation is the measurement of the number of cells that are dividing in a culture. Cell viability can be defined as the number of healthy cells in a sample. Whether the cells are actively dividing or are quiescent is not distinguished (54). It was observed that when supplemented with low doses of different AEDs, cellular behavior didn't significantly change. At high doses, all AEDs caused a dose-dependent non-significantly decrease on cellular proliferation / viability. In line with this, supplementation with carbamazepine, lamotrigine, topiramate and valproic acid elicited a dose-dependent increase of caspase-3 activity. Supplementation with gabapentin caused a small decrease of caspase-3 activity at 10^{-6}M concentration, while at 10^{-4}M it was observed an increase on cell response.

Osteoblastic cells were also tested for ALP activity. ALP was the first biochemical marker of bone turnover and is still the one most widely used in clinical

practice (55). The majority of the tested AEDs (carbamazepine, gabapentin, lamotrigine and topiramate) negatively modulated osteoblastogenesis, leading to a dose-dependent decrease of ALP activity. Valproic acid appeared as an exception since at lower doses caused an increase of ALP Activity.

Osteoblastic cell cultures were also tested for the involvement of several signaling pathways (MEK, NFkB, PKC and JNK). Some significant alterations on the contribution of the tested pathways on the response of osteoblastic cells were observed. PKC pathway appeared to be crucial in all tested conditions (control and presence of AEDs, except for gabapentin), which strongly suggests that, in our culture conditions, this one can be one of the major osteoblastogenic intracellular mechanism. NFkB signaling pathway did not significantly contribute to the cellular response in osteoblastic cell cultures treated with carbamazepine, although in the presence of the remaining AEDs it elicited a decrease on ALP activity slightly higher than the one observed in the control. MEK signaling pathway did not contribute to the cellular response in osteoblastic cell cultures treated with carbamazepine and lamotrigine, while in the presence of the remaining AEDs it elicited a decrease on ALP activity slightly higher than the one observed in the control. JNK signaling pathway affected the behavior of cultures maintained in the presence of all tested AEDs, although at a lower significance than the one observed in the absence of the drugs.

Osteoclast-osteoblast communication occurs at the initiation, transition and termination phases of bone remodeling. There are at least three modes of communication between these cells types. Osteoclasts and osteoblasts can make direct contact, form gap junctions or communicate through diffusible paracrine factors (14). In co-cultures, TRAP activity was stimulated by valproic acid however, the remaining AEDs caused a decrease in osteoclastogenesis. Number of TRAP+ multinucleated cells supported these results. The same profile was observed in PBMC cultures, for TRAP activity and number of TRAP+ multinucleated cells. Co-cultures also showed a high osteoblastic response at low doses of valproic acid. The remaining AEDs caused a decrease in osteoblasts differentiation. The same results were observed in osteoblastic cell cultures, for ALP activity.

Caspase-3 activity test was not performed in co-cultures because, since both cells were present, it was impossible to know which cell was contributing to the result.

Co-cultures were also analyzed for the involvement of several signaling pathways, either for osteoclastic and osteoblastic parameters. Concerning the osteoclastic response, it was observed that, in the control, the tested inhibitors caused a decrease in TRAP activity, which indicates that all the signaling pathways influence osteoblastic differentiation. The MEK signaling pathway seemed to be closely involved in osteoclastogenesis in the presence of all the tested AEDs, except in the presence of carbamazepine which appeared not to interfere with. NFkB and JNK signaling pathways appeared to be major intracellular mechanisms, being the latter up regulated by all the tested AEDs. PKC signaling pathway seemed not to be involved in osteoclastogenesis in the presence of carbamazepine, while in the presence of the remaining AEDs it elicited a decrease on TRAP activity.

Regarding the intracellular signaling pathways involving osteoblastic response, it was observed that, in the control, the tested inhibitors caused a decrease in ALP activity, which indicates that all the signaling pathways influence osteoblastic differentiation. Cell cultures treated with carbamazepine and lamotrigine were not significantly affected by U0126, which suggests that in these conditions MEK pathway may not be important for osteoblastogenesis. Also NFkB seemed not to be important in the presence of carbamazepine. Cultures treated with all the tested AEDs, except gabapentin, were significantly affected by GO6983, which suggests that in these conditions PKC signaling pathway may be important for osteoblastogenesis. JNK signaling pathway appeared to be important for osteoblastogenesis. This pathway affected the behavior of cultures maintained in the presence of all tested AEDs, although at a lower significance than the one observed in the absence of the drugs.

Taken together, AEDs seemed to cause a direct effect on human osteoclastic and osteoblastic differentiation. Interestingly, some of the tested molecules increased while others inhibited the processes in a dose-dependent profile. Furthermore, since in the co-culture, the AEDs influence in both cellular activities was somehow different, this suggests that AEDs may also modulate the reciprocal communications established between osteoclasts and osteoblasts.

It is important to analyze the therapeutic level of the tested AEDs, so that, we can compare with the tested concentrations and draw more conclusions. These values are presented in *Table 5*.

Table 5 - Therapeutic level for the AEDs

AED	Tested concentrations (mol/dm^3)	Tested concentrations (mg/L)	Therapeutic level (mg/L)	Ref
CARBAMAZEPINE	10^{-8} - 10^{-4}	0,00236 – 23,6	4 – 12	(56)
GABAPENTIN	10^{-8} - 10^{-4}	0,00171 – 17,1	2 – 20	(57)
LAMOTRIGINE	10^{-8} - 10^{-4}	0,00256 – 25,6	3 – 14	(57)
TOPIRAMATE	10^{-8} - 10^{-4}	0,00336 – 33,6	5 – 20	(57)
VALPROIC ACID	10^{-8} - 10^{-4}	0,00144 – 14,4	50 – 100	(56)

Therapeutic level within the range of tested concentrations

Part of the therapeutic level within the range of tested concentrations

Therapeutic level out of the range of tested concentrations

Looking at *Table 5*, it is possible to understand that the therapeutic levels of all the tested AEDs, except valproic acid, are within the range of tested concentrations. The therapeutic levels represent the AED serum range and it is important to note that bone concentrations might be lower. However, we also tested the cells for concentrations lower than the therapeutic level, which may reflect bone concentrations of these drugs.

Taken together, the present results revealed that the tested AEDs interfere with bone cells development, at doses that are below the plasma levels found in patients undergoing AEDs therapeutics.

AED-induced disturbances of bone integrity are mainly influenced by the type, dosage and duration of the AED therapy. A dose-dependent increase in the risk of fractures was particularly observed during therapy with carbamazepine (33). Several studies have concluded that AEDs such as carbamazepine have long been known to have an adverse effect on bone health (58, 59) which maybe can be justified by the decrease on osteoclastogenesis and osteoblastogenesis, observed in our study. In addition to the negatively modulation of these processes, Lee R. H. , *et al* (60) also summarized that carbamazepine led to increased metabolism of 25-hydroxyvitamin D to inactive metabolites, which resulted in metabolic bone disease. Thus, AEDs seem not only to affect bone cells, but also other molecules involved in bone remodeling process. In 2008, this fact was also documented by Bortolini L., *et al* (61). They suggested that the AEDs may interfere in the mineral bone metabolism by different mechanisms including direct effects over the bone cells, changes in the metabolism and/or in the action of the vitamin D in target organs, hypocalcemia and actions over the sexual steroids. Besides, Feldkamp J., *et al* (62) suggested that AEDs are recognized to have direct effects on bone cells, inhibiting the bone formation process, which supports our results. In their experimental studies, Feldkamp J., *et al* (62)also concluded that carbamazepine revealed no proliferation stimulating properties, which supports our results obtained by MTT assay: for concentrations lower than 10^{-5} M, carbamazepine did not significantly affect the cellular proliferation. In a study performed by Valimaki M., *et al* (63) they concluded that antiepileptic treatment with carbamazepine accelerates skeletal turnover. They also indicated that AEDs may directly affect the function of bone cells, in *in vitro* studies, which supports our results. Despite several studies have concluded that carbamazepine negatively affect bone metabolism, Sheth R. (18) summarized that carbamazepine has not been shown to have a predictable effect on bone mineral density.

According to previous studies (38, 43, 59) gabapentin therapy may cause bone loss, which can be justified by the results obtained in our study: as osteoclastogenesis is affected, bone remodeling does not occur properly. Gabapentin is also not free from this important adverse effect (59). However, only a few studies on this drug were performed.

Data regarding lamotrigine effects on bone is mixed (64). Other studies indicated that lamotrigine monotherapy may not have significant impact on bone

metabolism, which does not support our results (24, 65). However Sheth R. (18) and Verrotti A. (59) indicated that lamotrigine was associated with reduced bone formation, which supports our results. Osteoblasts are bone-forming cells and its function was decreased in the presence of this drug, as it is possible to see in *Figure 9*.

Topiramate effects on bone were the subject of a limited amount of studies (64). Topiramate also does not seem to be associated with alterations of bone metabolism (58, 59), however, a recent study demonstrated that long-term topiramate treatment could have adverse effect on bone, increasing bone turnover (39), that supports our results.

Concerning the effects of valproic acid on bone metabolism, our results are compatible with previous publications (28, 59, 66-69), and disagree with some others (35, 70, 71). Zare M., *et al* concluded that their findings indicate that bone mineral metabolism may not be affect by valproic acid. They admitted that real causes of discrepancies among different studies are not clear, but a long list of different factors could be suspected. Age, duration of treatment, type of concurrent drugs used, lifestyle, and socioeconomic and geographic conditions are some of the factors that can influence valproic acid's adverse bone effects (70). Either a recent study performed by Triantafyllou N., *et al* (35) showed no correlation between valproic acid monotherapy and bone mineral density measurements. However, Verrotti A., *et al* (66) have reported that markers of bone formation such as ALP have been significantly increased with this drug, which is compatible with the results obtained for ALP activity. An *in vitro* study performed by Hatakeyama Y., *et al* (67) cell morphology alterations were examined observing F-actin-stained cytoskeleton content at various concentrations of valproic acid. That data suggests that valproic acid was able to maintain normal cell shape, which supports our results obtained for osteoblastic cell cultures assessed by CSLM. No changes in cell morphology were observed. Moreover, they also performed a cell proliferation test concluding that the effect of valproic acid on cell proliferation is dependent on the maturation level of the cells, namely that immature cells have resistance to high concentrations of valproic acid. Another study performed by Sato Y., *et al* (72) suggested that valproic acid directly acts over the osteoblasts, which also corroborates our results. Also Samaniego E. *et al* (30) agreed that AEDs may affect bone density by possible altering osteoblastic function and Valsamis H., *et al* (69)

refered that valproic acid may cause an imbalance between bone formation and resorption, contributing to bone loss. A study referenced by Tsiropoulos I., *et al* (73) concluded that this drug enhance osteoblast viability and differentiation in low concentrations. Results obtained for ALP activity were in conform with this conclusion, because valproic acid at lower doses caused an increase of ALP activity. Sheth R. (18) suggested that bone turnover increased with valproic acid, and we can justified this with the increased observed in our results for ALP activity. Considering these results, it is possible to think that, if valproic acid increase ALP activity, it means that, probably this drug might stimulate the osteoblastogenesis process. However, we should consider that excess remodeling reduces bone mass, while suppressed bone remodeling increases it. Bone strength is determined not only by mass but by bone quality. Suppressed bone remodeling likely reduces bone quality due to accumulation of micro fractures and reduced restructuring of bone architecture (14). So, even though we have obtained an increase on ALP activity in the presence of valproic acid, it is important to keep in mind that, for the same concentration that caused this increase, we observed a decreased of TRAP activity. Therefore, a proper balance between bone formation and bone resorption may not exist, which makes this increase an adverse effect. According Sheth R., *et al* (74) the pathogenesis of valproic acid-associated reduction in bone mineral density remains undefined. This drug does not interfere with the absorption of either calcium or phosphorus. Thereafter, the most likely reason for the negative impact of valproic acid on bone metabolism seems to be the direct effect on bone cells, as our study proves. They also suggested that valproic acid negatively affect bone metabolism by stimulating osteoclast activity and causing imbalance between bone formation and resorption.

Considering the results obtained with co-cultures results, some differences were found comparatively to the results obtained for PBMC and osteoblastic cell cultures, suggesting that AEDs might also indirectly modulate the osteoclastogenic and osteoblastogenic properties of osteoblasts and osteoclasts, respectively. These differences can be justified by the osteoclast-osteoblast communication, as described by Matsuo K., *et al* (14) “Osteoclasts and cells in the osteoblast lineage can communicate through cell-cell contact to achieve “coupling” of bone resorption to formation. (…) Initiation of osteoclastogenesis largely depends on interaction between osteoclasts

precursors and cells in the osteoblast lineage." In our study we cannot say how they influence each other, but we observed that it happens.

Regarding the intracellular mechanisms involved in osteoclast and osteoblast response to AEDs, no studies were found with *in vitro* assays with signaling pathways. Thus, our study is the first one that goes into this issue.

Fitzpatrick L. (41) resumed some information about *in vitro* studies of AEDs. Although the studies summarized were just about phenytoin, a drug that was not used in our study, some conclusions are important. Not only with the drugs that we study, but also with phenytoin the mechanism(s) of drug-induced bone loss remains unexplained and appears to be multifactorial. The *in vitro* studies performed with this drug also reported that at micro molar concentrations phenytoin interfere with proliferation and differentiation of bone cells and may act on the resorption side of the bone remodeling process. This drug seemed to inhibit bone resorption at high doses and may have a more direct action on bone formation. Fitzpatrick L. (41) suggested that the discrepancy in findings of *in vitro* studies may be due to the higher doses of drug that are required to inhibit bone formation, whereas lower doses may inhibit bone resorption. Besides, it was suggested a toxic effect on bone formation at doses that are given to patients. Inhibition of osteoblast-like cells could impair bone formation, leading to bone loss.

We should keep in mind that unraveling the mechanisms beneath these observations might help to explain the adverse effects on bone tissue described for this drug class.

According Pack A., *et al* (52) several theories have been proposed to explain the link between AEDs and bone disease. Possible mechanisms include: a) hepatic induction of the cytochrome P450 leading to increased catabolism of vitamin D; b) a direct effect on bone cells, including an impairment of absorption of calcium and inhibition of response to PTH; c) hyperparathyroidism and d) calcitonin deficiency. In general, literature affirms that cytochrome P450 enzyme-inducing AEDs (such as carbamazepine) are most commonly associated with a negative impact on bone instead of the new-generation AEDs (such as gabapentin, lamotrigine and topiramate). However, no standard were found.

Taken together, the published studies were performed in different experimental models, of different origins and mainly *in vivo* models, which can help to explain the

contradictions found among the data obtained. Besides, Beerhorst K., *et al* (68) also refers that there are several AEDs on the market, but long-term follow-up of possible side effects on bone is still lacking.

CHAPTER 6 – Conclusion and future directions

6.1 Conclusion

Several risk factors have been suggested to contribute to bone disease in patients with epilepsy: polytherapy, age above 50 years, use of AEDs for more than 2 years and use of enzyme-inducing AEDs (37). Multiple therapies for bone disease are available including high-dose vitamin D supplementation (improved bone mineral metabolism), calcium supplementation (slowed the rate of bone loss), bisphosphonates (potent inhibitors of bone resorption), hormone replacement therapy (efficient in stopping bone loss but with side effects), selective estrogen receptor modulators (increased bone mass and reduces the risk of fractures), calcitonin (reduced the rate of fractures) and vitamin K supplementation (retardation of bone loss). However, international guidelines concerning screening for osteoporosis in people with epilepsy have not been established due to the lack of convincing studies. Triantafyllou N., *et al* (35) performed an *in vivo* study to evaluate the effect of long-term valproate monotherapy on BMD concluding that there are no correlation between duration or dosage of valproate monotherapy with BMD measurements. Moreover, scores were similar between patients and age and sex-matched controls. Thus, studies of the effect of AEDs on bone metabolism have yielded contradictory results.

A recent study performed by Beerhorst K., *et al* (37) strongly suggest that long-term AED use is associated with an extreme high prevalence of osteoporosis and osteopenia. The prevalence rate of low BMD of 80% in this study underscores the need for more bone health awareness in patients with epilepsy. Taking into account our and other studies that corroborate this relation between AEDs and bone metabolism, maybe the early screening, careful guidance of lifestyle factors, supplementation of vitamin D and calcium, and a critical or even restrictive attitude towards long-term treatment with a high drug load of AEDs should be justified.

The adverse metabolic effects of AEDs are probably currently underestimated, and thus represent an area of legitimate concern.

A long way still has to be traversed. More *in vitro* studies must be performed, to help in the understanding of direct effect of AEDs on bone cells.

More studies are needed and the newer AEDs may be less likely to have a negative impact on bone, although this remains to be studied too. Physicians and epileptologists

should consider the endocrine effects of AEDs and concerns about epilepsy itself. It is important to monitor blood parameters of metabolism, like bone markers (51). It is imperative to act on the improvement of the quality of life of patients and their health status in general. Neurologists often do not think about bone health, although there may be good reason to (75).

The aims of this study were fulfilled. The effects of AEDs in human bone cells were studied, firstly, by the focus on the influence of different concentrations of five different AEDs in the osteoclastogenic and osteoblastogenic process. AEDs differently modulated the osteoclastic and the osteoblastic behavior of PBMC, osteoblasts and co-cultures of both cell types. With the purpose of had some insights on the mechanisms of action of AEDs on bone cells, the influence of several signaling pathways on cellular response was performed.

In summary, this work provides new insights on the *in vitro* effects of AEDs on human bone cells and opens the doors towards a better understanding about this up till now poorly clarified issue.

6.2 Future directions

In the future, we should try to introduce other molecules, for example vitamin K2. In the review written by Plaza S., *et al* (76) it stated that vitamin K2 exerts a powerful influence on bone building and, thus, may be regarded as a potential partner in AEDs therapeutics, in order to counteract deleterious effects in bone tissue.

Another idea is to perform, at the same time, a clinical and an experimental study, analyzing patients and their cells. The same strategy was used by Feldkamp J., *et al* (62).

Furthermore, the duration of the study should be expanded, so that we can evaluate long-term effects. It is important to keep in mind that, during an *in vitro* study, it's possible to follow-up in real time the direct effects of AEDs on bone cells. These micro effects probably may take years to reveal symptoms, to be evidenced by our body. Regarding the referenced *in vivo* studies that affirmed that there is no relationship between AEDs and bone metabolism, this could be the proper reason: If they performed a 2 years follow-up, AEDs already started to change bone cells however, these changes

are not visible through bone mineral density yet. Also, Samaniego E., *et al* (30) also suggested that in mild cases, patients are asymptomatic and have normal bone mass.

It will also be interesting to try to test bone cells with combined AEDs. Tsiropoulos I., *et al* (73) declared that polytherapy was associated with a higher risk than monotherapy.

In order to have a more accurate perspective of the effects of AEDs on human bone metabolism, it is imperative to evaluate the way human bone cells behave in the presence of those molecules. There is no data regarding this issue so far. On the other hand, it is important to note that this study represents only the first step towards a physiological understanding of how AEDs may affect bone tissue. A correlation to the clinical situation cannot be directly predicted, due to the complex *in vivo* environment.

REFERENCES

1. Datta HK, Ng WF, Walker JA, Tuck SP, Varanasi SS. The cell biology of bone metabolism. Journal of Clinical Pathology. 2008;61:577-87.
2. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Archives of Biochemistry and Biophysics. 2008;473:139–46.
3. Guntur AR, Rosen CJ. Bone as an Endocrine Organ. Center for Clinical and Translational Research.1-15.
4. Salgado AJ, Coutinho OP, Reis RL. Bone Tissue Engineering: State of the Art and Future Trends. Macromolecular Bioscience 2004;4:743-65.
5. Kneser U, Schaefer DJ, Polykandriotis E, Horch RE. Tissue Engineering of bone: the reconstructive surgeon's point of view Journal of Cellular and Molecular Medicine 2006;10(1):7-19
6. Seeley, Stephens, Tate. Chapter 6: Skeletal System: Bones and Bone Tissue. Anatomy and Physiology. 6th ed: The McGraw−Hill Companies; 2004. p. 167-89.
7. Damjanov I. Chapter 19: Bones and Joints. Pathology for the Health Professions 4th ed: Elsevier Saunders 2012. p. 411-32.
8. Hill PA. Bone Remodeling British Orthodontic Society 1998;25:101-7.
9. Zuo C, Huang Y, Bajis R, Sahih M, Li Y-P, Dai K, et al. Osteoblastogenesis regulation signals in bone remodeling Osteoporosis International 2012;23:1653-63.
10. Robling AG, Castillo AB, Turner CH. Biomechanical and Molecular Regulation of Bone Remodeling Annual Review of Biomedical Engineering 2006;8:455-98.
11. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003;423:337-42.
12. Jensen ED, Gopalakrishnan R, Westendorf JJ. Regulation of gene expression in osteoblasts BioFactors 2010;36(1):25-32.
13. Sims NA, Gooi JH. Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption. Seminars in Cell & Developmental Biology 2008;19:444-51.
14. Matsuo K, Irie N. Osteoclast-osteoblast communication. Archives of Biochemistry and Biophysics. 2008;473:201-9.
15. Koide M, Kinugawa S, Ninomiya T, Mizoguchi T, Yamashita T, Maeda K, et al. Diphenylhydantoin Inhibits Osteoclast Differentiation and Function Through Suppression of NFATc1 Signaling. JOURNAL OF BONE AND MINERAL RESEARCH. 2009;24(8):1469-80.
16. NICHOLSON GC, MALAKELLIS M, COLLIER FM, CAMERON PU, HOLLOWAY WR, GOUGH TJ, et al. Induction of osteoclasts from CD14-positive human peripheral blood mononuclear cells by receptor activator of nuclear factor kB ligand (RANKL). Clinical Science. 2000;99:133-40.
17. Sila-Asna M, Bunyaratvej A, Maeda S, Kitaguchi H, Bunyaratavej N. Osteoblast Differentiation and Bone Formation Gene Expression in Strontium-inducing Bone Marrow Mesenchymal Stem Cell Kobe Journal of Medical Sciences. 2006;53(1):25-35.
18. Sheth RD. Bone health in pediatric epilepsy. Epilepsy & Behavior. 2004;5:S30-S5.
19. Lo¨scher W, Schmidt D. New horizons in the development of antiepileptic drugs. Epilepsy Research. 2002;50:3-16.
20. Phabphal K, Geater A, Limapichart K, Sathirapanya P, Setthawatcharawanich S, Natthawan Witeerungrot, et al. The association between BsmI polymorphism and bone mineral density in young patients with epilepsy who are taking phenytoin. Epilepsia. 2013;54(2):249-55.
21. Nakken KO. Adverse Metabolic Effects of Antiepileptic Drug Treatment Novel Treatment of Epilepsy 2011:83-94.
22. Elliott JO, Jacobson MP, Haneef Z. Homocysteine and bone loss in epilepsy. Seizure. 2007;16:22-34.
23. Petty SJ, O'Brien TJ, Wark JD. Anti-epileptic medication and bone health. International Osteoporosis Foundation and National Osteoporosis Foundation. 2007;18:129–42.
24. Meier C, Kraenzlin M. Antiepileptics and bone health. Therapeutic Advances in Musculoskeletal Disease. 2011;3(5):235-43.
25. Rauchenzauner M, Griesmacher A, Tatarczyk T, Haberlandt E, Strasak A, Zimmerhackl L-B, et al. Chronic antiepileptic monotherapy, bone metabolism, and body composition in non-institutionalized children. Developmental Medicine & Child Neurology. 2009;52:283–8.

26. Mattson RH, Gidal BE. Fractures, epilepsy, and antiepileptic drugs. Epilepsy & Behavior. 2004;5:S36–S40.
27. Sheth RD, Harden CL. Screening for bone health in epilepsy. Epilepsia. 2007;48(9):39-41.
28. Gniatkowska-Nowakowska A. Fractures in epilepsy children. Seizure. 2010;19:324-5.
29. Drezner MK. Treatment of anticonvulsant drug-induced bone disease. Epilepsy & Behavior. 2004;5:S41-S7.
30. Samaniego EA, Sheth RD. Bone Consequences of Epilepsy and Antiepileptic Medications. Seminars in Pediatric Neurology. 2007:196-200.
31. Pack A, Gidal B, Vazquez B. Bone disease associated with antiepileptic drugs. Cleveland Clinic Journal of Medicine. 2004;71:S42-S8.
32. Nicholas JM, Ridsdale L, Richardson MP, Grieve AP, Gulliford MC. Fracture risk with use of liver enzyme inducing antiepileptic drugs in people with active epilepsy: Cohort study using the General Practice Research Database Seizure. 2013;22:37-43.
33. Grober U, Kisters K. Influence of drugs on vitamin D and calcium metabolism Dermato-Endocrinology 2012;4(2):158-66.
34. Pack AM, Morrell MJ. Epilepsy and bone health in adults. Epilepsy & Behavior. 2004;5:S24–S9.
35. Triantafyllou N, Lambrinoudaki I, Armeni E, Evangelopoulos E-M, Boufidou F, Antoniou A, et al. Effect of long-term valproate monotherapy on bone mineral density in adults with epilepsy. Journal of the Neurological Sciences 2010 290:131-4
36. Ecevit Çd, Aydog˘an A, Kavakli T, Altinöz S. Effect of Carbamazepine and Valproate on Bone Mineral Density. Pediatric Neurology. 2004;31(4):279-82.
37. Beerhorst K, Tan I, Krom MD, Verschuure P, Aldenkamp A. Antiepileptic drugs and high prevalence of low bone mineral density in a group of inpatients with chronic epilepsy. Acta Neurologica Scandinavica. 2013:1-8.
38. Pack AM. Treatment of Epilepsy to Optimize Bone Health. Current Treatment Options in Neurology. 2011;13:346–54.
39. Heo K, Rhee Y, Lee HW, Lee SA, Shin DJ, Kim W-J, et al. The effect of topiramate monotherapy on bone mineral density and markers of bone and mineral metabolism in premenopausal women with epilepsy. Epilepsia. 2011;52(10):1884–9.
40. Chen L-A, Cheng S-J, Jou S-B. Epilepsy in the Elderly. International Journal of Gerontology. 2012;6:63-7.
41. Fitzpatrick LA. Pathophysiology of bone loss in patients reveiving anticonvulsant therapy. Epilepsy & Behavior. 2004;5:S3-S15.
42. Costa-Rodrigues J, Moniz KA, Teixeira MR, Fernandes MH. Variability of the Paracrine-Induced Osteoclastogenesis by Human Breast Cancer Cell Lines. Journal of Cellular Biochemistry. 2012;113:1069–79.
43. Andress DL, Ozuna J, Tirschwell D, Grande L, Johnson M, Jacobson AF, et al. Antiepileptic Drug-induced Bone Loss in Young Male Patients Who Have Seizures Archives of Neurology 2002;59:781-6.
44. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry Journal 1976;72:248-54.
45. Costa-Rodrigues J, Fernandes A, Fernandes MH. Reciprocal Osteoblastic and Osteoclastic Modulation in Co-Cultured MG63 Osteosarcoma Cells and Human Osteoclast Precursors. Journal of Cellular Biochemistry. 2011;112:3704–13.
46. YAMAGUCHI A, KOMORI T, SUDA T. Regulation of Osteoblast Differentiation Mediated by Bone Morphogenetic Proteins, Hedgehogs, and Cbfa1. Endocrine Reviews. 2000 21(4):393-411.
47. Costa-Rodrigues J, Teixeira CA, Sampaio P, Fernandes MH. Characterisation of the Osteoclastogenic Potential of Human Osteoblastic and Fibroblastic Conditioned Media. Journal of Cellular Biochemistry. 2010;216:205-16.
48. Costa-Rodrigues J, Reis S, Teixeira S, Lopes S, Fernandes MH. Dose-dependent inhibitory effects of Proton pump inhibitors on human osteoclastic and osteoblastic cells activity. The FEBS Journal 2013:[Epub ahed of print]
49. Kruger NJ. The Bradford method for protein quantitation. Methods in Molecular Biology 1994;32:9-15.

50. Lian JB, Stein GS, Javed A, Wijnen AJv, Stein JL, Montecino M, et al. Networks and hubs for the transcriptional control of osteoblastogenesis. Reviews in Endocrine and Metabolic Disorders. 2006;7(1-2):1-16.
51. Luef G, Rauchenzauner M. Epilepsy and hormones: A critical review. Epilepsy & Behavior. 2009;15:73-7.
52. Pack AM, Morrell MJ. Adverse Effects of Antiepileptic Drugs on Bone Structure: Epidemiology, Mechanisms and Therapeutic Implications. CNS Drugs. 2001;15(8):633-42.
53. Ballanti P, Minisola S, Pacitti MT, Scarnecchia L, Rosso R, Mazzuoli GF, et al. tartrate-resistant acid phosphate activity as osteoclastic marker: sensitivity of cytochemical assessment and serum assay in comparison with standardized osteoclast histomorphometry. Osteoporosis International 1997;7(1):39-43.
54. RocheAppliedScience. B: Cell Proliferation and Viability. Apoptosis, Cell Death and Cell Proliferation Manual. 3rd ed: Roche p. 74-109.
55. Singer FR, Eyre DR. Using biochemical markers of bone turnover in clinical practice. CLEVELAND CLINIC JOURNAL OF MEDICINE. 2008;75(10):739-48.
56. Shakya G, Malla S, Shakya KN, Shrestha R. Therapeutic Drug Monitoring of Antiepileptic Drugs. Journal of Nepal Medical Association. 2008;47(171):94-7.
57. Krasowski MD. Therapeutic Drug Monitoring of the Newer Anti-Epilepsy Medications. Pharmaceuticals. 2010;3:1909-35.
58. Koo DL, Joo EY, Kim D, Hong SB. Effects of levetiracetam as a monotherapy on bone mineral density and biochemical markers of bone metabolism in patients with epilepsy. Epilepsy Research. 2013;104:134-9.
59. Verrotti A, Coppola G, Parisi P, Mohn A, Chiarelli F. Bone and calcium metabolism and antiepileptic drugs. Clinical Neurology and Neurosurgery. 2010;112:1–10.
60. Lee RH, Lyles KW, Colón-Emeric C. A Review of the Effect of Anticonvulsant Medications on Bone Mineral Density and Fracture Risk The American Journal of Geriatric Pharmacotherapy. 2010;8(1):34-46.
61. Bortolini LGC, Kulak CAM, Boguszewski CL. Efeitos Endócrinos e Metabólicos dos Antiepilépticos Journal of Epilepsy and Clinical Neurophysiology 2008;14(2):32-8.
62. Feldkamp J, Becker A, Witte OW, Scharff D, Scherbaum WA. Long term anticonvulsant therapy leads to low bone mineral density evidence for direct effects of phenytoin and carbamazepine on human osteoblast-like cells Experimental and Clinical Endocrinology & Diabetes. 2000;108:37-43.
63. VALIMAKI MJ, TIIHONEN M, LAITINEN K, TAHTELA R, KARKKAINEN M, LAMBERG-ALLARDT C, et al. Bone Mineral Density Measured by Dual-Energy X-ray Absorptiometry and Novel Markers of Bone Formation and Resorption in Patients on Antiepileptic Drugs. JOURNAL OF BONE AND MINERAL RESEARCH. 1994;9(5):631-7.
64. Pack A. Bone health in people with epilepsy: Is it impaired and what are the risk factors? Seizure. 2008;17:181-6.
65. Pack AM, Morrel MJ, Marcus R, Holloway L, Flaster E, Done S, et al. Bone mass and turnover in women with epilepsy on antiepileptic drug monotherapy. Annals of Neurology 2005;57:252-7.
66. Verrotti A, Agostinelli S, Coppola G, Parisi P, Chiarelli F. A 12 month longitudinal study of calcium metabolism and bone turnover during valproate monotherapy. The Journal of the Neurological Sciences. 2010 290:131-4.
67. Hatakeyama Y, Hatakeyama J, Takahashi A, Oka K, Tsuruga E, Inai T, et al. The Effect of Valproic Acid on Mesenchymal Pluripotent Cell Proliferation and Differentiation in Extracellular Matrices. Drug Target Insights 2011;5:1-9.
68. Beerhorst K, Huvers FC, Renier WO. Severe early onset osteopenia and osteoporosis caused by antiepileptic drugs. The Netherlands Journal of Medicine 2005;63:222-6.
69. Valsamis HA, Arora SK, Labban B, MacFarlane SI. Antiepileptic drugs on bone metabolism Nutrition and Metabolism 2006;3(36):1-11.
70. Zare M, ghazvini MRA, Dashti M, Najafi MR, Alavi-Naeini AM. Bone turnover markers in epileptic patients under chronic valproate therapy. Journal of Research in Medical Sciences. 2013;18:338-40.
71. Fuleihan GE-H, Dib L, Yamout B, Sawaya R, Mikati MA. Predictors of bone density in ambulatory patients on antiepileptic drugs. Bone. 2008;43:149–55.
72. Sato Y, Kondo I, Ishida S, Motooka H, Takayama K, Tomita Y, et al. Decreased bone mass and increased bone turnover with vaproate therapy in adults with epilepsy. . Neurology. 2001;57:445-9.

73. Tsiropoulos I, Andersen M, Nymark T, Lauritsen J, Gaist D, Hallas J. Exposure to antiepileptic drugs and the risk of hip fracture: A case-control study. Epilepsia. 2008;49(12):2092–9.
74. Sheth RD, Wesolowski CA, Jacob JC, Penney S, Hobbs GR, Riggs JE, et al. Effect of carbamazepine and valproate on bone mineral density The Journal of Pediatrics. 1995;127(2):256-62.
75. Gidal B, Sheth RD. Epilepsy and bone health: is there cause for concern? Epilepsy & Behavior. 2004;5 1-2.
76. Plaza SM, Lamson DW. Vitamin K2 in Bone Metabolism and Osteoporosis. Alternative Medicine Review. 2005;10(1):24-33.