Doctoral Thesis

Cholinergic regulation of bone turnover: hip fracture risk in Alzheimer patients treated with acetylcholinesterase inhibitors.

Iskandar Ahmad Tamimi Mariño

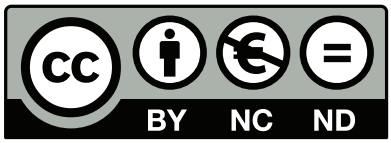
Málaga, 16 de Mayo 2014

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

AUTOR: Iskandar Tamimi Mariño

EDITA: Publicaciones y Divulgación Científica. Universidad de Málaga



Esta obra está sujeta a una licencia Creative Commons:
Reconocimiento - No comercial - SinObraDerivada (cc-by-nc-nd):
Http://creativecommons.org/licences/by-nc-nd/3.0/es
Cualquier parte de esta obra se puede reproducir sin autorización
pero con el reconocimiento y atribución de los autores.
No se puede hacer uso comercial de la obra y no se puede alterar, transformar o hacer obras derivadas.

Esta Tesis Doctoral está depositada en el Repositorio Institucional de la Universidad de Málaga (RIUMA): riuma.uma.es

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Index:

Abbreviations…… 5

I Introduction …...……...10

1. General aspects……11

2. Background……..13

2.1 The autonomic nervous system……..13

2.1.1 Structure and distribution……13

2.1.2 Neurotransmitter receptors……..16

2.1.3 Types of adrenergic receptors…….20

2.1.3.1 Alfa receptors…….……..21

2.1.3.2 Beta receptors……...22

2.1.3.3 Adrenergic receptors and bone tissue……...23

2.1.4. Types of cholinergic receptors…….....27

2.1.4.1 Muscarinic receptors…..……...28

2.1.4.2 Nicotinic receptors……...31

2.1.4.3 Cholinergic receptors and bone tissue……..35

2.2 Bone remodeling……37

2.2.1 Systemic regulation of bone remodeling……40

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.2.1.1 The hypothalamic-pituitary-axis……..40

2.2.1.2 Energy-bone-adipose-tissue-adrenergic sympathetic nervous system axis…….46

2.2.1.3 The parathyroid-vitamin D-calcitonin axis……..50

2.2.1.4 The bone interleukin1-cholinergic sympathetic nervous system axis……..52

2.2.2 Local regulation of bone remodeling……...……54

2.2.2.1 Wolff´s law and bone remodeling……54

2.2.2.2 Piezoelectic charges…….55

2.2.2.3 Hueter-Volkmann law……..56

2.2.2.4 Bone signaling and receptor activator of nuclear factor kappa-B ligand ……..57

2.3 Bone abnormalities associated with alterations in the autonomic nervous system…….60

2.4 Pathology of bone remodeling: osteoporosis…….……64

2.4.1 The pathophysiology of osteoporosis……..65

2.4.2 Risk factors……..67

2.4.3 Osteoporotic fractures…….69

2.4.7 Hip fractures…….……...70

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.4.7.1 Intracapsular hip fractures……70

2.4.7.2 Extracapsular hip fractures……...73

2.4.8 Pharmacology of osteoporosis “medication subgroups”….74

2.4.8.1 Bisphosphonates……...75

2.4.8.2 Vitamin-D supplements and calcium ……..76

2.4.8.3 Estrogen replacement therapy……..76

2.4.8.4 Salmon calcitonin…….76

2.4.8.5 Parathyroid hormone derivatives…...……...77

2.4.8.6 Monoclonal immunoglobulins…….77

2.5 Alzheimer disease …….77

2.5.1 Epidemiology……...…77

2.5.2 Classification……...78

2.5.3 Pharmacology of Alzheimer disease ……..……79

2.5.4 Acetylcholinesterase inhibitors……...……79

2.5.5 Memantine……...…82

II Hypothesis……82

III Materials and Methods…….……..86

1. Demographic information of the study population……..……87

2. Exposure assessment and statistical analysis…….……88

IV Results ……...90

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

1. Demographic information……91

2. Hip fracture incidence in the general population and Alzheimer patients..…91

3. Assessment of risk factors in cases and controls…..…….……100

4. Comparison between different acetylcholinesterase inhibitors……..…107

V Discussion……..……112

1. General aspects……..……113

2. Methodological considerations……..……117

3. Most relevant aspects……..…119

4. General conclusions……...122

5. Limitations…….……124

VI Conclusion……128

VII References……...……130

VIII Article I.....…..162

VIII Article II…….…….....172

IX Thesis Summary in Spanish…….....…...184

X Addendum……..212

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Abbreviations:

Acetylcholinesterase: AChE

Acetylcholinesterase inhibitors: AChEIs

Acetylchoine receptor: AChR

Adenosine triphosphate: ATP

Adrenocorticotropic hormone: ACTH

Alkaline phosphatase: ALP

Alzheimer disease: AD

Autonomic nervous system: ANS

Basic multicellular unit: BMU

Bio-med central: BMC

Blessed dementia scale: BDS

Body mass index: BMI

Bone mineral density: BMD

Bone morphogenetic proteins: BMP

Bone morphogenetic protein receptor: BMPR

Charlson comorbidity score: CCS

Central nervous system: CNS

Cholera toxin: CTX

Colony forming unit: CFU

Clinical dementia rating scale: CDR

Confidence interval: CI

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Corticoid releasing hormone: CRH

Cyclic adenosine 3, 5 monophosphate; cAMP

Dihydrotestosterone: DHT

Drosophila protein, mothers against decapentaplegic: SMAD

Dual energy X-ray absorptiometry: DEXA

Estrone 1,2, 3: E1 E2 E3

Excitatory postsynaptic potential: EPSP

Falls risk assessment tool: FRAT

Follicle stimulating hormone: FSH

Gamma-Aminobutyric acid: GABA

Gonadotropin releasing hormone: GnRH

Growth hormone: GH

Growth hormone releasing hormone: GHRH

Guanosine triphosphate hydrolase enzyme: GTPase

Hormone replacement therapy: HRT

Human osteosarcoma derived cells: SaOS-2, HOS, MG6

Human periosteum derived osteoblastic cells: SaM-1

Inhibitory postsynaptic potential: IPSP

Inositol trisphosphate: IP3

Insulin-like growth factor: IGF

Interleukin: IL

Kilodalton: KDa

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Leutinizing hormone: LH

Mesenchymal cells: MSCs

Messenger ribonucleic acid: mRNA

Mini-mental state examination: MMSE

Nicotinic acetylcholine receptors: nAChR

N-methyl-D-aspartic acid antagonists: NMDA

Odd ratio: OR

Osteoprotegerin: OPG

Parasympathetic nervous system: PSNS

Parathyroid hormone: PTH

Parathyroid hormone related peptide (PTHrp)

Paraventricular nucleus of the hypothalamus: PVN

Peripheral nervous system: PNS

Pertussis toxin: PTX

Platelet derived growth factor: PDGF

Polymerase chain reaction: PCR

Protein kinase A: PKA

Protein kinase C: PKC

Receptor activator of nuclear factor kappa-B ligand: RANKL

Reverse transcription polymerase chain reaction: RT-PCR

Selective serotonin reuptake inhibitor: SSRI

Sympathetic nervous system: SNS

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Thiazolidinediones: TZD

Thyroxin releasing hormone: TRH

Thyroxin stimulating hormone: TSH

Triiodothyronine-3: T3

Thyroxin-4: T4

Transforming growth factor β: TGF- β

Tumor necrosis factor α: TNF-α

United States: US

World Health Organization: WHO

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Introduction

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

1. General aspects

Clinical conditions and drugs that alter the acetylcholine signaling system have been found to have interesting effects on bone function (Walker et al, 2001) (Hoogduijn et al, 2009) (En-Nosse et al, 2009) (Warner et al, 2006) (Grimston et al, 2007) (Alkondon et al, 2000) (Hollinger et al, 1999) (Glowacki et al, 2008) (Warner et al, 2006) (Kliemann et al, 2012) (Bajayo et al, 2012) (Kondo et al, 2013) (Pouwels et al, 2013). At the pre-synaptic level, botulinum neurotoxin inhibits the release of acetylcholine from motor neurons, and has been found to impair bone healing and induce a decrease in bone mineral content. However, bone density does not improve after the recovery of muscle function, indicating an effect of botulinum toxin on bone tissue, unrelated to muscle function (Glowacki et al, 2008) (Warner et al, 2006). Poliomyelitis destroys motor neurons that use acetylcholine neurotransmitters. Patients suffering from Polio experience impaired bone growth in the limbs affected by nerve depletion. However, many years after recovery of muscle activity, polio patients tend to develop osteoporosis in a much larger proportion than the rest of the population (Mohammad et al, 2009).

At the postsynaptic level, smokers are well known to have an impaired bone healing, in part due to the effect of nicotine (Alkondon et al, 2000) (Glowacki et al, 2008). Smokers have high concentrations of nicotine in blood that interacts with the nicotinic acetylcholine receptor (nAChR) causing a desensitization of these receptors. All these observations suggest that acetylcholine and its receptors in bone tissue may play certain roles in the process of bone remodeling (Alkondon et al, 2000) (Glowacki et al, 2008).

Recent research has revealed the presence of a number of acetylcholine receptors (AChR) subunits in bone cells. Nicotinic subunits have been detected in human primary bone cells, mesenchymal stem cells (MSCs) and osteoblasts, while muscarinic receptors

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

have been detected in MSCs. Moreover, it has been found that osteoblasts express specific AChR and cholinergic components that may play a possible role in regulating the alkaline phosphatase (ALP) activity as well as the proliferation and differentiation of osteoblasts (Walker et al, 2001) (Hoogduijin et al, 2009) (En-Nosse et al, 2009) (Alkondon et al, 2000) (Sato et al, 2010).

Altogether, the available literature seems to indicate that the inhibition of the AChR at the bone level seems to cause a reduction in bone turnover. However, it is not clear whether or not boosting up of the acetylcholine activity might have an anabolic effect on bone formation (Ma et al, 2010).

Acelycholinesterase inhibitors (AChEIs) are a group of drugs that cause AChR stimulation by inhibiting the action of AChE and increasing the levels of acetylcholine in the synaptic space. These drugs have been widely used in the treatment of Alzheimer's disease (AD) and other dementias since the mid nineties (Pepeu et al, 2009) (Massoud et al 2010).

We hypothesize that the administration of AChEIs may have a beneficial effect on bone turnover that could translate into a reduction in the bone fracture risk. In order to test this hypothesis we have designed a retrospective case-control study in which we compare the incidence of hip fractures in a group of AD patients under AChEIs treatment, with AD patients receiving no AChEIs. But before discussing the methodological details and results of this study and in order to reach a better understanding of this thesis we will undergo a focused review of some fundamental principles of bone remodeling, the autonomic nervous system (ANS), osteoporosis and Alzheimer disease.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2. Background

2.1 The autonomic nervous system

2.1.1 Structure and distribution

The central nervous system (CNS) includes the brain in the cranial cavity and the spinal cord in the vertebral canal. The CNS is organized into regions of gray matter and white matter:

Gray matter contains the cell bodies of neurons, their dendrites, and the proximal parts of their axons. Groups of anatomically or functionally similar neuron cell bodies in the gray matter may be found in a nucleus, a lamina, or a layer (Netter, 1987).

White matter contains axons of neurons. Groups of anatomically or functionally similar axons may be found in a peduncle, a funiculus, a fasciculus, or a tract. Both gray and white matter also contains different types of glial or supporting cells (Netter, 1987).

The peripheral nervous system (PNS) is composed of spinal nerves, cranial nerves, and autonomic nerves. Nerves usually contain different combinations of axons of motor neurons and processes of sensory neurons. Groups of neuronal cell bodies in the PNS are found in sensory or autonomic ganglia. Spinal nerves contain both motor and sensory fibers. There are thirty one pairs of spinal nerves in the human body that enter or exit segmentally from the spinal cord (8 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 1 coccygeal). Spinal nerves supply structures in the limbs, trunk, and neck. (Purves et al, 2012) (Netter, 1987).

The sensory branches of a spinal nerve supply a specific dermatome, the area of skin supplied by the branches of a single spinal nerve. The muscular branches on the other

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

hand supply a specific myotome, the muscle mass supplied by the branches of a single spinal nerve (Netter, 1987).

Twelve pairs of cranial nerves emerge mainly from the parts of the brain stem. Cranial nerves mainly supply structures in the head, neck, and viscera in the thorax and abdomen (Purves et al, 2012).

Autonomic nerves are organized into sympathetic and parasympathetic divisions that provide motor innervation to smooth muscle, glands, and cardiac muscle (figure 1). The sympathetic and parasympathetic divisions use 2 neurons in series to innervate target structures. The cell body of the first neuron (the preganglionic neuron) is found in the gray matter of the CNS; the second neuron (the postganglionic neuron) is in an autonomic ganglion in the parasympathic nervous system (PSNS). The preganglionic signal is transmitted by nicotinic receptor in both the PSN and sympathetic nervous system (SNS) (figure 1). (Purves et al, 2012) (Netter, 1987).

The sympathetic division functions as an emergency or catabolic system involved in fight or flight responses and has a widespread distribution throughout the body. Preganglionic sympathetic neuron cell bodies are found in the thoracic and upper lumbar segments of the spinal cord from T1 through L2 and exit in the ventral roots of spinal nerves from T1 through L2. The SNS is divided into three parts (dopaminergic, cholinergic, and adrenergic) according to the acting neurotransmitters on the target organs (figure 2).

The parasympathetic division functions to conserve energy and restore body resources and has a restricted distribution. Preganglionic parasympathetic cell bodies are found either in the brainstem where they exit the brain in 1 of 4 cranial nerves (III, VII, IX,

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

and X), or at sacral spinal cord segments S2, 3, and 4 and exit the spinal cord with the ventral roots of S2 through S4 spinal nerves. The PSNS supplies 2 smooth muscles in the orbit, salivary and mucous glands in the head, smooth muscles and glands in the thoracic cavity, and abdomen- pelvic viscera (Purves et al, 2012)**.**

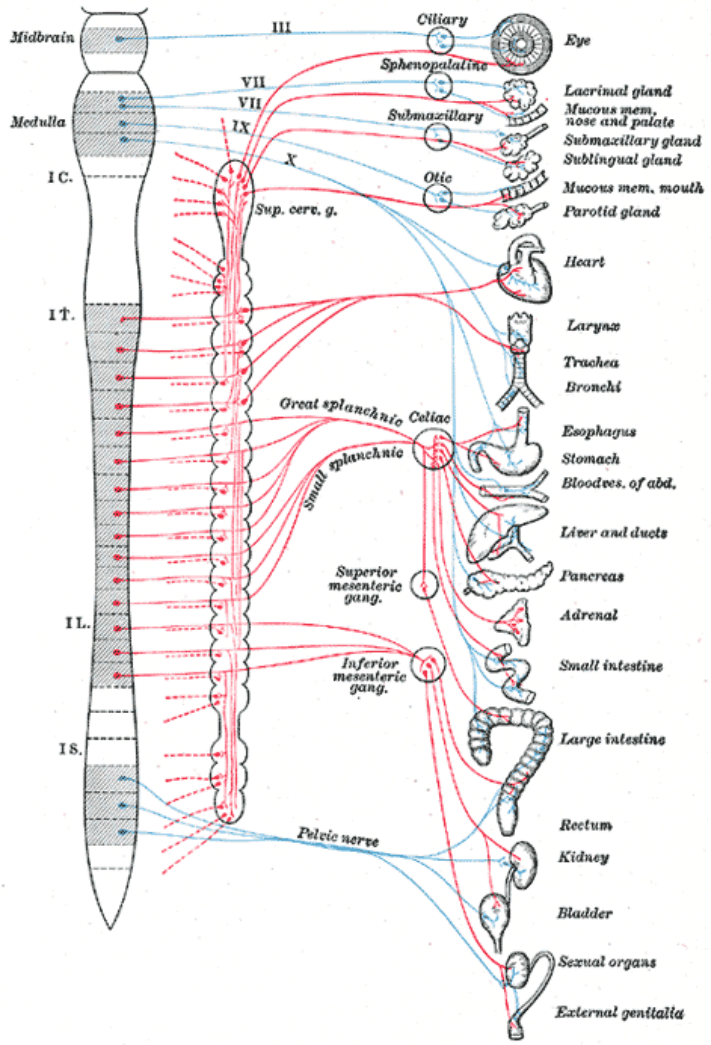


Figure 1: The distribution of the autonomic nervous system (including the parasympathetic and sympathetic braches). (Gray H. 1918).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

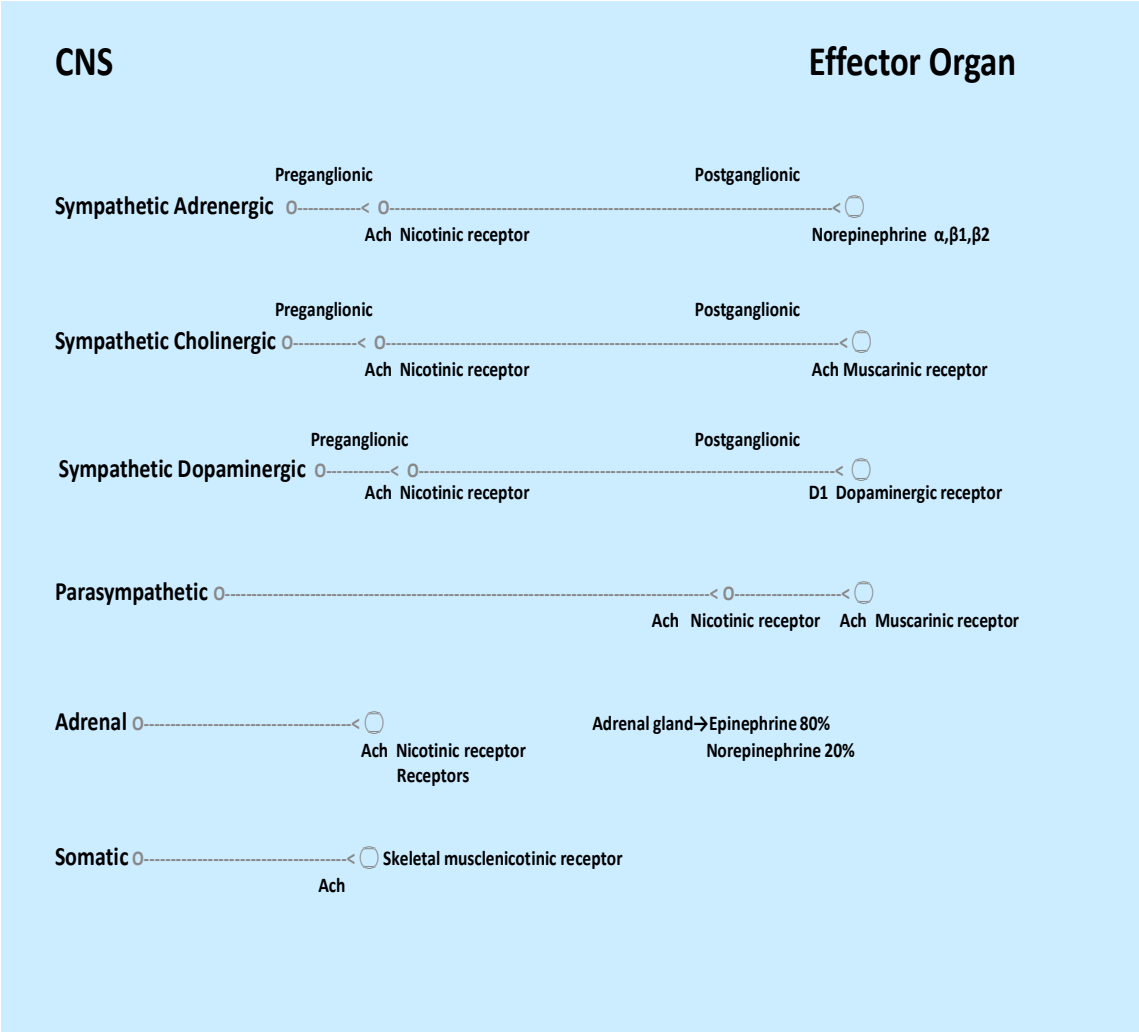


Figure 2: Neurotransmitter receptors in the peripheral nervous system. Abbreviations: Ach, acetylcholine.

2.1.2 Neurotransmitter receptors

Neurotransmitter receptors are membrane receptor proteins that can be activated by specific neurotransmitters. A membrane receptor protein interacts with a chemical in the cells external environment and a membrane protein interacts with the phospholipid bilayer that encloses the cell. Membrane receptor proteins that are present in neuronal and glial cells, enable cells to communicate with each other

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

through chemical signals. The postsynaptic response can be excitatory or inhibitory (table 1) (Levitan et al, 2002).

An excitatory postsynaptic potential (EPSP) is a temporary depolarization of the postsynaptic membrane, which increases the likelihood of the generation of an action potential in a postsynaptic axon. It results from a net inward depolarizing current caused by increased Na^+ and Ca^{2+} conductance at postsynaptic receptors. Glutamate and acetylcholine are the common CNS excitatory neurotransmitters that generate EPSPs. (Hall, 2010).

An inhibitory response can cause an inhibitory postsynaptic potential (IPSP), which decreases the likelihood of the generation of an action potential. IPSPs result from a net outward hyperpolarizing current caused by increased outward conductance of K^+ and inward conductance of Cl^- at postsynaptic receptors. Gamma-Aminobutyric acid (GABA) and glycine are the common CNS inhibitory neurotransmitters that generate IPSPs (Purves et al, 2012).

There are two types of neurotransmitter receptors; ionotropic receptors and metabotropic receptors. Ionotropic receptors can be excited by neurotransmitters (ligands) like glutamate and aspartate, and inhibited by GABA and glycine. On the other hand metabotropic receptors are neither excitatory nor inhibitory. They regulate the actions of excitatory and inhibitory neurotransmitters. Most neurotransmitters receptors are metabotropic (Purves et al, 2012) (Levitan et al, 2002).

Ionotropic receptors (ligand-gated) typically consist of 5 protein subunits that form or are linked to an ion channel. When a neurotransmitter binds to an ionotropic receptor, it results in a direct increase or decrease in the ion conductance of the channel. Ionotropic

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

receptors are located on postsynaptic elements where a fast response to the presynaptic signal is required. Nicotinic acetylcholine receptors are ionotropic receptors in the membranes at the neuromuscular junctions in skeletal muscle, on postganglionic autonomic neurons, and in the CNS. When acetylcholine binds to the nicotinic receptor Na^+ ions flow into the cell while K^+ ions flow outwards causing a depolarization of the cell membrane. These receptors are named "nicotinic" because nicotine binds to the receptor and mimics the effects of acetylcholine (Hall et al, 2010).

Metabotropic receptors (G protein-coupled) form a large protein family of transmembrane receptors that sense molecules outside the cell, activate intracellular transduction pathways and eventually cellular responses. These receptors can be found only in eukaryotes, including animal cells, yeast and choanoflagellates. G proteins contain an α-subunit that is critical to the correct functioning of these receptors. There are four main classes of G-proteins: G_s, G_i, G_q, and $G_{12/13}$ (Simon et al, 1991). These proteins are classified according to their susceptibility to cholera toxin (CTX) and pertussis toxin (PTX). G_s and some subtypes of G_i ($G_{\alpha t}$ and $G_{\alpha g}$) are sensible to CTX. G_i proteins are sensible to PTX, with the exception of the subtype G_i ($G_{\alpha z}$) which is immune. G-protein subunits have different effects on secondary messengers, for example some of them up-regulate phospholipases while others down-regulate cAMP (Dell´ Acqua et al, 1993).

The ligands that attach to and activate these receptors include odors, pheromones, hormones, and neurotransmitters, that can range from small molecules to large proteins. Metabotropic receptors are involved in the pathophysiology of many diseases, and are the target of approximately 30% of all modern therapeutic drugs (King et al, 2003) (Filmore, 2004) (Overington et al, 2006).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Neurotransmitter receptors are present on both postsynaptic and presynaptic neurons. When present at the presynaptic level they prevent further release of a given neurotransmitter, and when they are localized at the postsynaptic membrane they receive the incoming signal. Neurotransmitter receptors are also found in various immune and muscle tissues. Many neurotransmitter receptors are categorized as serpentine receptor because they can span the cell membrane up to 7 times. Neurotransmitter receptors can become unresponsive to the type of neurotransmitter they receive when exposed for prolonged periods of time, this phenomenon is known as down regulation or ligand-induced desensitization (Purves et al, 2012).

Neurotransmitter receptor type	Subtypes
Adrenergic	α_{1A}, α_{1b}, α_{1c}, α_{1d} α_{2a}, α_{2b}, α_{2c}, α_{2d} β_1, β_2, β_3
Dopaminergic	D_1, D_2, D_3, D_4, D_5
GABAergic	$GABA_A$, $GABA_{B1a}$, $GABA_{B1\delta}$, $GABA_{B2}$, $GABA_C$
Glutaminergic	NMDA, AMPA kainate, $mGluR_1$, $mGluR_2$, $mGluR_3$, $mGluR_4$, $mGluR_5$, $mGluR_6$, $mGluR_7$
Histaminergic	H_1, H_2, H_3
Cholinergic	Muscarinic: M1, M2, M3, M4, M5 Nicotinic: muscle, neuronal (α-bungarotoxin-insensitive), neuronal (α-bungarotoxin-sensitive)
Glycinergic	Glycine
Serotonergic	5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, 5-HT_7
Opioid	μ, δ_1, δ_2, κ

Table 1: Neurotransmitter Receptors, different types and subtypes.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.1.3 Types of adrenergic receptors

These receptors were first described by Raymond Ahlquist, in 1948. He observed that a single sympathetic mediator could cause both an excitatory and an inhibitory response; and that adrenergic receptors were of two different types based on the potency of activation created by the agonists (Ahquist et al, 1948).

Adrenergic receptors are G protein-coupled receptors that are targets of catecholamines, such as norepinephrine and epinephrine. These receptors are present in many cells, and when they bind to a catecholamine they usually lead to the stimulation of the SNS. The sympathetic response causes a widening of the pupils, mobilization of energy, and diverts blood flow from less essential organs to skeletal muscle (Hall, 2010).

There are two main groups of adrenergic receptors, α and β, each have several subtypes. Alpha receptors are divided into the subtypes α1 (aG_q coupled receptor) and α2 (aG_i coupled receptor). On the other hand β receptors are divided into the subtypes β_1, β_2 and β_3. All three are linked to G_s proteins (although β_2 also couples to G_i), which in turn are linked to adenylate cyclase (Chen-Izu et al, 2000). Epinephrine interacts with both α and β receptors, causing vasoconstriction and vasodilatation, respectively. However the overall effect of epinephrine is a circulatory vasoconstriction. This can be explained by the fact that there are more peripheral α1 receptors than β adrenoreceptors. On the other hand α receptors are less sensitive to epinephrine, thus at lower levels of circulating epinephrine, β-adrenoreceptor stimulation dominates, producing vasodilatation followed by decrease of peripheral vascular resistance. At higher levels of circulating epinephrine α receptors stimulation becomes predominant resulting in vasoconstriction. Further details will be discussed in the following sections (Table2) (Hall, 2010).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.1.3.1 Alfa Receptors

Alpha receptors have several functions in common such as vasoconstriction of the coronary arteries, and decrease smooth muscle motility in the gastrointestinal tract, but each subtype also has its own specific effects (Woodman et al, 1987) (Elliott, 1997) (Sagrada et al, 1987).

Alpha-1 receptors are members of the G protein-coupled receptor family. When stimulated G_q proteins activate phospholipase C, which causes an increase in inositol 1,4,5-triphosphate (IP_3) and calcium. This will trigger further effects, through the activation of protein kinase C (PKC) that will lead to the phosphorylation of other enzymes causing their activation, or by the phosphorylation of specific channels that will cause an increase or decrease of electrolyte transfer in or out of the cell. Alpha-1 receptors can be stimulated by substances such as norepinephrine, epinephrine, metaraminol and phenylephrine. When activated these receptors can induce vasoconstriction of the coronary arteries, vasoconstriction of veins, and decrease motility of smooth muscle in the gastrointestinal tract (Woodman et al, 1987) (Elliott, 1997).

In the smooth muscles of blood vessels the main effect is vasoconstriction. Blood vessels with α1 receptors are present in the skin, the sphincters of gastrointestinal system, renal arteries and brain. During situations of stress, vasoconstriction will cause a decrease in the blood flow to these organs. Alpha-1 stimulation can also induce contraction of the urinary bladder, and a decrease in appetite (Sagrada et al, 1987) (Rang, 2003) (Schmitz et al, 1981) (Purves et al, 2012) (Cheng et al, 2003).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Alpha-2 receptors are also members of the G protein-coupled receptor family that are associated with the G_i protein. There are 3 homologous subtypes; α2A, α2B, and α2C. Catecholamines such as epinephrine and nor-epinephrine stimulate the α2-receptors in the central and peripheral nervous systems (Ruuskanen et al, 2004).

In α2 subunits the inhibitory G_i protein dissociates from the G protein causing an inactivation of adenyl cyclase, a decrease of cyclic adenosine 3,5-monophosphate (cAMP) production and therefore a decrease of intracellular cAMP. Protein kinase A (PKA) is not able to be activated by cAMP, so proteins such as phosphorylase kinase cannot be phosphorylated by PKA. Phosphorylase kinase is required for the phosphorylation and activation of glycogen phosphorylase, which is necessary for glycogen breakdown. Thus the final effect of this pathway is a decreased breakdown of glycogen (Schaak et al, 2000).

The α2 receptors are classically located on vascular prejunctional terminals where they inhibit the release of norepinephrine by a negative feedback mechanism. They are also present on the vascular smooth muscle cells of some blood vessels (i.e. skin arterioles and veins) where they coexist with α1 adrenergic receptors. Alpha-2 receptors can bind to both norepinephrine released by sympathetic postganglionic fibers and epinephrine released by the adrenal medulla. The stimulation of α2-adrenergic receptor can cause muscle relaxation and pain relive through effects on the CNS (Hall, 2010).

2.1.3.2 Beta receptors

The activation of these receptors triggers a sympathomimetic response. They are G-protein coupled receptors that act by activating a Gs protein which activates adenylyl cyclase, causing an increase in the levels of intracellular cAMP. Elevated levels of

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

cAMP cause PKA activation, which leads to the phosphorylation of cellular proteins. Beta receptors can respond to epinephrine, norepinephrine and isoproterenol stimulation. They are subdivided into three subgroups, β1, β2, and β3. This division is based on their affinities to adrenergic agonists and antagonists (Purves et al, 2012).

Beta-1 receptors are associated with the Gs protein. They are expressed predominantly in cardiac tissue and when stimulated they can increase the cardiac output, the heart rate through sino-atrial node stimulation, atrial cardiac muscle contractility, contractility and automaticity of the ventricular cardiac muscle, and the conduction and automaticity of atrioventricular node. They also stimulate amylase secretions from salivary glands, renin release from juxtaglomerular cells, and lipolysis. These receptors are also present in the cerebral cortex. (Rang, 2003) (Purves et al, 2012).

Isoprenaline has higher affinity for β1 than epinephrine, which, in turn, has a higher affinity than norepinephrine. There are other selective agonists to the β1 receptor such as denopamine and dobutamine (Rang, 2003).

Beta-2 receptors are directly associated with the class C L-type calcium channel $Ca_V1.2$. This receptor-channel complex is linked to the G_s protein, which activates adenylyl cyclase, inhibiting the formation of cAMP which activates PKA, and the counterbalancing protein phosphatase (Rubenstein et al 2006).

Beta-2 receptors have been found to couple with G_i protein, and appear to provide a mechanism of response localized within cells. On the other hand β1 receptors are coupled only to G_s protein, and their stimulation causes a more diffuse cellular response (Chen-Izu, et al 2000) (Zamah et al, 2002). Beta-2 receptors appear to increase heart muscle contraction, increase cardiac output (lesser degree compared to β1),

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

increase heart rate in sinoatrial node, increase atrial cardiac muscle contractility, and increases contractility and automaticity of ventricular cardiac muscle. They also appear to dilate the hepatic artery and arteries to skeletal muscle (Purves et al, 2012).

In the human eye, β2 stimulation by salbutamol increases intraocular pressure. These receptors also regulate glycogenolysis and gluconeogenesis in liver, glycogenolysis and lactate release in skeletal muscles, contract sphincters of the gastrointestinal tract, and thickens secretions from salivary glands. Substances that stimulate β2 receptors include salbutamol , bitolterol mesylate and isoproterenol (Hall, 2010).

Beta-3 receptors are located mainly in adipose tissue and are involved in the lipolysis process and thermogenesis. Some β3 agonists have demonstrated to induce anti-stress effects in the CNS. These receptors can be found in the urinary bladder, gallbladder and in brown adipose tissue. They seem to play a role in lipolysis and thermogenesis in brown fat. In the urinary bladder they are thought to cause the relaxation of the bladder and prevention of urination (Table 2) (Ferrer-Lorente, 2005).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Adrenergic receptor	Functions	Drugs affecting this receptors	Medical use
α1-receptor	Noradrenaline mediated smooth muscle contraction (mainly vascular, urogenital and sphincteric)	Prazosin (antagonist)	Hypertension
α2-receptor	Presynaptic control of noradrenaline, ATP, and Ach release at nerve terminals.	Yohimbine (antagonist)	Ejaculation delay
β1-receptor	Stimulating effect of adrenaline and noradrenaline on the heart	Atenolol (antagonist)	Hypertension
β2-receptor	Noradrenaline mediated smooth muscle relaxation (Mainly the digestive tract, respiratory tract and the urogenital system)	Salbutamol (agonist)	Bronchodilator
β3-receptor	Stimulates fatty acid release in adipose tissue	None	Potential treatment in obesity

Table 2: Types of adrenergic receptors their function and medical use (Masaaki et al, 2006).

2.1.3.3 Adrenergic receptors and bone tissue

As we mentioned earlier adrenergic receptors are well known for their role in the regulation of cardiovascular, uterine and airway smooth muscle functions. However, some histological studies have demonstrated the presence of norepinephrine fibers on bone, and pharmacological studies have shown that both osteoblastic and osteoclastic cells contain receptors for norepinephrine. Gene expression of α1 and α2 receptors and/or β2 receptors has been detected by reverse transcription polymerase chain reaction (RT-PCR) in human periosteum-derived osteoblastic cells (SaM-1), human

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

osteosarcoma-derived cells (SaOS-2, HOS, MG6), mouse primary osteoblasts and human osteoclasts. Among all postsynaptic adrenergic receptors β2 receptors appears to be the main, and possibly the only functional adrenergic receptor expressed in osteoblasts. The presence of both β2 receptor messenger ribonucleic acid (mRNA) and protein has been confirmed in rat calvarial osteoblasts by polymerase chain reaction (PCR) and confocal microscopy. However neither β1 nor β3 receptor expression was detected in these cells. Moreover β2 receptor mRNA was expressed in extracts from mouse femur, whereas β1 and β3 receptor genes were not identified (Elefteriou et al, 2005) (Togari et al, 1997) (Togari, 2002) (Kellenberger et al, 1998) (Moore et al, 1993) (Bonnet et al, 2008) (Pierroz et al, 2006).

Several clinical conditions in which there is an overstimulation of the adrenergic system have been associated with a low bone mass and increased bone fragility. Beta-2 agonists used in obstructive lung disease patients have 2 x fold increased risk of hip fracture. In reflex sympathetic dystrophy (also known as Sudeck Syndrome), characterized by a hyperactivity of the adrenergic pathway, localized osteopenia is caused by an increase in bone resorption. Athletes that consume β2 agonists for their anabolic effect on muscle mass show bone loss and an increased risk of suffering fatigue fractures (Pierroz et al, 2006) (De Vries et al, 2007) (Schwartzman, 2000). This osteopenic effect caused by an increased adrenergic signaling could be explained by the osteoclastogenic effect associated with β2 receptor stimulation (Kondo et al, 2012).

Mice deficient for the β2 adrenergic receptor have a lower bone resorption and increased bone formation, for this reason they appear to maintain greater trabecular bone microarchitecture as they grow older. A recent study has demonstrated that mice deficient for β1 receptors do not have increased histomorphometric

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

bone forming indices or bone density. On the other hand these values were increased in mice deficient for β2 receptors. Moreover, bone mass and microarchitecture were increased in femurs and vertebrae of β2 receptor deficient mice, whereas β1 receptors deficient mice had a lower femur bone mineral density (BMD), cortical size, and cortical thickness compared to wild type mice. These results suggest that β1 and β2 adrenergic signaling induces different effects on bone. Beta-1 signaling leads to a predominant anabolic stimulus in response to mechanical stimulation whereas β2 adrenergic receptor signaling mainly regulates bone resorption during aging (Pierroz et al, 2012).

In a case control study performed by Schlienger and cols. the use of β-blockers appeared to be associated with a reduced risk of fractures, taken alone and in combination with thiazide diuretics. However this study did not discriminate between different types of β-blockers. Many elderly patients with hypertension who are at risk of developing osteoporosis could potentially benefit from combined therapy with blockers and thiazides (Schlienger et al, 2004).

2.1.4. Types of cholinergic receptors

Langley and Dickinson were the first to observe that the stimulation of the superior cervical ganglia with nicotine caused a synaptic transmission. Dale and Dixon described the initial classification of these receptors; nicotinic and muscarinic. These receptors are present in both the SNS and PSNS (Purves et al, 2012).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.1.4.1 Muscarinic Receptors

Muscarinic receptors belong to the metabotropic receptor group; they form G-protein complexes that activate other ion channels via second messengers. These receptors play several roles, including acting as the main target receptor stimulated by acetylcholine released from postganglionic fibers in the PSNS. They are named muscarinic because they are more sensitive to muscarine than to nicotine. Muscarinic receptors vary in the G protein to which they are bound (Ishii et al, 2006).

Muscarinic receptors play a variety of roles in the human body. They function as recovery receptors in the postganglionic neuron. This effect is caused by a hyperpolarization and slow depolarization that is mediated by type M2 and M1 muscarinic receptors (Purves et al, 2012). Another function of these receptors is at the junction between the target tissue and the postganglionic neuron in the parasympathetic division of the ANS.

On the other hand only a few parts of the SNS contain cholinergic receptors that form what is also known as the cholinergic SNS: some postganglionic fibers terminating on sweat glands, skeletal arterioles, and in piloerectile muscles of the body hairs.. (Hall, 2010).

Muscarinic acetylcholine receptors are also present in different locations throughout the CNS, in post-synaptic and pre-synaptic positions. There is also some evidence for the presence of postsynaptic muscarinic receptors on sympathetic neurons allowing the PSNS to inhibit sympathetic effects (Purves et al, 2012).

These receptors are also present on the presynaptic membrane of somatic neurons at the neuromuscular junction, where they regulate the release of acetylcholine release.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Muscarinic receptors also seem to control a number of cellular pathways that appears to regulate the inflammatory process, growth and proliferation of the cell. These pathways include protein Ga/i/o- and Gaq/11- (Caulfield et al, 1998) (Eglen, 1998) (Ishii et al, 2006) (Purves et al, 2012).

Types of Muscarinic receptors:

Five subtypes of muscarinic receptors have been identified (M_1-M_5) using selective radioactively labeled agonist and antagonist substances. M1, M3 and M5 receptors increase the intracellular concentration of Ca^{2+}. Protein G_i forms part of a cellular pathway that includes M2 and M4 receptors and seems to modulate K^+ ion conduction (figure 3) (Caulfield, 1993).

M1 receptor is common in exocrine glands and in the CNS. It appears to mediate the slow EPSP at the ganglion in the postganglionic nerve and it is predominantly found bound to G_q proteins, which use upregulation of phospholipase C as a signaling pathway (Caulfield, 1993).

M2 receptors are present in the heart, where they down regulate the heart rate by slowing the speed of depolarization and reducing the conduction velocity of the atrioventricular node. These receptors also decrease the contractile forces of the atrial cardiac muscle, and to a lesser extent those of the ventricular muscle. M2 receptors act via a G_i type receptor, which induces a decrease in intracellular cAMP, inhibits the Ca^{2+} channels, and increases the efflux of K^+, which generally causes inhibitory effects (Purves et al, 2012).

M3 receptors are present in the smooth muscles of the blood vessels and in the lungs. These receptors are G_q coupled and when stimulated they lead to an increase in

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

intracellular calcium which causes smooth muscle contraction (i.e. bronchoconstriction). On the other hand, the activation of M3 on vascular endothelial cells leads to an increase in the synthesis of nitric oxide, which extends to proximal vascular smooth muscle cells and causes their relaxation. This mechanism would explain the paradoxical effect of parasympathetic stimulation on vascular tone and bronchiolar tone. However, a direct stimulation of vascular smooth muscle by M3 receptors leads to vasoconstriction in pathologies where the vascular endothelium is disrupted. The M3 receptors are also located in many glands, where they stimulate active secretion (i.e. the salivary glands, as well as other glands of the body) (Parker et al, 2006).

A recent study has demonstrated that M3 receptor exert stimulatory effects on the production of bone matrix and cancellous bone microarchitecture (Kliemann et al, 2012). Moreover, mice with knockout M3 receptors are osteoporotic due to a decrease of osteoblast numbers and an increase of osteoclast numbers (Shi et al, 2010).

M4 receptors are present in the CNS. The mechanism of action of these receptors is mediated by G_i proteins that decrease intracellular cAMP and lead to a generally inhibitory effect. Bronchospasm may result from the stimulation of M4 receptors by certain muscarinic agonists (Johnson et al, 2001).

The location of M5 receptor is not well known. These receptors, like M1 and M3 muscarinic receptors are coupled with G proteins of class G_q that up regulate phospholipase C, IP3 and intracellular calcium (Johnson et al, 2001) (Burford et al, 1996).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Muscarinic subtypes M1, M2, M3, M4, and M5 are expressed at different levels in human osteosarcoma cells, rat femur, and human rib bone tissue. M1, M4, M5 were successfully in cultured mouse femur bone cells and cultured mouse calvarial bone cells; M2, M3, M4 were in bovine bone. It appears that muscarinic receptors are present in bone tissue to evoke calcium signaling and modulate cell proliferation. Different muscarinic receptor subtypes can be identified in various parts of the animal skeletal system. Bone remodeling involving osteoblast proliferation could be related with the activity of muscarinic receptors (Table 3) (Liu et al, 2011).

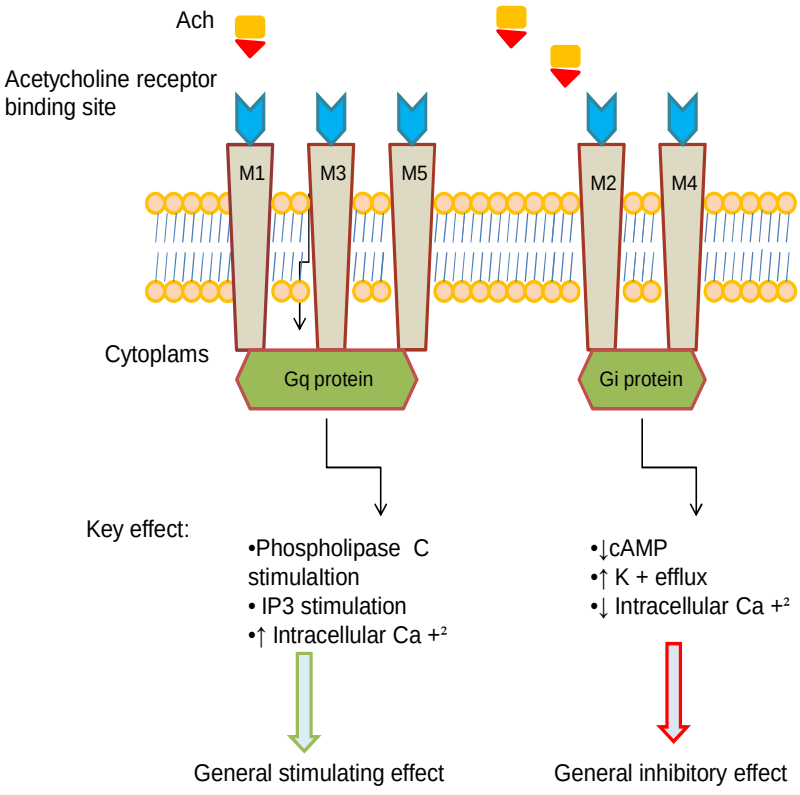


Figure 3: Achetylcholine muscarinic receptors. Abbreviations: Ach, acetycholine; IP3, 1, 4, 5-triphosphate; cAMP, cyclic adenosine 3, 5-monophosphate.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.1.4.2 Nicotinic receptors

Nicotonic receptors are ionotropic receptors that contain cholinergic ionic channels that open when a specific chemical messenger binds to the receptor. They can be found in the autonomic ganglia of the SNS and PSNS, at the neuromuscular junction and in the adrenal medulla. The receptors at these different locations are similar but not identical. They can be stimulated by both acetylcholine and nicotine, and blocked by hexamethonium in the autonomic ganglia. They are formed from the union of 5 different AchR subunits. So far, a total of 17 subunits (divided into muscular and neural subtypes) have been identified, that can be united in a large number of possible combinations. The most frequent combinations are; $(\alpha 1)_2$-$\beta 1$ $\delta\varepsilon$ (muscular type), $(\alpha 3)_2$-$(\beta 4)_3$ (ganglionar type), $(\alpha 4)_2$-$(\beta 2)_3$ (CNS) and $(\alpha 7)_5$ (CNS). The molecular weight of these pentamers is of 290 kilodaltons (KDa) (table 4) (Itier et al, 2001).

The union point of acetylcholine is composed of 2 α subunits or 1 α +1 β subunits. When the channels are opened, a net flow of positive ions (Na^+, Ca^{2+}) into the interior of the cell begins, which causes a depolarization of the plasma membrane, and the stimulation of the postsynaptic neuron (figure 4). On the other hand the influx of Ca^{2+} ions would act on the different intracellular cascades and on certain genes. The prolonged stimulation of the nicotinic receptors would eventually cause a desensitization of these receptors (tables 3 and 4) (Purves et al, 2012).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Cholinergic receptor	Functions	Drugs affecting these receptors	Medical use
Nicotinic-cholinergic receptors	Rapid excitation of the postganglionic neurons at the autonomic ganglia	Hexamethonium (antagonist)	Hypertension (in the past)
M1-receptor	Inhibition of Ach and noradrenaline release from the autonomic nerve terminals	Pirenzepine (antagonist)	Anti-ulcer
M2-receptor	Effects of Ach on the heart and smooth muscles	Atropine (non selective antagonist)	Mydriasis
M3-receptor	Glandular stimulation induced by Ach	Atropine (non selective antagonist)	Reduction of salivary secretion in Parkinson disease

Table 3: Types of cholinergic receptors their function and medical use (Masaaki et al, 2006). Abbreviations: Ach, acetylcholine.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Neural type nicotinic subunits	Muscle type nicotinic subunits
Type I: $\alpha_{9,}\ \alpha_{10}$	Type IV : $\alpha_{1,}\ \beta_1$, δ, γ, ε
Type II: $\alpha_{7,}\ \alpha_8$	
Type III-1: $\alpha_{2,}\ \alpha_{3,}\ \alpha_{4,}\ \alpha_6$	
Type III-2: $\beta_{2,}\ \beta_4$	
Type III-3: $\beta_{3,}\ \alpha_5$	

Table 4: Nicotinic receptor subfamilies.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

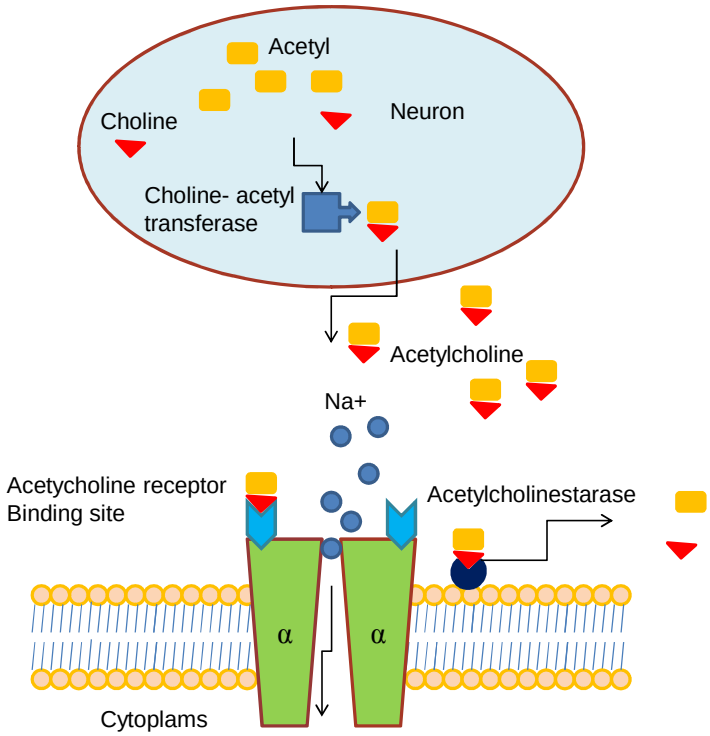


Figure 4: Achetylcholine receptor (nicotinic α- α subunits).

2.1.4.3 Cholinergic receptors and bone tissue

The wide expression of cholinergic components in bone tissue points toward the important role they could play in bone remodeling. Bone cells have been shown to express all components of the cholinergic system (acetylcholine, cholinergic receptors and AChE) (Bajayo et al, 2012) (Shi et al, 2010) (Dani et al, 2007) (Liu et al, 2011) (En-Nosse et al, 2009) (Vogel-Hopker et al, 2012) (Grisaru et al, 1999) (Inkson et al, 2004) (Fujii et al, 2009). For instance, acetylcholine has been reported in osteosblasts (Sato et al, 2010). Cholinergic receptors, both nicotinic and muscarinic, have been identified in human primary bone cells, mesenchymal stem cells, osteoblast and osteoclasts. AChE is expressed in bone cells such as bone marrow-derived monocytes,

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

osteoclasts and osteoblasts (Bajayo et al, 2012) (Genever et al 1999) (Inkson et al, 2004).

Previous studies have shown that acetylcholine might regulate the migration of bone marrow mesenchymal stem cells (Tang et al, 2012). These are multipotent cells able to differentiate into bone cells. Cholinergic receptors, both nicoting and muscarinic, have shown to affect bone turnover. The only nicotinic receptor that shows an effect on the bone remodeling pattern is subtype α2 (Alkondon et al, 2000) (Klienmann et al, 2012) (Rothem et al, 2009). Indeed, knockout nicotinic receptor subtype α2 mice are osteoporotic due to up-regulation of osteoclasts (Bajayo et al, 2012). Stimulation of nicotinic receptors in wild type mice has been shown to induce bone mass gain as a result of an increase in osteoclast apoptosis (Bajayo et al, 2012).

Muscarinic receptors, more specifically M3 receptors, have been shown to influence bone remodeling favoring bone formation and decreasing bone resorption. Knockout M3 receptors mice are osteoporotic due to an increase in the number of osteoclasts and a decrease in the number of osteoblasts. Stimulation of muscarinic receptors with cholinergic agonists *in vitro* increases osteoblast proliferation (figure 5) (Shi et al, 2010) (Klienmann et al, 2012) (Liu et al, 2011).

The activity of cholinergic receptors in bone is terminated by AChE and its capability to break down acetylcholine in bone. AChE can play significant roles during bone development. It has been identified at sites of new bone formation, suggesting that AChE could act as a bone matrix protein. Indeed, it has been reported that AChE can regulate cell proliferations, differentiations, cell-cell contact as well as mesenchymal stem cells migration (Vogel-Hopker et al, 2012) (Inkson et al, 2004) (Genever et al, 1999) (Fujii et al, 2009).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

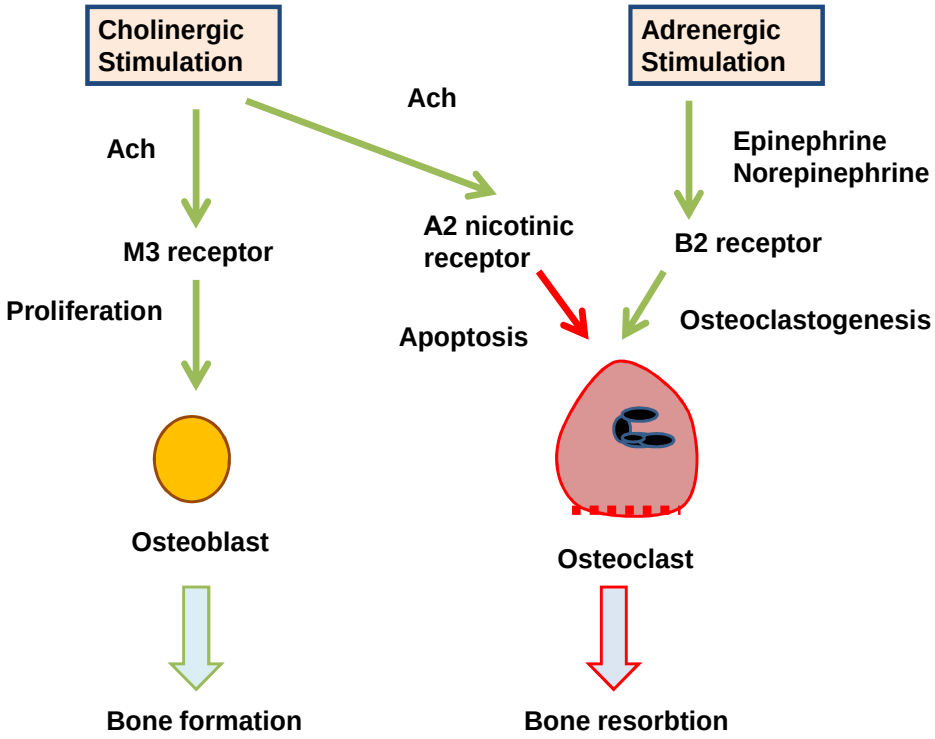


Figure 5: Summary of the most important cholinergic and adrenergic known pathways in bone. Abbreviations: Ach, acetylcholine; A2, alpha-2; B2, beta-2; M3, muscarinic-3.

2.2 Bone remodeling

Bone remodeling allows the conservation of normal bone mass despite constant internal and external environments changes that affect cortical and cancellous bone throughout life that may affect osteoclastic and osteoblastic activity. Osteoclasts bind to bone surfaces through cell attachment or "anchoring" proteins (Nijweidi et al, 1986). Integrins, seal the space below osetoclasts which then produce hydrogen ions that increase the solubility of hydroxyapatite crystals. The organic matrix is then removed by proteolytic digestion through the activity of lysosomal enzyme cathepsin K (Netter, 1987) (Hall, 2010). Osteoclast mediated bone resorption takes approximately 2-4 weeks during each cycle (Bart, 2008).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Osteoclastic tunneling is followed by layering of osteoblasts and the deposition of layers of lamellae. The initial stage of bone production is the secretion of collagen molecules (collagen monomers) and ground substance (mainly proteoglycans) by osteoblasts. The collagen monomers are linked together to from collagen fibers, the main component of osteoid. Some of the osteoblasts become entrapped in the osteoid and become osteocytes (Mark et al, 2012). A few days later calcium salts begin to precipitate on the surfaces of the collagen fibers. The precipitates initially appear at the intervals along each collagen fiber, forming small nidi that rapidly grow into hydroxyapatite crystals within a period of days and weeks. However a few per cent of the deposited crystals may remain permanently in the amorphous from. This is important because these amorphous salts can be absorbed rapidly in situations where there is an increased need for extra calcium in the extracellular fluid (Hall et al, 2010) (Ramachandran, 2007). The process of bone tissue remodeling is extremely complex and only partially understood, never the less some important discoveries have been made in the last decade, which have lead to a better comprehension of this important process. Generally speaking bone remodeling appears to be regulated at systemic and local levels which closely interact with each other.

Mechanism of bone remodeling:

Bone remodeling occurs in local groups of osteoblasts and osteoclasts called bone multicellular units (BMU). Each unit is organized into a "cutting cone" of osteoclasts reabsorbing bone that are followed by a group of osteoblasts that fill the bone defect left by osteoclasts. The end result is the production of a new osteon. Each BMU has a limited lifetime, so new units are continuously forming as old units disappear. In normal bone the number of BMUs, the bone resorption rate, and the bone formation rate are all

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

relatively stable (Martin, 2007). Therefore a steady-state situation develops in which the total amount of missing bone due to bone remodeling at any time is generally constant. Any changes in the number of BMUs, bone resorption rate, or formation rate will alter this balanced situation. In adults, bone removed by a BMU is not completely restored and a small amount of bone is permanently lost, this gradual loss of bone is a physiological process in humans and it is responsible for the gradual bone loss related to age. Some pathologic conditions may increase the amount of irreversible bone loss by either increasing the total number of BMUs or by increasing the amount of irreversible bone loss in each BMU. Irreversible bone loss per BMU can result from an increase in the osteoclastic activity or a decrease in the osteoblastic activity. Irreversible bone loss occurs both in cortical and trabecular bone. A permanent increase in bone mass does not normally occur in adult life, except in situations of periosteal bone formation due to exercise (Hall et al, 2010) (Ramachandran, 2007).

A leading cause of metabolic bone disease is an increase in number of BMUs, there are many different types of stimuli that lead to this increase. The best studied example is parathyroid hormone (PTH); in mild hyperparathyroidism the number of BMUs is increased, but the rates of bone resorption and formation in each BMU are not altered and the amount of irreversible bone loss is relatively small. In severe hyperparathyroidism, an increase in the total bone resorption can be so high that bone formation cannot keep up with the large quantities of bone that is lost. In high turnover osteopenia both the formation and the resorption rates are increased, but the formation can´t keep up with the resorption. Most patients with postmenopausal osteoporosis have a low turnover osteopenia. In this situation, the rate of resorption is usually unchanged, but the rate of formation is decreased (Smith et al, 2012).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

The process of bone remodeling depends on a balanced interaction between a series of hormones, inflammatory mediators, neurotransmitters, regulated both systemically and locally. There are four different systemic pathways involved in bone regulation: the energy-bone-adipose tissue-SNS axis, the hypothalamic-pituitary-thyroid axis, the IL1-cholinergic SNS axis and the parathairoid-vitamin D-calcitonin axis. We will discuss these pathways with more detail below; the local regulating factors involved in the process of bone remodeling will be also addressed.

2.3.1 Systemic regulation of bone remodeling:

2.2.1.1 The hypothalamic-pituitary-axis

The hypothalamus regulates bone formation through its indirect control on end organs such as the thyroid gland, adrenal glands, gonads, and liver. This process is mediated by a group of releasing and stimulating hormones (figure 6).

The thyroid pathway:

Thyrotropin releasing hormone (TRH) is a tropic, tripeptidal hormone that stimulates the release of thyroid-stimulating hormone (TSH) and prolactin from the anterior pituitary. TRH can also be found in other sites of the body including the gastrointestinal system and pancreatic islets (Zhang et al, 2001).

TRH gene expression is continuously regulated in the medial and periventricular regions of the paraventricular nucleus (PVN) of the hypothalamus in response to physiological changes such as illness, starvation, coldness, and pituitary or thyroid disease (Hollenberg et al, 1995).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

TSH is a glycoprotein-hormone secreted by thyrotrope cells in the anterior pituitary gland. It stimulates the thyroid gland to produce thyroxine (T_4), and then triiodothyronine (T_3), these hormones regulate the metabolism of almost every tissue in the body (Sacher et al, 2000).

Human studies demonstrate an inverse relationship between T3 and TSH. Thus the skeletal effects of T3 excess cannot be distinguished from low TSH and vice versa. T3 appears to increase bone turnover while high TSH levels have an inhibitory effect. In Grave´s disease there is a production of antibodies that activate the TSH receptors and consequently T3 production. However, Graves disease causes osteoporosis, suggesting the dominant effect of high T3 on the skeleton rather than TSH. Animal models help reinforce this concept, for instance mice with deletion of the gene for TR-α with normal circulating T3, display growth retardation in early age and delayed endochondral ossification. Adult mice show increased trabecular bone mass and reduced osteoclast numbers. Mice lacking TR-β are similar to thyroid hormone-resistant patients with elevation of circulating TSH and T3 (Bassett et al, 2008). Young mice have advanced endochondral and intramembranous ossification and increased bone mineral deposition, while adults have osteoporosis with reduced mineralization and increased osteoclast numbers. This is explained if TR-α is the predominant thyroid receptor in skeletal cells. Since T3 could not affect these cells, there is delayed ossification and reduced bone remodeling. In contrast, TR-α deficient mice have increased circulating T3 with anabolic actions in children and catabolic actions in adults. These and other supportive findings raise the possibility that T3 may be an anabolic agent in childhood (Bassett et al, 2008).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

In summary thyroid hormones stimulate bone resorption more than bone formation, and large doses of thyroxine can cause osteoporosis. Thyroid hormones also regulates the skeletal growth of the physis, stimulates chondrocyte growth, type X collagen production, and alkaline phosphatase activity (Ballock et al, 1994).

The Gonadotropin releasing hormone pathway

Gonadotropin releasing hormone (GnRH) is a trophic peptide hormone synthesized and released from neurons within the hypothalamus. GnRH stimulates the anterior pituitary gland to release of follicle stimulating hormone (FSH) and luteinizing hormone (LH).

Both FSH and LH are produced and secreted by gonadotrophs of the anterior pituitary gland. FSH regulates the recruitment of immature ovarian follicles, pubertal maturation, estrogen production and reproductive processes of the body. In males FSH stimulates primary spermatocytes to undergo the first division of meiosis, to form secondary spermatocytes. LH triggers ovulation and development of the corpus luteum in females. In males it stimulates Leydig cell production of testosterone. FSH and LH act synergistically in reproduction (Louvet et al, 1975).

Estrogens, in females, are produced primarily by the granulose cells that are present in the ovaries, and during pregnancy by the the placenta. Estrogens are also produced in smaller quantities by other tissues such as the breasts, liver and adrenal glands. These additional sources of estrogens are of significant importance in postmenopausal women. Fat cells produce estrogen as well (Nelson et al, 2001). There are 3 main types of estrones; estrone (E1), estradiol (E2), and estriol (E3). Estradiol is the predominant estrogen during the reproductive years. During menopause, estrone becomes the

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

predominant circulating estrogen and during pregnancy estriol is the predominant circulating estrogen in terms of serum levels (Hall et al, 2010).

Estrogens play a fundamental role in the development of female secondary sexual characteristics and are also involved in regulating the menstrual cycle. On the other hand, estrogen also regulates certain functions of the reproductive system in males; it is important to the maturation of sperm and it may be necessary to maintain a healthy libido. (Hess et al, 1997) (Martin-Du Pan et al, 2011).

Estrogen deficiency was identified as the causative factor in postmenopausal osteoporosis over 60 years ago (Albright et al, 1940), and since that time estrogen has been recognized as the main gonadal steroid regulator of bone turnover in women. Estrogen prevents bone loss by inhibiting bone resorption. Bone formation and resorption are coupled, therefore estrogen therapy can also decrease bone formation. Supplementation is helpful in postmenopausal women only if started within 5 to 10 years after onset of menopause (Ramachandran et al, 2007). Prolactin is a protein secreted by the pituitary gland in response to estrogens, feeding and nursing. High levels of prolactin over long periods of time could also lead to loss of bone mineral density (Abraham, et al 2003).

Androgen is the generic term for any natural or synthetic compound, usually a steroid hormone that regulates the development and maintenance of male characteristics in humans by stimulating androgen receptors. They are involved in the activity of the male sex organs and in the development of male sex characteristics. Androgens are also the primary anabolic steroids and the precursor of all estrogens, which are stress hormones. The primary androgen is testosterone, dihydrotestosterone (DHT) and androstenedione are less known, but are also of significant importance in male

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

development. DHT in the embryo life is responsible of the differentiation of the penis, scrotum and prostate. Later in life DHT also contributes to male balding, prostate growth and sebaceous gland stimulation (Hall et al, 2010) (Orwoll, 1998).

It had long been assumed that androgens played an analogous role to estrogens in male bone metabolism. This hypothesis was based largely on the clinical observation that hypogonadism was associated with osteopenia in men and on unambiguous data demonstrating direct androgen action *in vitro* and in animal studies (Orwoll, 1998) (Kasperk et al, 1989) (Pederson et al, 1999) (Bellido et al, 1995). Over the past several years, however, the role of androgens in male bone physiology has been increasingly questioned as data have emerged suggesting an important role for estrogens in male skeletal development and homeostasis. Most recent studies suggest that androgens and estrogens play independent and fundamental roles in regulating bone resorption in men. These studies also suggest that androgens may play an important role in the regulation of bone formation in men (Leder et al, 2003).

The CRH pathway:

Corticotropin releasing hormone (CRH) is a peptide hormone and neurotransmitter secreted by the PVN of the hypothalamus in response to stress. The portal system carries the CRH to the anterior lobe of the pituitary gland, where it stimulates corticotropes to secrete adrenocorticotropic hormone (ACTH) and other biologically active substances (i.e. β-endorphin)(Santos et al, 1999).

ACTH stimulates the secretion of glucocorticoid steroid hormones from adrenal cortex cells, mainly in the fasciculate zone of the adrenal glands. It acts by binding to

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

specific ACTH receptors that are mainly situated on adrenocortical cells of the adrenal cortex (Raikhinstein et al, 1994).

Glucocorticoids are a class of steroid hormones that bind to the glucocorticoid receptors, which are present in almost every vertebrate animal cell. Glucocorticoids play a major role in the regulation of glucose metabolism by the stimulation of gluconeogenesis in the liver, mobilization of amino acids from extra-hepatic tissues, inhibition of glucose uptake in muscle and adipose tissue, and by the stimulation of fat breakdown in adipose tissue (Pazirandeh et al, 2002).

Corticosteroids increase bone loss by decreasing gut absorption of calcium, and decrease bone formation by inhibiting collagen synthesis and osteoblastic protein synthesis. Corticosteroids do not affect mineralization (Hall et al, 2010).

The GHRH pathway:

Growth hormone releasing hormone (GHRH) is a 44-amino acid peptide hormone produced in the arcuate nucleus of the hypothalamus. It is then carried to the anterior pituitary gland through the hypothalamic hypophyseal portal circulation where it stimulates the secretion of growth hormone (GH) (Hall et al, 2010).

GH is a peptide hormone secreted from the anterior pituitary gland. It is involved in the regulation of many metabolic pathways in the body. Some of its effects are mediated by the insulin-like growth factor-I (IGF-I), which is produced in the liver. Both GH and IGF-I play an important role in the regulation of growth, bone metabolism and bone mass. GH causes a direct and indirect (through IGF-1) stimulation of osteoblast production and activity, which leads to an increase in bone to formation. It also stimulates osteoclast differentiation and activity, promoting bone resorption. The end

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

result is an increase in the rate of bone remodeling, and bone accumulation. The absence of GH can cause a reduction in the bone remodeling rate and a progressive loss of BMD (Hall et al, 2010) (Mark et al, 2012) (Ramachandran et al, 2007). GH has a direct effect on chondrocytes present in the epiphyseal growth plates. This effect is regulated mainly by IGF-I, which stimulates the proliferation of these cells matrix production. GH deficiency causes a severe deceleration of bone growth and bone mass accumulation (Olney, 2003). GH causes positive calcium balance by increasing gut absorption of calcium more than it increases urinary secretion. Other growth factors such as transforming growth factor β (TGF- β), platelet derived growth factor (PDGF), monokines, and lymphokines have roles in bone and cartilage repair (Mark et al, 2012).

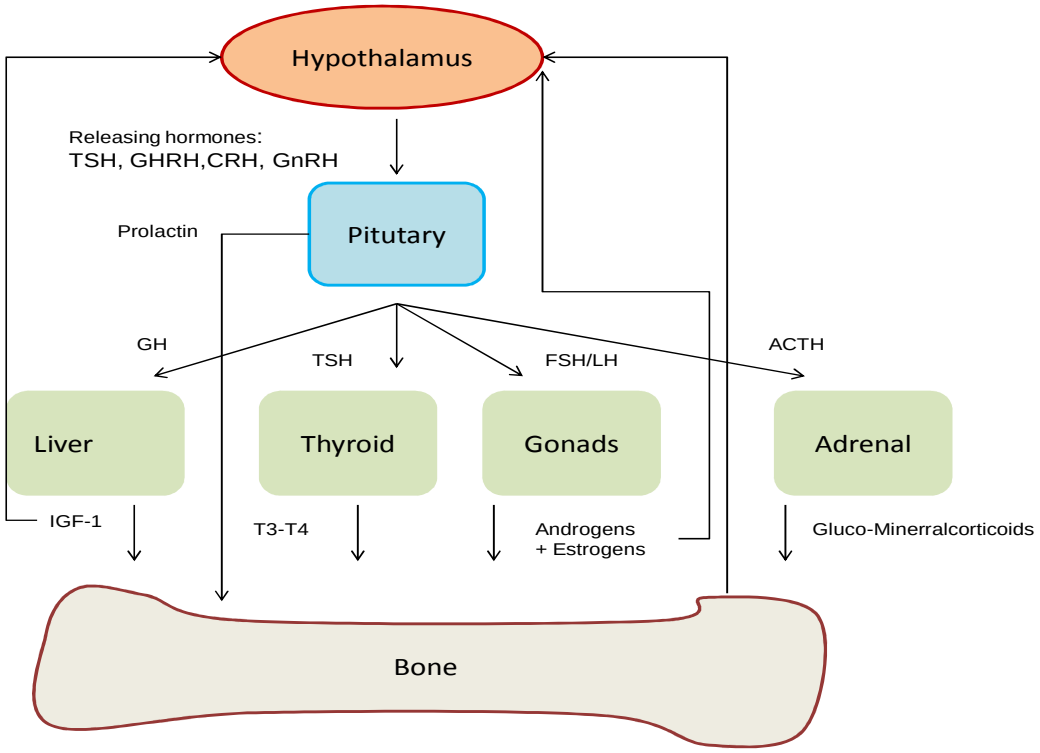


Figure 6: The hypothalamic-pituitary-axis. Abbreviations: TSH, thyroid-stimulating hormone; GHRH, growth hormone releasing hormone; CRH, corticotropin-releasing hormone; GnRH, gonadotropin-releasing hormone; GH, growth hormone; TSH, thyroid

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

stimulating hormone; LH, Luteinizing hormone; FSH, follicle-stimulating hormone; ACTH, adrenocorticotropic hormone.

2.2.1.2 Energy-bone-adipose tissue-adrenergic sympathetic nervous system axis

The association between body weight and bone density has pointed to the possible existence of a common factor that regulates both body weight and bone mass (Elefteriou et al, 2008). The hypothalamic and brainstem centers, as well as the SNS, have been identified as regulators of bone remodeling. However, the mechanism by which the afferent CNS stimuli regulate SNS centers involved in the control of bone remodeling remains unclear.

Primary osteoblasts express a functional β2 adrenergic receptor but none of the other post-synaptic adrenergic receptors (Takeda et al, 2002), this suggests that osteoblasts are receptive to sympathetic tone in vivo. Multiple clinical studies have demonstrated that the inhibition of the SNS can increase the BMD and reduce the risk for any fracture whereas the hyperstimulation of the SNS can have the opposite effect. Experimental studies have shown similar results. For example, a recent study has demonstrated that mice and rats treated with the non-selective β adrenergic blocker propranolol had an increase in their bone mass; whereas mice treated with the non-selective beta-agonist isoproterenol had a significant loss of their bone mass. Adrenergic receptor selective agonists such as clenbuterol or salbutamol appeared to lead to a low bone mass. These results demonstrate that the SNS controls bone formation via β receptors in osteoblasts (Bonnet et al, 2005).

The sympathetic regulation of bone remodeling also appears to be affected by the vestibular system. A recent study has shown that the induction of a bilateral vestibular

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

lesion in rats can generate a significant bone loss, which is limited to weight-bearing bones and associated with a significant decrease in bone formation (Vignaux et al, 2013).

Another fundamental component of the energy-bone-adipose tissue-sympathetic axis is Leptin. It is a 16 KDa adipocyte derived protein hormone that regulates major physiological functions such as body weight (appetite/hunger) and reproduction (through the hypothalamus and its ObRb receptor). Once released from adipose tissue, it can cross the blood–brain barrier and bind to its receptors in the brain, where it stimulates the SNS and regulates bone metabolism (Satoh et al, 1999). Leptin injections can lead to an increase in the sympathetic tone, and short-term administration of leptin into the cerebral ventricles can increase the renal sympathetic activity (Hall et al, 2001). In addition to its effects through the brain, it may act directly on cells in bone tissue to regulate bone metabolism. Leptin probably signals to bone on a local and systemic level balancing and regulating the final outcome. As a result, leptin acts to reduce cancellous bone and increase cortical bone. A number of theories have been postulated in order to explain this double function, including a recent theory suggesting that increased leptin during obesity may represent a mechanism for enlarging bone size and thus bone resistance to cope with increased body weight (Hamrick et al, 2008). The clinical utility of leptin for treatment of bone diseases remains open, but ongoing research may provide new therapies for stimulating bone formation (Takeda et al, 2002).

Bone marrow adipocytes can store significant amounts of fat and produce adipokines, leptin and adiponectin, which are all involved in the regulation of energy metabolism. On the other hand osteoblasts produce osteocalcin, a bone specific hormone that regulates insulin production in the pancreas and adiponectin production in adipose

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

tissue. Both osteoblasts and bone marrow adipocytes express insulin receptors and respond to insulin-sensitizing anti-diabetic thiazolidinediones (TZD) in a manner, that tightly links bone with the energy metabolism system. Adipose tissue in bone marrow resembles that of both, white and brown fat. It can acquire different functions such as bone homeostasis and response to physiologic and pathologic changes in energy metabolism status by changing volume and metabolic activity (figure 7) (Lecka-Czernik, 2012).

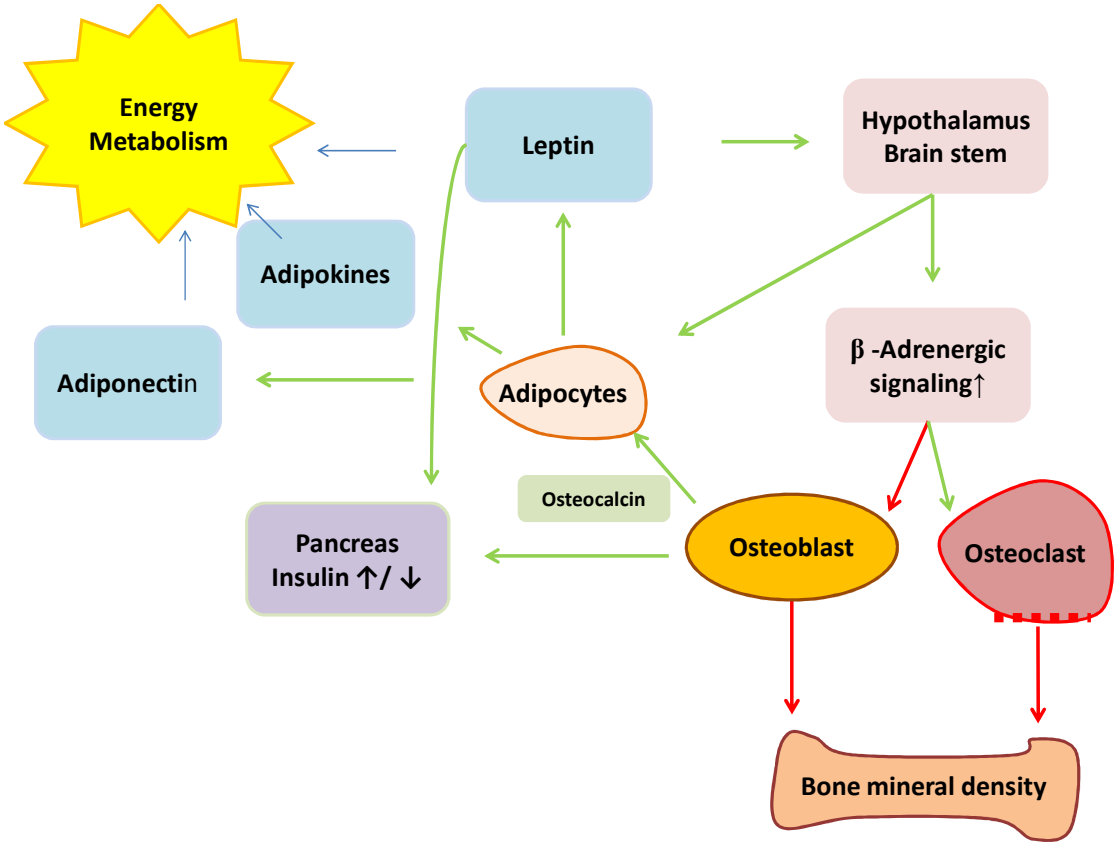


Figure 7: The energy-bone-adipose tissue-sympathetic nervous system axis.

The presence of multiple types of nerves within the bone microenvironment and periosteum suggests that additional neuropeptides and receptors might be involved in the regulation of bone remodeling. The expression of neuropeptide receptors in other cells than osteoblasts within bone tissue suggested that neuronal signaling may regulate

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

bone remodeling indirectly, through certain cell lineages such as osteoclasts, adipocytes or immune cells (Bonnet et al, 2005).

2.2.1.3 The parathyroid-vitamin D-calcitonin axis

PTH is an 84-amino acid peptide; it is synthesized in and excreted from the chief cells of the four parathyroid glands. The N-terminal fragment 1-34 is the active portion. The effect of the PTH is mediated by the cAMP second-messenger mechanism.

Decreased calcium and magnesium levels in the extracellular fluid stimulates chief cells to release PTH, which acts at the intestines (increasing absorption of calcium), kidneys (increasing calcitriol secretion and decreasing calcium excretion), and bones (releasing calcium and phosphorus). Osteoblasts have specific PTH receptors that when stimulated increase interleukin-1 (IL-1) cell secretion which increases osteoclastic activity. The end effect of PTH is an increase in calcium blood levels and a decrease in phosphate levels (Mark et al, 2012).

Vitamin D is a naturally occurring steroid that is activated by ultraviolet radiation from sunlight or utilized from dietary intake. Hydroxylated to 25 (OH)-vitamin D_3 in the liver and hydroxylated a second time in the kidneys to one of the following:

-24, 25$(OH)_2$ -vitamin D3, the inactive form.

- Calcitriol (1, 25 $(OH)_2$ -vitamin D3), the active form which stimulates intestinal absorption of calcium and phosphate, osteoblastic differentiation and osteoclastic resorption of bone. calcitriol production is regulated by PTH (Van-Driel et al, 2004) (Takeda et al, 1999).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Calcitonin is a 32-amino acid peptide hormone produced by clear cells in the parafollicular cells of the thyroid gland. Increase extracellular calcium levels cause secretion of calcitonin which inhibits osteoclastic resorption of bone and decreases calcium serum levels. However calcitonin has a limited role in calcium regulation. Osteoclasts have specific calcitonin receptors that when stimulated cause a decrease in the number and activity of the osteoclasts. Calcitonin may also play a role in the process of fracture healing (figure 8) (Ramachandran et al, 2007).

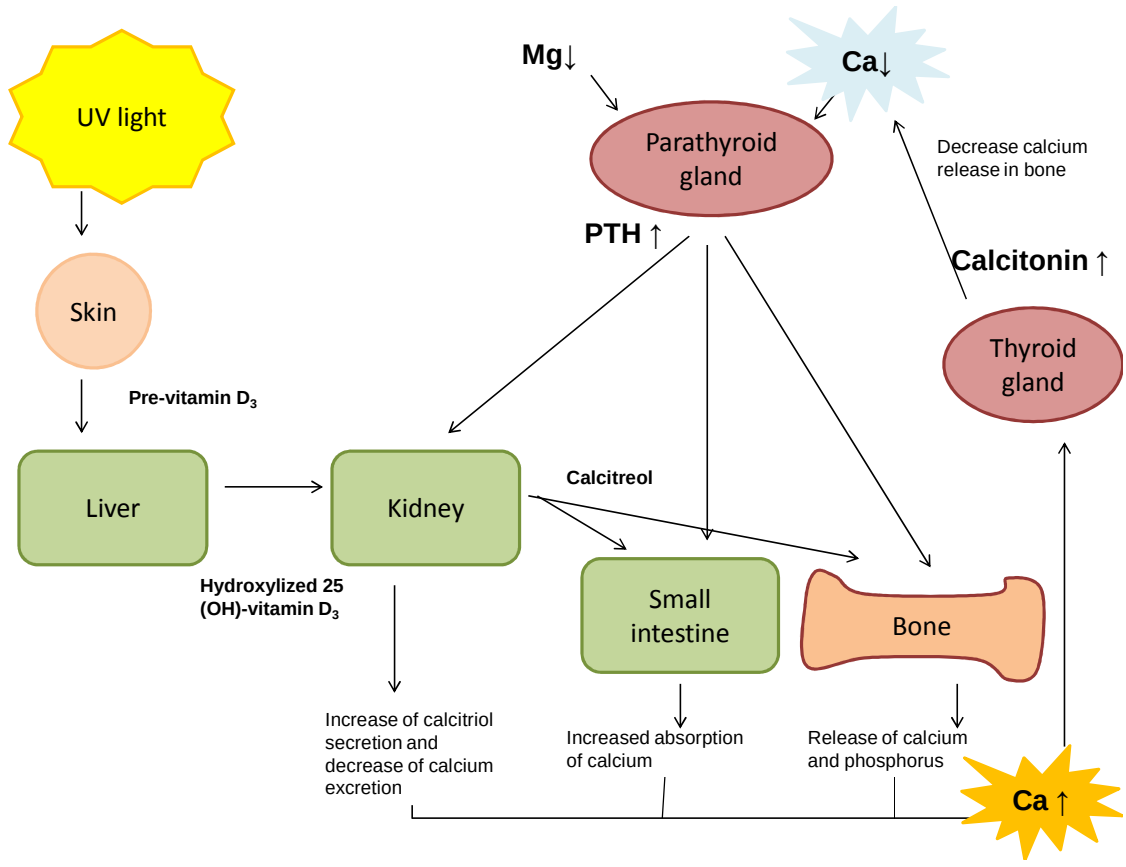


Figure 8: The parathyroid-vitamin D-calcitonin axis. PTH; parathyroid hormone.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.2.1.4 The bone IL1-cholinergic sympathetic nervous system axis

It has been shown that bone tissues are innervated by cholinergic fibers from the sympathetic cholinergic system. Fibers from the sympathetic cholinergic system transmit neuronal signaling from nuclei in the CNS into cholinergic receptors located in bone. It would be logical to expect that disruption in the function of these cholinergic fibers would affect bone. (Bajayo et al, 2012) (Sisask et al 1996) (Tien et al, 2003). Cholinergic fibers of the SNS have been shown to innervate progenitor cells that are present in the periosteum that evolve into osteoblasts, indicating a possible role of these neuronal fibers in regulating bone formation and remodeling. Indeed, the denervation of the periosteum results in poor bone healing in animal models, indicating that periosteal nerves are required for bone formation and fracture healing (Asmus et al, 2000) (Song et al, 2012) (Frymoyer et al, 1977) (Aro et al, 1981). The PSNS also seem to regulate the process of bone remodeling through the cholinergic fibers of the vagus nerve, it has been shown that mice subjected to subdiaphragmatic vagotomy suffer from low bone mass in their lumbar vertebrae (Bajayo et al, 2012).

The mechanism by which the cholinergic system affects bone remodeling is not well understood. It is still not known whether the cholinergic activity affects bone cells locally, through an autocrine-paracrine pathway or systemically, through a neuronal-endocrinal pathway. The presence of cholinergic components in bone, as many non-neuronal cells, might indicate that bone could be regulated by the cholinergic activity through an autocrine-paracrine pathway.

Central IL-1 is a proinflammatory cytokine produced by brain neuron cells, and it is known to regulate learning, memory, sleep patterns, and could also affect bone metabolism. Knockout central IL-1 mice are osteoporotic due to the down-regulation of osteoclasts apoptosis. A recent study has shown that the mechanism by which central

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

IL-1 regulates bone remodeling is mediated by the cholinergic nerve fibers of the PSNS. In addition to low bone mass, knockout central IL-1 mice express very low levels of skeletal acetylcholine even after the administration of cholinergic agonist. Accordingly, these findings suggest that central IL-1 controls bone remodeling through regulation of the cholinergic SNS (figure 9) (Bajayo et al, 2012).

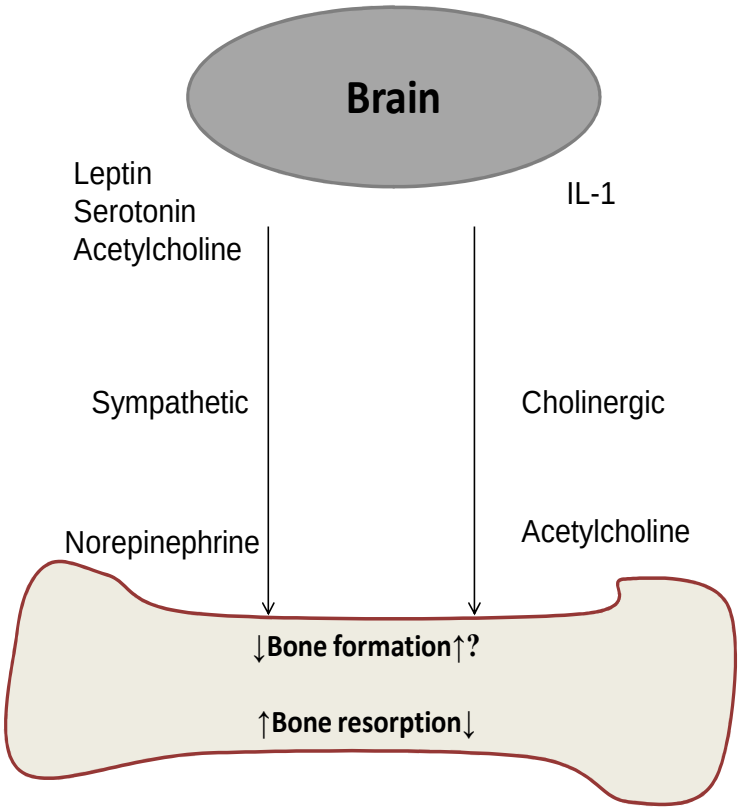


Figure 9: The bone- IL1- cholinergic sympathetic nervous system bone axis. IL; Interleukin-1.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.2.2 Local regulation of bone remodeling

2.2.2.1 Wolff´s law and bone remodeling

Bone remodels in response to mechanical stress. An increased load on a particular bone will stimulate bone remodeling and will therefore make it stronger (Frost et al, 1994). The internal architecture of the trabeculae initially undergoes adaptive changes; these are followed by secondary changes at external cortical bone. On the other hand, the bone will become weaker in the absence of a continuous stress due to an increased bone turnover (Wolff et al, 1986).

The mechanism by which bone remodeling is achieved in response to a mechanical stress is called mechano-transduction. This is a process in which mechanical signals are translated into biochemical and cellular signals (Huang et al, 2010). The process of mechano-transduction leads to bone remodeling in several steps: mechano-coupling, biochemical coupling, signal transmission, and cell response. The end effect on bone structure depends on the duration, magnitude and rate of the stress and it has been noticed that only cyclic loading can induce bone remodeling (Duncan et al, 1995). When loaded, fluid flows away from high pressure areas in the bone matrix (Turner et al, 1994). Osteocytes are the most abundant cells in bone tissue and are highly sensitive to changes in fluid flow caused by a mechanical stress. An increase in the matrix fluid flow will stimulate osteocytes to signal other cells and start the remodeling process. Moreover, osteoprogenitor cells are also mechano-sensitive and may differentiate into osteoblasts or osteoclasts depending on the stress condition (Chen et al, 2010).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.2.2.2 Piezoelectic charges

The direct piezoelectric effect is defined as the ability of a material to generate an electrical potential under tension or compression. On the other hand, the inverse piezoelectric effect is defined as the bending caused in piezoelectric materials, such as ceramics and crystals, when exposed to an electric field. It is believed that hydroxyapatite is responsible for piezoelectricity in bones. When collagen molecules are stressed, charge carriers move from the inside to the surface of the specimen, and produce an electric potential throughout the bone (McDonald, 1993).

The electronegative charge produced by the piezoelectric effect will stimulate osteoblasts formation. These cells will subsequently deposits minerals primarily calcium on the stressed side of the bone. Therefore, the piezoelectric effect increases bone density. On the other hand the negative charge created at the tension side will stimulate osteoclastic activity and decrease bone formation (McDonald, 1993) (Williams et al, 1996).

2.2.2.3 Hueter-Volkmann Law

This principle was first described by Hueter in 1862. It originally stated that compression and tension forces could alter growth at the level of the epiphyseal plate (Gocht, 1920) (Mehlman et al, 1997). This concept was later on generalized beyond joint growth and extended to describe growth and remodeling in long bones. It suggests that a moderate increase in stress would first lead to an increased growth while an excessive stress would cause to retardation (Frost, 1990) (Stokes, 2002) (Rauch, 2005) (Mau, 1984) (Stein et al, 1998).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

The mechanical modulation of longitudinal bone growth by compressive forces is widely accepted. However, tension, torsion and bending forces also seem to be involved in longitudinal, rotational and angular development of bone. While growth responds to a persistent load, bone remodeling responds mainly to transient loading. Therefore it would be reasonable to think that if bone growth responded to transient forces, then more active children could achieve a higher stature than less active infants. However, recent studies suggest that intermittent large compressive forces do reduce endochondral growth in the rat forelimb. A sustained compression of physiological magnitude inhibits growth by 40% or more (Stokes, 2002).

2.2.2.4 Bone signaling and RANKL

Factors such as IGFs, prostaglandins, TGF-β, bone morphogenetic proteins (BMP), and cytokines are also involved in the local regulation of bone remodeling. A large number of cytokines and growth factors that affect bone cell functions have been identified. The processes of bone resorption and formation are tightly coupled through the receptor activator of nuclear factor kappa B (RANK)/ RANK-ligand (RANKL)/ osteoprotegerin (OPG) system allowing a wave of bone formation to follow each cycle of bone resorption and maintaining skeletal integrity (Hadjidakis et al, 2006).

RANK, RANKL, and OPG interact with precursor cells, mature osteoblasts and osteoclasts to mediate bone resorption. RANKL is secreted by osteoblasts and binds to the RANK receptor on osteoclast precursor and mature osteoclast cells leading to the stimulation of bone resorption. PTH and 1,25 dihydroxy vitamin D stimulate RANKL expression. On the other hand OPG is a protein produced by osteoblasts and stromal cells that binds to RANKL inhibiting osteoclastic differentiation, fusion, and activation. Calcitonin inhibits osteoclastic activity by a direct interaction with the osteoclast via

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

cell-surface receptors. Estrogen stimulates bone production due to a decrease in RANKL and an increase in bone production. TGF-β increases in OPG production and thus decreases bone resorption. Interleukin 10 (IL-10) suppresses osteoclastic activity while IL-1 and IL-6 contribute to the recruitment of osteoclasts from the monocyte cell line (Barnes et al, 2008) (Athanasou, 1996) (Zaidi et al, 2003).

BMPs are a group of growth factors produced by osteoclasts known to induce the formation of bone and cartilage. They appear to be involved in a series of morphogenetic signals that regulate tissue architecture throughout the body (Reddi et al, 2009) (Bleuming et al, 2007). There are more than 20 distinct BMPs subunits which together constitute a sizable component of the larger TGF-β ligand family (Feng et al, 2005) (Miyazono et al, 2005).

BMPs interact with specific receptors on the cell surface, which are called bone morphogenetic protein receptors (BMPRs). Signal transduction through BMPRs results in mobilization of members of the sma & Mad genes (SMAD) family of proteins. The signal pathways that involve BMPs, BMPRs and SMADs are thought to be key regulators in certain physiologic processes such as embryonic osteogenesis, endochondral ossification, bone remodeling, fracture repair, and bone regeneration (Hogan, 1996) (Grimsrud et al, 1999) (Wozney et al, 1998) (Groeneveld et al, 2000) (Reddi, 1995) (Onishi et al, 1998) (Ripamonti et al, 1998). BMP-2 and BMP-7 are both osteoinductive, they have been demonstrated to potentially induce osteoblast differentiation in a variety of cell types (Marie et al, 2002). The implantation of BMPs in bone can result in a series of cellular events including mesenchymal cell infiltration, cartilage formation, vascularization, bone formation, and

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

remodeling of the new bone tissue along with hematopoietic bone marrow elements (Reddi, 1981).

Positive staining was seen in osteoclasts for BMP 2, 4, 6, and 7. PCR was used to quantitatively confirm the presence of transcripts for BMP-2, BMP-4, and BMP-6 mRNA in murine osteoclasts (Garimella et al, 2008). The action of BMPs can be inhibited by sclerostin which is a glycoprotein secreted by osteocytes and some chondrocytes (Li et al, 2005).

These observations suggest a possible direct role for osteoclasts in the stimulation of bone formation via the expression and production of BMPs that would play an important role in enhancing the recruitment, proliferation, and differentiation of osteoblasts at sites of bone resorption.

Finally, osteocalcin is a non collagenous protein exclusively secreted by osteoblasts that also appears to play a significant role in the body's metabolic regulation and bone formation. It has a pro-osteoblastic effect and seems to be implicated in bone mineralization and calcium ion homeostasis (figure 10) (Lee et al, 2007).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

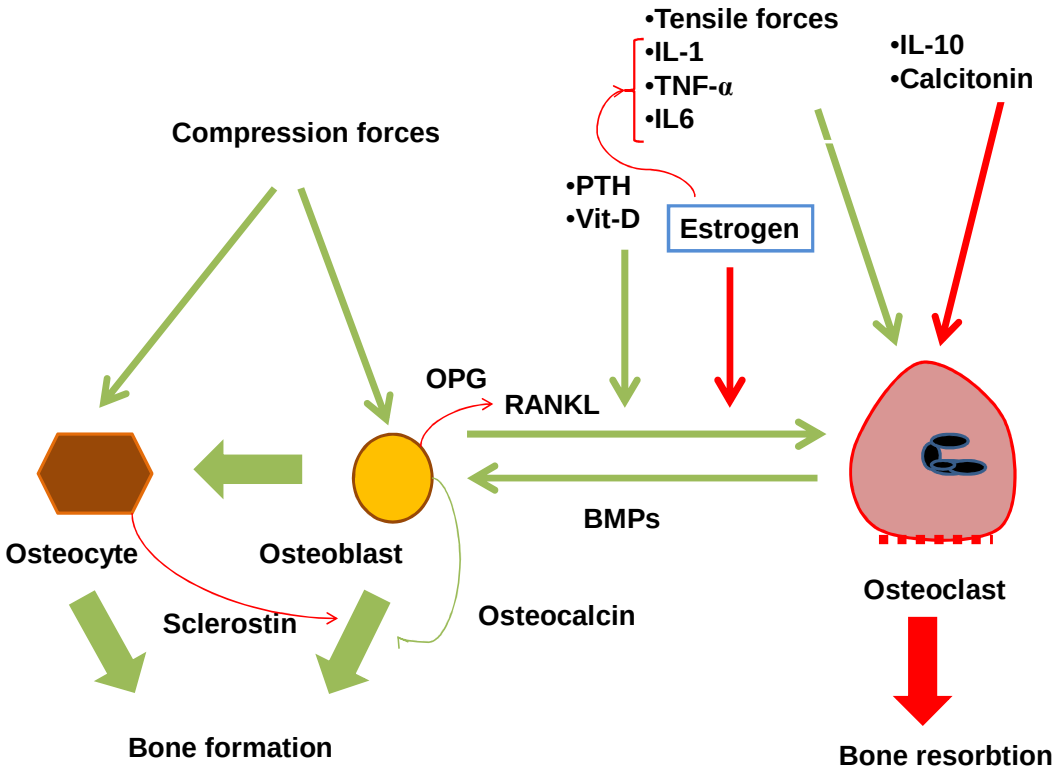


Figure 10: The most important pathway involved in the local regulation of bone remodeling. Abbreviations: IL, interleukin; PTH, parathyroid hormone; OPG, osteoprotegerin; RANKL; receptor activator of nuclear factor kappa B ligand; Vit-D, vitamin-D.

2.3 Bone abnormalities associated to alterations in the autonomic nervous system

Cholinergic-regulated tissues

The cholinergic activity regulates many organs in which cholinergic receptors have been identified such as: salivary cells, lacrimal cells, Langerhans cells, the respiratory system, the gastrointestinal system and the vestibular organ within the ear (table 3). A decrease in the cholinergic activity at the salivary glands results in a dry mouth (xerostomia) (Grisaru et al, 1999) (Caulfield, 1993) (Hegde et al, 1997) (Bacman et al,

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

1998) (Furness, 2000) (Gautman et al, 2007) (Hong et al, 1990) (Matsuoka et al, 1975) (Wang et al, 2004). Similarly, suppression of the cholinergic activity in the lacrimal glands results in dry eyes (xerophthalmia). Damage to M3 receptors in Langerhans cells result in diabetes type I. It is remarkable that several articles in the literature have reported a strong association of an unknown etiology between these medical conditions (xerostomia, xerophthalmia and diabetes type 1) and bone loss. However, the evidence we present in this study seems to indicate that bone loss in these medical conditions could be due to alterations in the cholinergic activity (Park et al, 2011) (Agha-Hosseini et al, 2011) (Moss et al, 2000) (Liu et al, 2003) (Tuominen et al, 1999) (Arends et al, 1988) .

The suppression of cholinergic activity is also known to cause the onset of serious pathologies such as obstructive pulmonary diseases, disruption in the body's circadian rhythm and learning memory deficits. Interestingly, all these conditions have as a common skeletal feature, a reduction in BMD. Another interesting condition which is related to cholinergic activity is vertigo. Vertigo patients are characterized by dizziness and being off-balanced as a consequence of a damage to the vestibular end organ in the ear, a cholinergic-regulated organ. Patients suffering from vertigo are associated with higher risk of osteoporosis (Bhattacharyya et al, 2011) (Gispen et al, 2000) (Mirakhur et al, 1978) (Kesler et al, 1999) (Katz et al, 2010) (Yaturu et al 2009) (Baloh et al, 1987).

Circadian rhythm

An interesting phenomenon that links cholinergic activity with bone remodeling is the circadian rhythm. The ANS follows a circadian pattern that matches the bone remodeling cycle. Sympathetic activity is dominant during day hours when bone resorption activity reaches its peak, while the parasympathetic activity is intense during

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

night hours when bone formation is more active (Nielsen et al, 1991) (Pressman et al, 1989).

Menopause

Another observation that links cholinergic activity to bone is the estrogen replacement therapy, a hormonal treatment for osteoporosis. Menopausic patients treated with estrogen replacement therapy have a higher bone mass and lower fracture risk when compared to control groups. Surprisingly, these patients express an increase in cholinergic activity (Nikolov et al, 1998) (No authors, 1996).

Smokers

Clinical studies have shown that heavy smoking is associated with a decreased bone mass and diminished fracture healing capacity. Even though many hypotheses have been postulated to explain this phenomenon, it has been found that the association between smoking and bone is strongly related to nicotine levels in the body. Even though low concentration of nicotine up-regulates osteoblasts through the activation of the nicotinic receptors, excessive levels down-regulate osteoblasts through the desensitization of the nicotinic receptors. Also excessive nicotinic levels up-regulate osteoclasts through the activation of the nicotinic receptors (Hollinger et al, 1999) (Wong et al, 2007) (Chen et al 2011) (Zheng et al, 2008) (Walker et al, 2001) (Rothem et al, 2009) (Bajayo et al, 2012).

Poliomyelitis, Botox injection and Myasthenia gravis

Cholinergic neurons innervate muscle fibers, and their activity can affect bone mass through mechanical stress. Indeed, it has been argued whether the muscle activity,

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

rather than the neuronal activity, is the main regulator of bone mass. However, the following observations argue strongly against a model where the muscle activity is more important than neuronal activity in regulating bone mass. Underneath, we discuss how three conditions, known to affect neuro-muscular synapses: Poliomyelitis, Botox injections and myasthenia gravis, affect bone health status. Poliomyelitis is a viral disease that destroys motor neurons that utilize acetylcholine neurotransmitter resulting in muscle paralysis. Patients diagnosed with poliomyelitis are known to suffer from impaired bone growth affected by nerve depletion. Interestingly, it has been found that after the recovery of muscle activity, polio patients develop osteoporosis in much higher rates than the rest of the population, indicating that the effect of polio virus on bone tissue is unrelated to muscle function (Koenen et al, 2005) (Maselli et al, 1995) (Mohammad et al, 2009).

Botox, also known as botulinum neurotoxin, prevents the release of acetylcholine from its membrane vesicles at the terminal ends of cholinergic neurons resulting in muscle paralysis. Botulinum neurotoxin causes bone loss that does not improve after the recovery of muscle function. Moreover, bone loss due to botulinum neurotoxin has been linked to an increase in bone resorption due to osteoclasts up-regulation, a phenomena that is recently associated to the inhibited activity of the cholinergic system. These findings indicate that the effect of botulinum neurotoxin on bone tissue is unrelated to muscle function.

Myasthenia gravis is a disorder that is characterized with suppressed muscular activity due to autoantibodies blocking the cholinergic receptors in muscle fibers. Surprisingly, despite the low muscular activity, patients diagnosed with myasthenia gravis are not associated with the risk of developing bone diseases. In contrast, the prevalence of

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

osteoporosis in myasthenia gravis patients is significantly lower compared to the general population. Myasthenia gravis only affects acetylcholine receptors in muscles leaving the cholinergic signaling in bone intact, which might explain why bone is not affected in these patients (Hambleton et al, 1992) (Warner et al, 2006) (Grimston et al, 2007) (Pouwels et al, 2013) (Wakata et al, 2004).

The linking findings described in the above three conditions indicate that the cholinergic inervation is necessary and might be more important than muscle activity for healthy bone remodeling. However, future studies will have to be performed in order to confirm the role of cholinergic activity in defining the bone phenotype of patients with poliomyelitis, myasthenia gravis or following Botox injections (table 5) (Hambleton et al, 1992) (Warner et al, 2006) (Grimston et al, 2007) (Pouwels et al, 2013) (Wakata et al, 2004).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Body tissue that express cholinergic receptors	Function of local cholinergic receptors	Effect of inhibition of local cholinergic receptors	Association between local inhibition of cholinergic receptors and bone mineral density (BMD)
Salivary Glands	Saliva secretion	Dry mouth	Patients with dry mouth are associated with low BMD
Lacrimal glands	Lacrimal secretion	Dry eyes	Patients with dry eyes are associated with low BMD
Pancreas	Insulin secretion	Diabetes type I	Patients with type I diabetes have low BMD
Respiratory system	Smooth muscle contraction and epithelial regulation	Obstructive lung disease	Patients with lung diseaases have low BMD
Gastrointestinal system	Smoth muscle contraction	Decrease muscle tone	Patients with certain Gastrointestinal diseases have low BMD
Vestibular organ within ear	Balance regulation	Vertigo	Patients with vertigo are associated with low BMD

Table 5: The relationship between cholinergic receptors in body tissues with bone turnover in various clinical conditions. BMD; Bone mineral density.

2.4 Pathology of bone remodeling: Osteoporosis

Osteoporosis is defined by the World Health Organization (WHO) as a BMD of 2.5 standard deviations or more below the mean peak bone mass (average of young, healthy adults) as measured by dual-energy X-ray absorptiometry (DEXA). In established osteoporosis a fragility fracture must also be present (WHO, 1994). The disease may be classified as primary type 1, primary type 2, secondary and idiopathic juvenile. Osteoporosis is characterized by a progressive loss of bone mass and microarchitecture which leads to increased risk of suffering fractures (mainly vertebrae, hip, proximal humerus and distal radius) (Brian et al, 2009) (Lippuner et al, 2012). It is

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

a widespread disease and around 200 million people worldwide are affected by this condition, making it a major health and economic problem.

The most common form of osteoporosis is typically present in postmenopausal women and it is referred to as primary type 1 osteoporosis. Primary type 2 osteoporosis occurs in elderly patients above 75 years of age and it can affect both sexes at a female-male ratio of 2:1. Secondary osteoporosis can appear at any age and affects men and women equally. This form results from predisposing medical conditions, or from the prolonged use of medications such as glucocorticoids (Mark et al, 2012).

Another form of osteoporosis is idiopathic juvenile osteoporosis which affects children between 8 and 14 years and is characterized by diffused bone pains, generalized osteoporosis and a negative calcium balance. This is a self limiting condition and a complete recovery achieved in most of the cases (Lapatsanis et al, 1971).

2.4.1 The pathophysiology of osteoporosis

Under physiologic conditions, bone formation and resorption are well balanced. An increased in bone resorption or decreased in bone formation may cause osteoporosis. Osteoporosis can result from a failure to reach an acceptable peak bone mass during young age and by bone loss later in life. As we mentioned earlier osteoporosis can result from an altered hormonal status or secondary to various diseases and medications. The ageing process and loss of gonadal function are the two most important factors related to the development of osteoporosis. In the first years after menopause the lack of gonadal hormones (mainly estrogen) is thought to increase the osteoclastic activity, due to an over expression of RANKL by osteoblasts and a decrease in the release of OPG (figure 10). The increase in RANKL results in recruitment of higher numbers of

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

preosteoclasts and an increase in the activity and lifespan of mature osteoclasts (Jilka et al, 1992) (Ramachandran et al, 2007).

Estrogen can also affect bones indirectly through cytokines and local growth factors. A lack of estrogen may stimulate T cells to promote the differentiation, recruitment and survival of osteoclasts via IL-1, IL-6, and tumor necrosis factor α (TNF-α) (figure 10) (De et al, 1992).

A study in which mice were ovarectomized, found that IL-6 and granulocyte-macrophage colony forming unit (CFU) levels were significantly increased. These results provided evidence that estrogen inhibits IL-6 secretion and that IL-6 contributes to the recruitment of osteoclasts from the monocyte cell line, thus contributing to osteoporosis (figure 10).

IL-1 has also been involved in the production of osteoclasts. IL-1 production is increased in bone marrow mononuclear cells in ovarectomized rats. The administration of IL-1 receptor antagonist to these rats can prevent bone loss secondary to ovarian dysfunction, but it does not prevent bone loss in early. The increase in IL-1 seems to take place as a result of the removal of the inhibitory effect of sex steroids on IL-6 and other genes that are directly regulated by sex steroids. IL-7 secreted by T cells also inhibits osteoblast differentiation and activity and cause premature apoptosis of osteoblasts (Jilka et al 1992).

Bone loss that accompanies the aging process is related to a decrease in the differentiation of osteoblasts in relation to the demand. This demand is determined by the frequency with which new BMU are created and new cycles of bone remodeling are initiated. Bone resorption starts to exceed bone formation after the third decade of life

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

this leads to osteopenia and, in some cases to osteoporosis. Women may lose up to 30-40% of their cortical bone and 50% of their trabecular bone over their lifetime. On the other hand men lose around 15-20% of their cortical bone and 25-30% of the trabecular bone (Hall et al, 2010) (Mark et al, 2012) (Ramachandran et al, 2007).

Insufficient calcium intake or impaired intestinal absorption of calcium due to aging or disease can lead to secondary hyperparathyroidism. PTH secretion is increased due to low serum levels of calcium, which will cause an increase in calcium resorption from bone, a decrease in the renal excretion of calcium, and an increase in the renal production of 1, 25-dihydroxyvitamin D which is necessary in calcium and phosphorus absorption. On the other hand vitamin D deficiency can result in a secondary hyperparathyroidism due to a decrease in intestinal calcium absorption (Bono et al, 2003). All these conditions will cause a decrease in bone calcium concentration and eventually lead to osteoporosis.

Prenatal and postnatal factors can also affect the bone mass in adult age. In one study, the mother's health during pregnancy, the infant's birth weight, and the child's weight at age 1 year were predictive of adult bone mass in the seventh decade for both men and women (Dennison et al, 2005). It is postulated that growth in the first year of life programs growth hormone which is then maintained into the seventh decade. Larger babies and rapid growth in the first year of life predicted increased bone mass in adults aged 65-75 years (Fall et al, 1998).

2.4.2 Risk factors

Non modifiable risk factors include female gender, postmenopausal estrogen deficiency, decreased testosterone levels in men, European or Asian ancestry, family history of

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

osteoporosis, and small stature. A total of 30 genes seem to be associated to the development of osteoporosis (Melton et al, 2003) (Raisz et al, 2005) (Ojo et al, 2007) (Brian et al, 2009).

Modifiable factors include vitamin D deficiency, tobacco smoking, soft drinks with an acidic composition, dietary deficiencies such as calcium, magnesium, zinc, iron, vitamins A, E, K, high protein diets and alcohol excess (WHO, 2003) (Poole et al, 2006) (Wong et al, 2007) (Jain et al, 2000) (Hegsted et al, 1981) (Shapses et al, 2006) (American Academy of Pediatrics, 2004).

Medical conditions associated with osteoporosis

Osteoporosis is associated with multiple medical conditions such as chronic renal failure, hypogonadism, hyperthyroidism, prolonged periods of immobilization, Cushing syndrome, hyperparathyroidism, myeloproliferative disorders, in addition to a variety of syndromes and hereditary conditions (i.e. Marfan syndrome, Turner syndrome, and osteogenesis imperfecta) (Simonelli et al, 2006) (WHO, 2003).

Medications associated with the development osteoporosis

A number of medications have been associated with osteoporosis when used for prolonged periods of time: glucocorticoids, barbiturates, phenytoin, thyroxine, proton pump inhibitors, heparin, warfarin, progesterone, methotrexate, lithium and TZD (Yang et al, 2006) (Murphy et al, 2007) (Gourlay et al, 2007) (Petty et al, 2007) (Gage et al, 2006).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.4.3 Osteoporotic fractures

Osteoporotic fractures represent the clinical presentation of osteoporosis. They can result both from low energy trauma (i.e. from sitting or standing position), and from high-energy trauma (i.e. motor vehicle accidents).

Fractures occur when bones are exposed to an excessive stress. The frequency and direction of the falls can influence the severity, and features of the fractures. The risk of falling may be increased by neuromuscular impairment due to vitamin D deficiency with secondary hyperparathyroidism or corticosteroids (Cummings et al, 2002).

An understanding of the biomechanics of bone provides greater understanding as to why the risk of fracture may increase in certain circumstances. A significant amount of the bone strength is derived from the horizontal trabecular intercrossing system. This system of horizontal intercrossing trabeculae assists in the support of vertical elements, therefore limiting lateral bowing and fractures that may occur with vertical loading (Mark et al, 2012).

The disruption of the trabecular connections is known to occur in patients with osteoporosis, particularly in postmenopausal women. Rosen and Tenenhouse studied the unsupported trabeculae and their susceptibility to fractures within each vertebral body and found a very high prevalence of trabecular fracture callus sites within vertebral bodies examined at autopsy, between 200-450 healing or healed fractures per vertebral body. These horizontal trabecular fractures are asymptomatic, and their accumulation reflects the impact of lost trabecular bone and greatly weakens the cancellous structure of the vertebral body (Rosen et al, 1998).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.4.7 Hip fractures

Hip fractures are becoming a major health and economic problem in many countries. Worldwide, the total number of hip fractures is expected to reach 6 million per year by 2050. A total of 310,000 individuals were hospitalized with hip fractures in the United States (US) alone in 2003, accounting for 30 percent of all hospitalized patients. Approximately one third (101,800) of these patients underwent a hip replacement. The estimated cost spent in the treatment of these patients is approximately $ 10.3 to $ 15.2 billion per year in the US (Kannus et al, 1996). Moreover, hip fractures substantially increase the risk of death in elderly patients and are also a major cause of morbidity. One year mortality rates have ranged from 12 to 37 percent in these patients (Bentler et al, 2009) (Panula et al, 2011).

Hip Fractures are divided into two main groups (intracapsular fractures, and extracapsular fractures) depending on the fracture pattern which is directly related to the blood supply compromise. The blood supply of the femoral head comes mainly from the medial femoral circumflex artery (through the lateral epiphyseal artery), and secondary from the lateral circumflex artery and ligamentum teres (Mark et al, 2012).

2.4.7.1 Intracapsular fractures

These fractures are more common in white elderly women, and are becoming more common due to the increasing elderly population worldwide. They occur due to high energy trauma in young patients and low energy falls in older patients. Intracapsular fractures have a limited healing capacity due to the lack of a periosteal layer in the femoral neck and limited callus formation. Moreover a displacement of the femoral neck fracture will disrupt the blood supply and cause a femoral head necrosis and an

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

intracapsular hematoma. An associated femoral shaft fracture can be present in 6-9% of the cases (Mark et al, 2012) (Jay et al, 2009).

Garden classified these fractures into four types according to the displacement:

- Type I: Incomplete, valgus impacted
- Type II: Complete. Non-displaced
- Type III: Complete, displaced < 50%
- Type IV: Complete, displaced

On the other hand these fractures can also be classification according to their location:

- Subcapital
- Transcervical
- Basicervical

In undisplaced fractures the patient usually complains from moderate pain in the groin or pain referred along the medial side of the thigh and knee, with no obvious clinical deformity and minor discomfort with passive or active movements. In displaced fractures the pain is more intense and affects the entire hip region. The leg appears in external rotation and abduction, with shortening. An anterior-posterior pelvic x-ray and an axial projection should be performed in all cases. CT scan is useful in determining displacement and degree of comminution in some patients. MRI and bone scan are helpful to rule out occult fractures (Mark et al, 2012) (Jay et al, 2009).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Several factors should be taken into consideration in the treatment of intracapsular fractures including, the patients age, the presence a hip arthritis, previous mobility and comorbidities, femoral neck-shaft angle (normal 130º +/- 7º) and anteversion angles (normal 10º +/- 7 º). Operative treatment is indicated for most of the displaced and undisplaced fractures. Exceptions include bedridden palliative patients with a limited life expectancy (Mark et al, 2012) (Jay et al, 2009).

Internal fixation with cannulated screws is indicated in young patients with displaced or undisplaced fractures. In the age group between 65-85 years, consider cannulated screws for type I and II fractures, and physiologically young patients with type III and IV. In patients aged >85 consider internal fixation only for type I and II fractures.

Sliding hip screw or cephalomedullary nail is indicated in basicervical fractures. Consider placement of an additional cannulated screw above sliding hip screw to prevent rotation. Hemiarthroplasty is indicated in debilitated elderly patients with a metabolic bone disease. Total hip arthroplasty is used in older active patients or in patients with preexisting hip osteoarthritis.

Complications related to femoral neck fractures include; osteonecrosis (in 10-45% of the cases, mainly in displace fractures), nonunion (in 5 to 30% of the cases, related to varus malreduction), dislocation (higher rate with total hip arthroplasty ~ 10%) and mortality (pre-injury mobility is the most significant determinant for post-operative survival) (Holt et al, 1994) (Watson et al, 2002).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.4.7.2 Extracapsular fractures

Extracapsular fractures have a bimodal distribution; older patients that suffer low energy falls, and younger patients following high energy accidents. These fractures are divided into; stable fractures (that will resist medial compressive loads once reduced) and unstable (reverse oblique pattern, large posteromedial fragment, subtrochanteric extension). Physical examination reveals pain in the trochanteric area and groin, and a shortened, externally rotated lower extremity.

Surgical options include the use of sliding hip compression screw which is indicated in stable intertrochanteric fracture. This therapeutic approach is contraindicated in reverse obliquity fractures (a 56% failure rate) and when there is a lack in the integrity of lateral femoral wall. Surgery within the first 48 hours is associated with a decreased in the mortality rate (Mark et al, 2012) (Jay et al, 2009).

Proximal femoral nails are indicated in fractures with a reverse obliquity pattern, and other unstable fracture patterns. Advantages of this technique include; a decreased reoperation rate at one year, a shorter operative time, prevents medialization of the shaft, fewer blood transfusions, and shorter hospital stay. On the other hand, fractures at the tip of the prosthesis can occur as a result of mechanical stress points. Blade plates can be indicated in reverse obliquity pattern fractures. This technique has improved outcomes for unstable peritrochanteric fractures as compared to sliding hip screws (Mark et al, 2012) (Jay et al, 2009).

The most common complication associated with intertrocanteric fractures is implant failure (cut-out). In young patients it can be treated with a corrective osteotomy or revision open reduction and internal fixation. In elderly patients it can be treated with total hip arthroplasty. Another complication is the anterior perforation of the distal

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

femur that can occur after intramedullary nailing due to a mismatch of the radius of curvature of the femur and implant (Mark et al, 2012).

2.4.8 Pharmacology of osteoporosis "medication subgroups"

Some of the drugs available to date have caused a reduction in vertebral and non-vertebral fracture risk. However, despite the use of these medications, more fractures happen today than are avoided in the elderly groups of industrialized countries. This highlights the need to develop new therapeutic lines and strategies. Recent studies focused on bone metabolism, have led to a better understanding of bone cell functions and communication between osteoblasts, osteoclasts, and osteocytes. Hopefully in the near future, the treatment options against osteoporotic fractures are likely to be enriched by new bone anabolic substances such as; antibodies directed against the endogenous inhibitors of bone formation sclerostin; PTH and PTH related-peptide (PTHrp) analogues; new inhibitors of bone resorption (i.e. cathepsin K inhibitors) which may suppress osteoclast function without impairing osteoclast viability and thus maintain bone formation by preserving the osteoclast-osteoblast crosstalk; denosumab an antibody against RANKL which inhibits osteoclast formation, function, and survival; and new therapeutic strategies based on an extended understanding of the pathophysiology of osteoporosis which may include sequential therapies with two or more bone active substances aimed at optimizing the management of bone capital acquired during adolescence and maintained during adulthood in terms of both quantity and quality (Ramachandran et al, 2007) (Mark et al, 2012).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Finally, a better understanding of the role the PSNS and CNS play in the regulation of bone remodeling could lead to the development of new medications that could be very useful in the treatment of osteoporosis (Lippuner et al, 2012).

2.4.8.1 Bisphosphonates

Bisphosphonates are still the most common medications used in the treatment of osteoporosis. There use is indicated in hip or vertebral fractures, patients with a T-score <2.5 at the femoral neck or spine (after exclusion of secondary causes), a low bone mass (T-score between -1.0 and -2.5), a 10-year probability of a hip fracture of 3% or greater, and 10-year probability of a major osteoporosis related fracture of 20% or greater (based on the WHO algorithm). (WHO, 1994)

Bisphosphonates have the capacity to accumulate at sites of bone remodeling and are incorporated into bone matrix. They are released into an acidic media once bone is reabsorbed, and are then taken up by osteoclasts causing a decrease in osteoclastic bone resorptive activity, flattening of the ruffled border and an increase in osteoclast apoptosis. Bisphosphonates are then excreted in the kidneys without undergoing metabolism. Nitrogen containing bisphosphonates inhibit osteoclast farnesyl pyrophosphate synthase enzyme, required in the mevalonate (cholesterol pathway) which inhibits guanosine-triphosphate hydrolase enzyme (GTPase) formation. Alendronate can reduce the rate of hip, spine and wrist fractures by 50% (over 3 years) while risedronate reduces vertebral-nonvertebral fractures by 40% (over 3 years) and zolendronic acid reduces the rate of spinal fractures by 70% and hip fractures by 40% (over 3 years). Non-nitrogen containing bisphosphonates such as etidronate, clodronate and tiludronate produce a toxic adenosine triphosphate (ATP) analog that causes a premature apoptosis of the osteoclasts. The use of bisphosphonates is associated with

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

complications such as avascular necrosis of the jaw and atypical subtrochanteric fractures, gastric ulcers, and esophagitis. They are contraindicated in cases of severe renal disease and following lumbar fusion (Capeci et al, 2009).

2.4.8.2 Vitamin-D supplements and calcium

They should be taken in all patients with type 2 osteoporosis. It has been shown that the use of these supplements can reduce the risk of hip fracture. Side effects associated with vitamin-D intake can cause hypercalcemia, renal stones and atherosclerosis (Sahota, 2010).

2.4.8.3 Estrogen replacement therapy

Conjugated estrogen-progestin hormone replacement therapy (HRT) is indicated in women with type 1 osteoporosis and women that underwent an oophorectomy. The treatment should be started within the first 6 years of menopause and should not be prolonged indefinitely. Taking unconjugated estrogen (without progesterone) increases the risk of endometrial hyperplasia and uterine cancer.

Raloxifene is an estrogen agonist that reduces osteoclastic resorption and antagonizes estrogen receptors in breast thus reducing breast cancer. It appears to exclusively reduce the risk of vertebral fractures. The use of raloxifene is associated with the development of hot flashes, leg cramps and is contraindicated in patients with a thromboembolic disease (Mark et al, 2012) (Ramachandran et al, 2007).

2.4.8.4 Salmon calcitonin

It binds to membrane receptors on osteoclasts causing an inhibition in bone resorption. The use of calcitonin is indicated in postmenopausal women with acute vertebral

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

compression fractures and it is associated with side effects such as intranasal rhinitis, nausea, vomiting and hypersensitivity reactions (Mark et al, 2012) (Jay et al, 2009).

2.4.8.5 Parathyroid hormone derivatives

Teriparatide is the 1-34 amino acid terminal residue of PTH that activates specific osteblast receptors and renal tubule cells causing an increase in osteblastic activity and in the intestinal absorption of calcium and phosphate. The use of teriparatide can reduce the vertebral fracture risk by 65% and non-vertebral fractures (hip, radius, and humerus) by 62%. Side effects associated with teriparatide include hypercalcemia, nausea, and headaches. Its use is contraindicated in Paget´s disease due to a potential risk of developing osteosarcoma (Neer et al, 2001) (Genant et al, 2005).

2.4.8.6 Monoclonal immunoglobulins

Denosumab is a monoclonal Ig2 that can bind to RANKL and causes a decrease in the osteoclastic activity. The use of denosumab can cause arthralgias, nasopharyngitis, back pain and it is contraindicated in severe hypocalcemia.

2.5 Alzheimer disease

2.5.1 Epidemiology

AD was first described by the German psychiatrist and neuropathologist Alois Alzheimer in 1907. It is a neurodegenerative condition which manifests as cognitive impairment, behavioral disorders and progressive memory loss. Patients that suffer from AD require a permanent and continuous care and a great degree of dedication.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

In a study published in bio-med central (BMC) neurology it is estimated that the number of patients suffering from AD in Spain is about 400,000. The annual cost of caring for an AD patient in the US in 1996 was between $ 18,400 and $ 36,100 / year. The total annual cost spent on AD patients in the US is $ 148.000 million / year (De Pedro-Cuesta et al, 2009).

The cognitive symptoms of AD include memory loss, disorientation, problems in oral and written communication, inability to recognize people, objects and other computing difficulties such as planning and reasoning. The behavioral symptoms include depression, irritability, hallucinations, delusions, aggression and wandering (De Pedro-Cuesta et al, 2009).

AD also has an impact on the functional activities of daily living such as leisure, hobbies, using transportation, financial affairs, ability to dress, walk and feed alone, and sphincteric control. The physical implications of the disease include nutritional problems, dysphasia, decubitus ulcers and infections (CEAFA, 2010).

2.5.2 Classification

The clinical evolution of AD is divided into three different phases according to the clinical dementia rating scale (CDR) (Huges et al, 1982) (Morris et al, 1993. In the initial mild phase the patient experiences an insidious development of recent memory loss, irritability, depression and deficient executive functions and calculation. In the moderate phase the patient experiences a severe memory loss that classically spares the long term memory, disorientation, inability to recognize close relatives, impoverished language with difficulty in understanding certain orders, and personal care neglect.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

When the disease enters the severe phase the patient suffers from an altered sleep rhythm, double incontinence, mutism, inability to walk, dysphagia, cachexia. In this phase patients need assistance for all the basic activities of daily life.

The diagnosis of the disease is mainly clinical based on the mini-mental state exam (MMSE) and blessed dementia scale (BDS) (Folstein et al, 1975). However other causes of dementia have to be excluded using neuroimaging studies and blood analysis. Specific treatments for AD include AChEIs such as donepezil hydrochloride, galantamine and rivastigmine. Other alternative medications that do not depend on AChE inhibition such as memantine have been recently used. Unfortunately, the evolution this disease is progressive despite the use of these medications and the median survival is of 10 years from the onset. Death usually occurs from pneumonia, urinary tract infections, and decubitus ulcers (De Pedro-Cuesta, 2009).

2.5.3 Pharmacology of Alzheimer disease

AChEIs and N-methyl-D-aspartic acid antagonists (NMDA) or memantine are the only two approved classes of drugs used in the treatment of AD.

2.5.4 Acetylcholinesterase inhibitors

AChEIs are chemicals that inhibit the acetylcholinesterase enzyme from breaking down acetylcholine, causing an increase in the level and duration of action of this neurotransmitter. AChEIs are also used in the treatment of conditions such as glaucoma, myasthenia gravis and smooth muscle atony. Others AChEIs have been used as insecticides and in chemical warfare. Compounds which function as reversible competitive or noncompetitive inhibitors of cholinesterase are most likely to have therapeutic uses. On the other hand compounds which function as irreversible

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

inhibitors of cholinesterase are mostly used as chemical weapons or pesticides (Pepeu et al, 2009).

The major side effects of AChEIs are related to their action on the PSNS these effects include bradycardia, hypotension, hypertension, bronchoconstriction, GI tract hypermobility, and decrease intraocular pressure. Actions on the neuromuscular junction will result in prolonged muscle contraction.

Most AChEIs able to cross the blood-brain barrier were introduced in the mid-1990s and eventually became the standard treatment for AD patients (Pepeu et al, 2009). Unfortunately, AChEIs can only lead to a marginal clinical improvement regarding the cognitive state and global assessment of dementia, and do not seem to alter the natural course of the disease (Raina et al, 2008).

The main AChEIs used in the treatment of AD are donepezil, galantamine and rivastigmine. These drugs are approved for use in mild to moderate AD, except for rivastigmine, which is also approved for Parkinson's disease dementia, and donepezil, which can be also used in severe AD (Jimenez et al, 2009). In addition, the use of donepezil is associated with a lesser degree of deterioration of the cognitive state and functional status.

Finally it is important to mention that galantamine does not alter significantly the activity of M1-M5 receptors. Its therapeutic action seems to be caused by a predominant allosteric stimulation of the nAChRs rather than by general cholinergic enhancement due to cholinesterase inhibition and it (Samochocki et al, 2003). On the other hand rivastigmine and donepezil appear to increase the cholinergic activity of both muscarinic and nicotinic receptors (Clayton et al, 2007) (Chen et al, 1998).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2.5.5 Memantine

The activation of NMDA receptors found on neurons by toxic neurotransmitters is believed to mediate neuronal apoptosis; this process contributes to the development of dementia (Jain, 2000). Memantine is currently the only drug used in the treatment of dementia that has an NMDA antagonistic activity. It has been approved for its use in moderate to severe AD. A recent study has reported that memantine was superior to placebo in controlling behavioral symptoms. Combination therapies have been considered potentially beneficial as dementia involves multiple pathological processes and not just a cholinergic deficit (Xiong et al, 2005). A cost-effectiveness study of combination therapy with memantine and donepezil in patients with moderate to severe AD showed that there was a net decrease in the cost of total lifetime AD-related care (Weycker et al, 2007). Unfortunately, the overall effect of these medications on the cognition levels and mortality rates of AD patients remain relatively limited and new lines of research should be created in order to reach a better understanding of this pathology and ultimately improve the general outcome of these patients.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Hypothesis

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Clinical conditions and drugs that alter the acetylcholine signaling system have been found to have interesting effects on bone function (Walker et al, 2001) (Hoogduijn et al, 2009) (En-Nosse et al, 2009) (Warner et al, 2006) (Grimston et al, 2007) (Alkondon et al, 2000) (Hollinger et al, 1999) (Glowacki et al, 2008) (Kliemann et al, 2012) (Bajayo et al, 2012) (Kondo et al, 2013) (Pouwels et al, 2013). Botulinum neurotoxin inhibits the release of acetylcholine from motor neurons at the pre-synaptic level, and has been found to impair bone healing and induce a decrease in bone mineral content (Warner et al, 2006) (Grimston e al, 2007). Poliomyelitis destroys motor neurons that use acetylcholine neurotransmitters and appears to be directly related to osteoporosis (Mohammad et al, 2009). Smokers have high concentrations of nicotine in blood that interacts with the nicotinic nAChR causing a desensitization of these receptors.

Moreover, recent research has revealed the presence of a number of AChR subunits in bone cells. Nicotinic AChR subunits have been detected in human primary bone cells, MSCs and osteoblasts, while muscarinic receptors have been detected in MSCs and human osteoblasts. It has also been found that osteoblasts express specific AChR and cholinergic components that may play a possible role in regulating the ALP activity as well as the proliferation and differentiation of osteoblasts (Walker et al, 2001) (Hoogduijn et al, 2009) (En-Nosse et al, 2009) (Alkongdon et al, 2000) (Sato et al, 2010).

Altogether, the available literature seems to indicate that the inhibition of the AChR at the bone level seems to cause a reduction in bone turnover. However, it is not clear whether or not boosting up of the acetylcholine activity might have an anabolic effect on bone formation (Ma et al, 2010).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

We hypothesize that the administration of AChE inhibitors may have a beneficial effect on bone turnover that could translate into the reduction of the bone fracture risk. In order to test this hypothesis we have designed a retrospective case-control study in which we compare the incidence of hip fractures in a group of AD patients under AChEIs treatment, with AD patients not treated with AChEIs.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Publicaciones y
Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Materials and Methods

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Approval from the ethical committee of "Carlos Haya Hospital" was obtained to carry out a retrospective case-control study on AD patients living within our local health area. Data was obtained from the Neurology Department and Orthopedic Surgery Department computerized databases. The overall study period was of 5 years, between January 1st, 2004 and December 31st, 2008. All the hip fractures in AD patients that occurred within the study period were reviewed.

1. Demographic information of the study population

Study groups

The epidemiological data of the studied population was obtained using Carlos Haya Hospital local statistical records. All the hip fractures that were treated between January 2004 and December 2008 were reviewed; all these cases were registered in the computerized database of the orthopedic surgery department. From this initial group we identified all the AD patients with an incident diagnosis of a hip fracture recorded in the computerized medical records within the study period. Cases were identified without any exposure information. Potential cases had to be aged between 75 to 95 years at the date of the fracture (case index date), and to be recorded in the neurology department database for at least 2 years, in order to ensure an adequate record of the treatment received for AD. Case patients with a medical disorder known to substantially affect bone metabolism (ie, a history of osteoporosis diagnosed by DEXA scan, osteomalacia, Paget disease, cancer [excluding non-melanoma skin cancer], or alcoholism) as well as patients who were on corticosteroids, anti-epileptic drugs or bisphosphonates (as treatment for osteoporosis or bone metastases), prior to the index date were excluded from the analysis. Data regarding the socioeconomic status of the patients was not

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

registered because the Spanish public healthcare system provides universal free healthcare, and all of our patients have equal access to the drugs assessed in this study.

Controls included all AD patients in our local health area registered in the Neurology Department database between January 2004 and December 2008 and who did not present any incidence of hip fracture prior to December 31st 2008 (control index date). Controls had to be alive on the index date and had to be recorded in the database for at least 2 years. The same exclusion criteria applied to cases were also applied to controls. All the patients included in the study group were subject to an additional standardized questionnaire in order to complement any missing information in the medical records.

2. Exposure assessment and statistical analysis

Exposure assessment

The following parameters were retrieved from the patient's computerized records and standardized questionnaires: patients age, gender, mobility, institutionalization, history of hip fractures (including subcapital, transcervical, basicervical, pertrocanteric, and subtrocanteric femoral fractures), history of other osteoporosis related fractures (including, vertebral, distal radius, proximal humerus, and previous hip fractures), body mass index (BMI), fall risk status according to the falls risk assessment tool (FRAT) (Huges et al, 1982) **,** Charlson comorbidity score (CCS) (Charlson et al, 1987), uptake of selective serotonin reuptake inhibitors (SSRI) and smoking. The grade of AD according to the CDR scale and the cognitive state according to the MMSE and BDS were also retrieved (Morris, 1993) (Folstein et al, 1975). The specific treatment for AD at the index date; (AChEIs [i.e. donepezil hydrochloride, galantamine, rivastigmine, or

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

no AChEI treatment] and/or memantine) including the dose, duration (0.5-2 years, >2 years).

Statistical analysis

We conducted unconditional logistic regression analyses using the software IBM SPSS Statistics 19^{TM} (SPSS Inc. Chicago, IL)**.** The annual incidence of hip fractures in the general population during the period January 2008 and December 2008 was calculated with 95% confidence intervals (CI), on the other hand the average annual incidence of hip fractures during the period between January 2004 and December 2008 was also calculated. Risk estimates were presented as odd ratios (ORs) with 95% CIs. P values were 2-sided and were considered statistically significant if less than 0.05. Odd ratios were adjusted for the potential confounders: age, gender, mobility, institutionalization, BDS (<9,9-16.9, ≥17), CDR score (1,2,3), MMSE score (≤20, >20), FRAT score (=1,>1), other fractures in the body, CCS score (0-1,2,>3), uptake of SSRI, BMI (<25,25-29.9,≥30), family history of osteoporosis and smoking habits (smoker, non smoker). Missing values for BMI and FRAT were treated as a separate group in the analysis. Differences between users and nonusers of AChEIs were assessed with student t-test (for continuous variables, i.e.: age, MMSE, CDR, BDS, CCS, BMI, and FRAT), and with Chi square test (for nominal variables, i.e.: gender, other fractures, use of SSRI and smoking). P values were 2-sided and were considered statistically significant if less than 0.05.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Results

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

1. Demographic information

The general population registered in Carlos Haya Hospital health care area in 2008 was of 607217 inhabitants, from these a total of 40011 individual [14111 males and 25900 females, (35% and 65 % respectively)] were aged above 75 years at the index date (table 6). A total of 2794 AD patients [994 males and 1800 females (35.5% and 64.5 % respectively)] without hip fractures were identified in our health care area during the year 2008, among these patients 2178 fulfilled our control group inclusion criteria (table 7). The total number of hip fractures treated in our centre during the year 2008 was of 381 [108 males and 273 females, (28% and 72% respectively)]. The distribution of these cases within their corresponding age groups is illustrated in (table 8). We also identified 135 cases of hip fractures in patients with AD and other dementias between 2004 and 2008, and among whom 80 patients [14 males and 66 females (17.5 % and 82.5% respectively)] fulfilled our case group inclusion criteria (table 9). The selection of cases and control subjects is illustrated in figure 11. Hip fractures appeared to be correlated with the age and gender of the patients in the general population and in AD patients (female-male ratio; 3:1 in the general population and 4:1 in the AD group) (table 8- 9).

2. Hip fracture incidence in the general population and Alzheimer patients

The annual incidence of hip fractures (per 1000 persons) in the general population in the year 2008 appeared to increase with age following a linear pattern (Figure 12). The hip fracture incidence ranged from 1.4×10^{-3} (CI 95% 1.0-2.0) in the age group between 70-74 years (including both genders) and 17.3×10^{-3} (CI 95% 12.6-23.1) in patients ≥ 90 years (table 10) (figure 12). However this tendency was more obvious in the female population; the hip fracture incidence in female and in male patients ≥ 90 years was

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

28.9 x 10^{-3} (CI 95% 20.5-39.4) and 4.9 (CI 95% 1.8-10.6) respectively (table 10) (figure 12).

In contrast to what was observed in the general population the average incidence of hip fractures (per 1000 persons) in AD patients in the period between 1/1/2004-31/12/2008 followed a negatively-skewed distribution (figure 13), increasing in younger age groups and decreasing in older groups; ranging from 2.1 x 10^{-3} (CI 95% 0.1-11.9) in the age group between 70-74 years (including both genders), 8.0 x 10^{-3} (CI 95% 2.9-18.4) in the group between 75-79 years, and 1.6 x 10^{-3} (CI 95% 0-8.0) in patients ≥ 90 years (table 11) (figure 13); However, the global average hip fracture incidence for all the age groups remained higher in the female population; 6.4 x 10^{-3} (CI 95% 3.6-10.5) in females and 2.6 (CI 95% 0.5-7.6) in males (table 11) and (figure 13).

When overlapping figures 12 and 13, we can appreciate a clear difference in the evolution of the incidence curves. In the general population hip fractures tend to progressively increase with age (SD 6.1), on the other hand hip fractures in AD reach a peak at the age group between 74-79 years and then they progressively decrease (SD 2.8). However the total hip fracture incidence for both genders was almost equal in both groups: 5.0 x 10^{-3} (CI 95% 3.0-8.0) in the AD group, and 5.1 x 10^{-3} (CI 95% 4.7-5.8) in the general population ($p>0.05$) (tables 10 and 11). We can appreciate the evolution of the hip fracture incidence in the general population and in AD patients in (figure 14).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Age Groups	Male population	Female population	Male and female population living in our local health area in 2008
≤69	270054 (49,6%)	274993 (50,4%)	545047
70-74	9444 (42,6%)	12715 (57,4%)	22159
75-79	7322 (39,4%)	11266 (60,6%)	18588
80-84	4091 (34,0%)	7938 (66,0%)	12029
85-89	1465 (21,4%)	5379 (78,6)	6844
≥90	1233 (48,4%)	1317 (51,6%)	2550
Total	293609 (48,4%)	313608 (51,6%)	607217

Table 6: The age distribution of the general population registered in Carlos Haya Hospital health care area in 2008.

Age groups	Male /Alzheimer population	Female/ Alzheimer population	Total Alzheimer population
70-74	158 (34,3%)	302 (65,7%)	460
75-79	162 (26,6%)	448 (73,4%)	610
80-84	286 (31,9%)	610 (68,1%)	896
85-89	277 (48,6%)	293 (51,4%)	570
≥90	111 (43,0%)	147 (57,0%)	258
Total	994 (35,6%)	1800 (64,4%)	2794

Table 7: The Alzheimer population (≥70 years) in Carlos Haya Hospital health care area in 2008. A total of 2178 controls were selected from this group.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Age groups	Hip fractures in males	Hip fractures in females	Total number of hip fractures
≤69	36 (55,4%)	29 (44,6%)	65
70-74	7 (21,9%)	25 (78,1%)	32
75-79	21 (28,8%)	52 (71,2%)	73
80-84	27 (27,0%)	73 (73,0%)	100
85-89	11 (36,6%)	56 (63,4%)	67
≥90	6 (13,6%)	38 (86,4%)	44
Total	108 (28,3%)	273 (71,7%)	381

Table 8: The total number of hip fractures (in all the age groups) treated in the Carlos Haya Hospital in 2008 distributed in different age groups.

Age groups	Hip fractures in male /Alzheimer population	Hip fractures in female/ Alzheimer population	Hip fractures /Total Alzheimer population
70-74	1 (20,0%)	4 (80,0%)	5
75-79	1 (4,5%)	21 (95,5%)	22
80-84	5 (17,9%)	23 (82,1%)	28
85-89	7 (28,0%)	18 (72,0%)	25
≥ 90	1 (20,0%)	4 (80,0%)	5
Total	15 (17,6%)	70 (82,4%)	85

Table 9: The age distributions within the study group n=80 (the AD patients that suffered a hip fracture within the period 1/1/2004- 31/12/2008 and met our inclusion-exclusion criteria). The age group between 70-74 years is also illustrated (it is not part of the study group, but was used to calculate the hip fracture incidence in AD patients).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

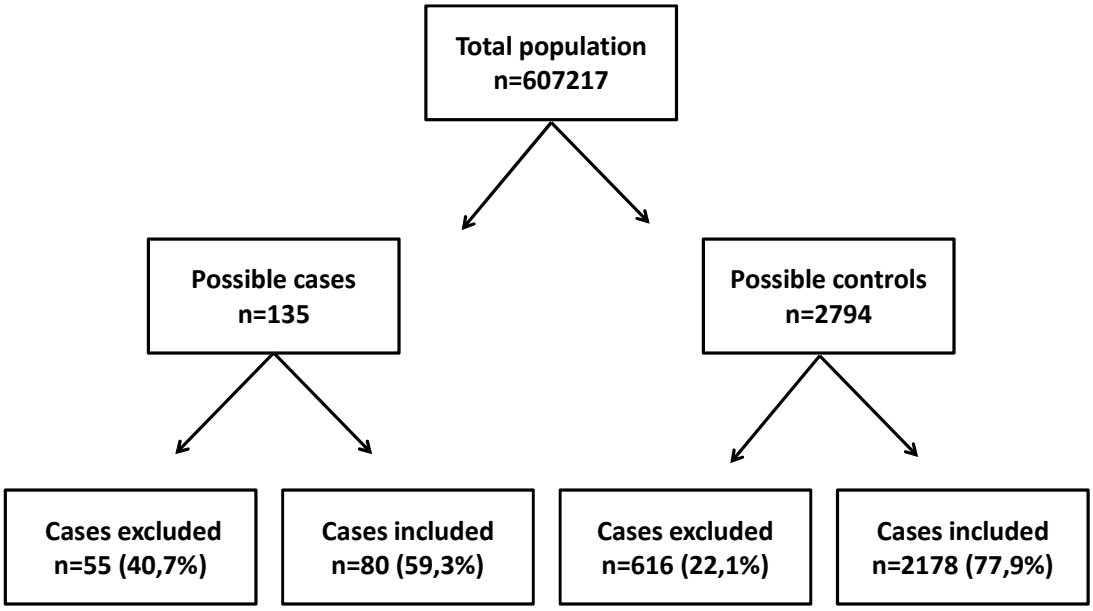


Figure 11: Flow chart describing the selection for case and control subjects.

Age groups	Hip fractures incidence in males per 1000 (CI 95%)	Hip fractures incidence in females per 1000 (CI 95%)	Total incidence of hip fractures per 1000 (CI 95%)
70-74	0.74 (0.3-1.5)	2.0 (1.3-2.8)	1.4 (1.0- 2.0)
75-79	2.9 (1.8-4.4)	4.6 (3.5-6.0)	3.9 (3.1-4.9)
80-84	6.6 (4.4-9.6)	9.2 (7.2-11.6)	8.3 (6.8-10.1)
85-89	7.5 (3.8-13.4)	10.4 (7.9-13.5)	9.8 (7.6-12.4)
≥90	4.9 (1.8-10.6)	28.9 (20.5-39.4)	17.3 (12.6-23.1)
Total	3.1 (2.4-3.9)	6.3 (5.6-7.2)	5.1 (4.5-5.7)

Table 10: The annual incidence (CI 95%) of hip fractures in the general population (patients ≥70 years) per 1000 in the year 2008 (n=316). The age group between 70-74 years is also illustrated (which is not part of the study group).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Age groups	Hip fractures incidence/ male- AD population per 1000 (CI 95%)	Hip fractures incidence/ female- AD population per 1000 (CI 95%)	Hip fractures incidence/ total- AD population per 1000 (CI 95%)
70-74	1.2 (0.2-2.2)	2.7 (0.1-12.2)	2.1 (0.1-11.9)
75-79	1.0 (0.1-.1.7)	11.6 (3.2-29.3)	8.0 (2.9-18.4)
80-84	3.4 (0.1-18.7)	8.3 (3.0-19.1)	6.5 (2.7-14.1)
85-89	6.8 (1.2-26.6)	5.8 (1.9-14.9)	4.8 (1.4-11.0)
≥90	0.7 (0.1-1.3)	2.4 (0.1-10.9)	1.6 (0.0-8.0)
Total	2.6 (0.5-7.6)	6.4 (3.6-10.5)	5.0 (3.0-8.0)

Table 11: The average annual incidence of hip fractures in the AD population (patients ≥70 years) per 1000 (CI 95%) in the period 1/1/2004- 31/12/2008. The age group between 70-74 years is also illustrated (which is not part of the study group).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

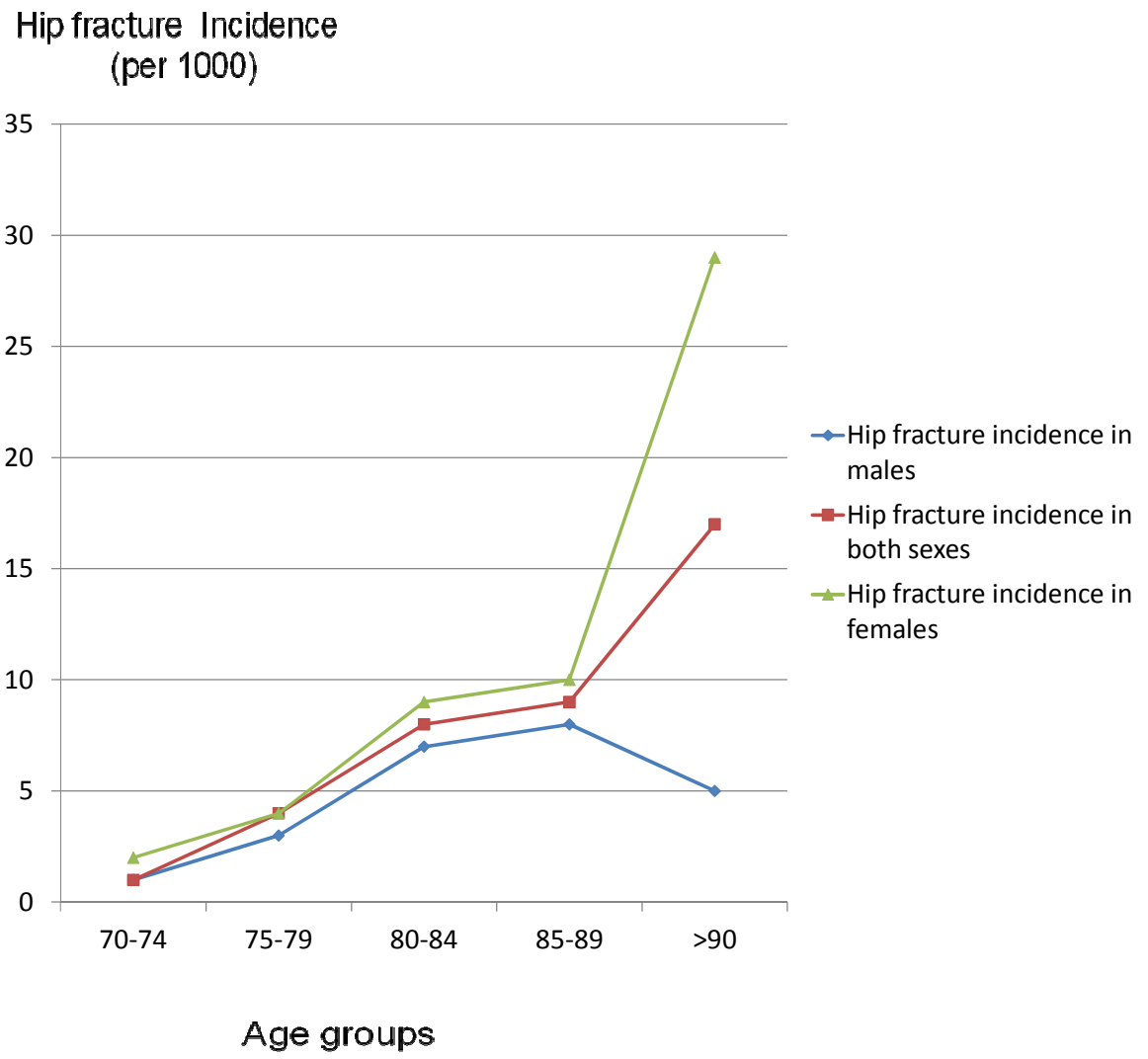


Figure 12: The annual incidence of hip fractures in the general population (patients ≥70 years) per 1000 in the year 2008, according to gender and age.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

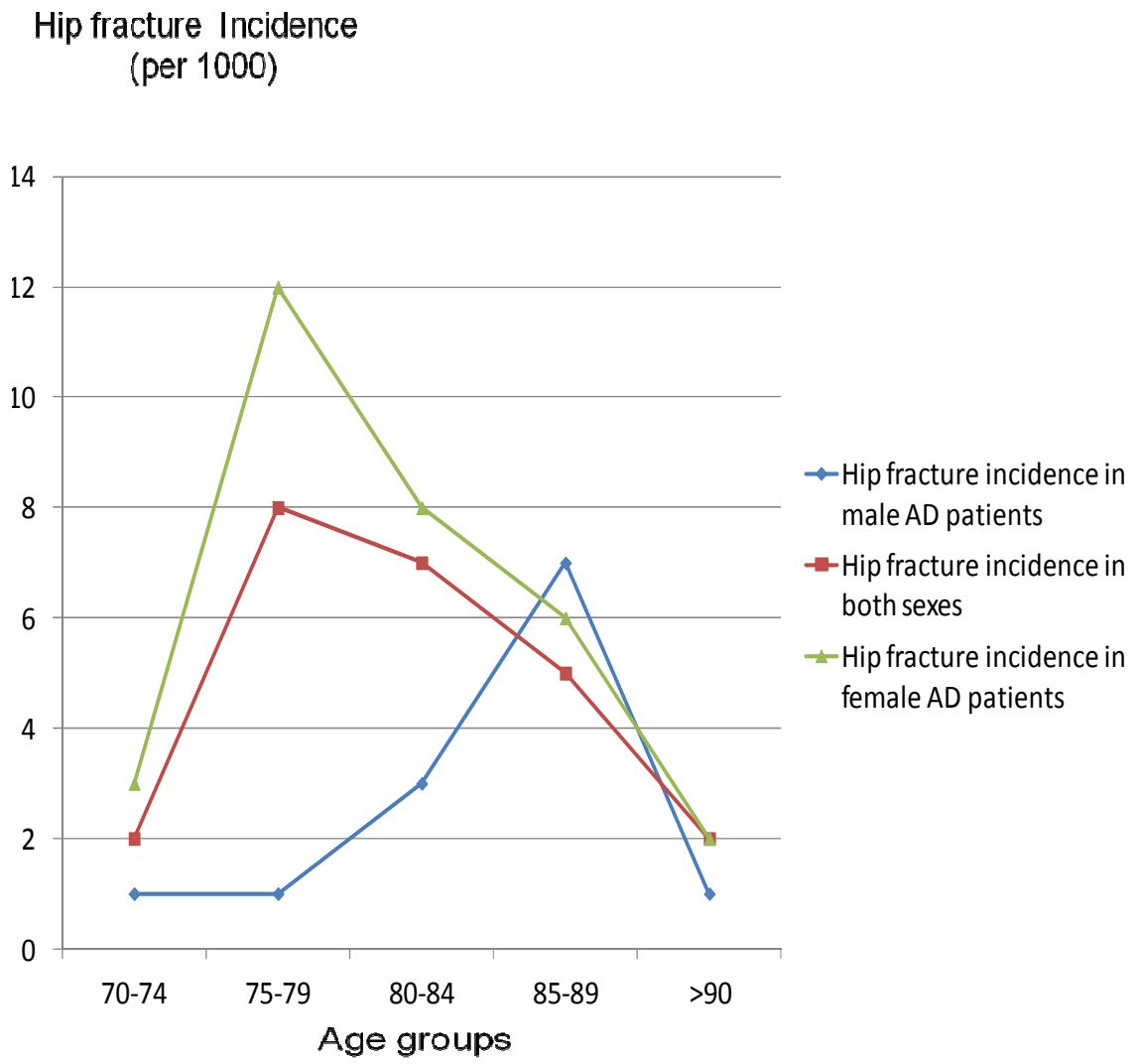


Figure 13: The average annual incidence of hip fractures in the AD population (patients ≥70 years) per 1000 during the period 1/1/2004 to 31/12/2008, according to gender and age.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

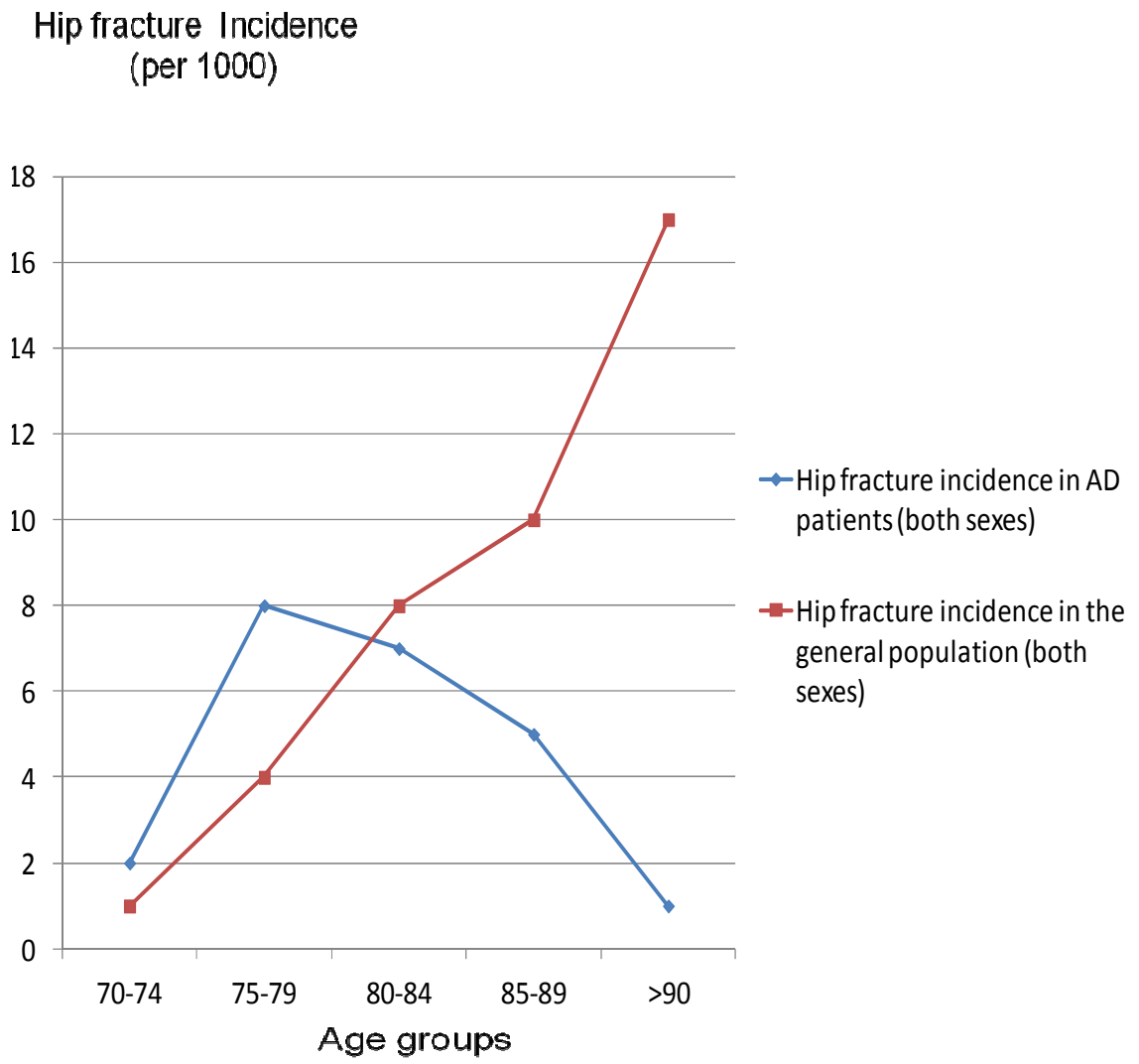


Figure 14: The average annual incidence of hip fractures in the AD population (patients ≥70 years) during the period 1/1/2004 to 31/12/2008 and in the general population (patients ≥70 years) per 1000 during the year 2008; according to gender and age.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

3. Assessment of risk factors in cases and controls

A total of 55 patients were excluded from the study group; 30 patients were less than 75 years old at the index date, 6 were on treatment with bisphosphonates, 10 were on corticosteroids due to pulmonary obstructive disease, 3 had chronic renal disease, there were 4 cancer cases (excluding non-melanoma skin cancer), 1 case of alcohol abuse and 1 was on anti-epileptic drugs. None of the patients were diagnosed with Paget's disease or osteomalacia (figure 11).

A total of 616 patients were excluded from the control group; 460 patients were less than 75 years old at the index date, 30 were on treatment with bisphosphonates, 61 were on corticosteroids due to pulmonary obstructive disease, 6 had chronic renal disease, there were 34 cancer cases (excluding non-melanoma skin cancer), 21 cases of alcohol abuse and 1 was on anti-epileptic drugs. There were 2 cases of Paget´s disease and one case of vitamin D deficiency (figure 11).

The distribution of hip fractures in the study groups as a function of age, gender, BMI, MMSE, FRAT, CDR, BDS, CCS, SSRI, smoking, institutionalization and the presence of other fractures are displayed in table 12. Results were adjusted to all confounders. There were a total of 80 cases (21 between 75-79 years, 28 between 80-84, and 31 above 85 years) and 2178 controls (594 between 75-79 years, 846 between 80-84, and 738 above 85 years). Among these, there were 66 female cases, 14 male cases, 1548 female controls and 630 male controls.

Patients were divided according to their BMI in 3 groups; <25 [included 53 (66.3%) cases and 396 (18.2%) controls], 25-29.9 [included 21 (26.3%) cases and 468 (21.5%)

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

controls] and >30 [included 6 (7.5%) cases and 216 (9.9%) controls]. There were 1098 (50.4%) controls with missing information.

Regarding the cognitive status of the patients, the CDR scores were I, II and III for 13 (16.3%), 33 (41.3%), and 34 (42.5%) cases respectively, and 288 (13.2%), 1062 (48.8%), 828 (38.0%) controls respectively. Patients were also divided according to the MMSE into 2 groups: scores between 21-30 [included 4 (5%) cases and 234 (10.7%) controls], and 0-20 [included 76 (95%) cases and 1944 (89.2%) controls]. BDS scores were divided into 3 separate groups: 0-9 [included 1 (1.3%) case and 78 (3.6%) controls], 9-16 [included 42 (52.5%) cases and 900 (41.3%) controls], 17-28 [included 37 (46.3%) cases and 1200 (55.1%) controls] (table 12).

The FRAT scores were divided in 2 subgroups: 1 and 2-3. A total of 51 (63.7%) cases and 810 (37.2%) controls scored 1 point according to the FRAT scale, while 29 (36.3%) cases and 270 (12.4%) controls scored between 2-3 points, there were 1098 (50.4%) controls with missing information.

Only 4.5% of the cases were bedridden; there was 1 (1.3%) institutionalized case and 152 (7.0%) institutionalized controls. However the large bulk of the patients were not institutionalized 79 (98.7%) cases and 2026 (93.0%) controls). The CCS scores were divided in 3 subgroups: 0-1, 2 and >3. A total of 30 (37.5%) cases and 753 (34.8%) controls scored 0-1 points, 23 (28.8%) cases and 672 (30.9%) controls scored 2 points, and 27 (33.8%) cases and 753 (34.6%) controls scored >3 points (table 12).

The non smoking group included 74 (92.5%) cases and 2163 (99.3%) controls; smokers on the other hand included 6 (7.5%) cases and 15 (0.7%) controls. A total of 70 (87.5%) cases and 2003 (92.0%) controls did not take SSRI, while 10 (12.5) cases 175

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

(8.0%) controls were SSRI users. Patients with other fractures included 21 (26.3%) cases and 162 (7.4%) controls; on the other hand 59 (73.8%) cases and 2016 (92.6%) controls did not have a fracture history.

Female patients had a slightly higher risk of hip fracture than male patients, although not significant, and patients between the age of 80 to 85 years had a lower risk of hip fracture than patients between the age of 75–79 and >85 years. On the other hand, the risk of hip fracture was not associated with CDR, BDS, institutionalization, MMSE, and SSRI treatment. Patients with a CCS ≥ 3 had a lower risk of hip fracture than those with a CCS = 1 (table 12). Smokers, patients with a FRAT score >1, and patients with BMI <25 showed a higher risk of hip fracture compared to nonsmokers, patients with FRAT score = 1, and patients with BMI >25, respectively.

Potential confounders such as age, gender, BMI, MMSE, FRAT, CDR, BDS, CCS, SSRI, smoking, institutionalization and the presence of other fractures were compared between of users and non users AChEIs (table 13). The mean age among the users of AChEIs was 82.15 (SD 4.49), and 83.49 (SD 4.16) in non users. The female: male ratio was 2.8 for users and 2.55 for non users. The BMI in users of AChEIs and nonusers was [26.0 (SD 3.5) and 24.56 (SD 4.3) respectively]. Regarding the cognitive scores, the average CDR score was 2.16 (SD0.67) for users and 2.59 (SD 0.58) for non users, BDS [15.72(SD 3.48) for users and 17.31 (SD 3.37) for non users], MMSE [15.66 (SD 3.98) for users and 14.52 (SD 2.31) for non users]. The FRAT score in users and nonusers was [1.33 (SD0.55) and 1.24 (SD5.68) respectively]. The average CCS score in users and non users was [2.52 (SD 1.57) and 2.38 (SD 1.35) respectively). The presence of other fractures was of [15.97 (CI 95% 13.34- 18.61) in users and 16.25 (CI 95% 11.87-20.62) in non-users] (table 13).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Patients treated with AChEIs had better mental indices (CDR, BDS, and MMSE) than nonusers (table 13). The BMI was also higher in AChEIs users than in nonusers. The use of SSRI appeared to be higher in non users than in users. Gender, smoking habits, CCS and incidence of other fractures were not significantly different between users and nonusers of AChEIs (table 13). Interestingly, users of AChEIs were associated with a lower risk of hip fracture than nonusers even though they had a higher risk of falling (FRAT) than nonusers [1.33 (SD0.55) and 1.24 (SD5.68) respectively] (table 13). Values of *p* were two-sided and were considered statistically significant if less than 0.05.

Characteristics		Cases (n=80)a	Controls (n=2178)a	Crude Odds Ratio (95%CI)	Adjusted Odds Ratio (95%CI) b	P Value
Age, y	75-79	21(26.3)	594(27.3)	1.00	1.00	-
	80-84	28(35.0)	846(38.8)	0.94(0.53-1.7)	0.39(0.18-0.85)	0.018
	>85	31(38.8)	738(33.9)	1.19(0.68-2.09)	1.730.81-3.69)	0.159
Gender	Women	66(82.5)	1548(71.1)	1.00	1.00	-
	Men	14(17.5)	630(28.9)	0.52(0.29-0.94)	0.41(0.15-1.08)	0.072
BMI c	<25	53(66.3)	396(18.2)	1.00	1.00	-
	25-29.9	21(26.3)	468(21.5)	0.34(0.20-0.57)	0.40(0.21-0.74)	0.004
	>30	6(7.5)	216(9.9)	0.21(0.09-0.49)	0.16(0.06-0.41)	<0.001
	Missing	0(0.0)	1098(50.4)	0.27(0.24-0.29)	0.47(0.23-0.96)	0.039

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

CDR	1	13(16.3)	288(13.2)	1.00	1.00	-
	2	33(41.3)	1062(48.8)	0.69(0.36-1.33)	0.59(0.22-1.16)	0.304
	3	34(42.5)	828(38.0)	0.91(0.47-1.75)	0.40(0.07-2.14)	0,282
MMSE	21-30	4(5.0)	234(10.7)	1.00	1.00	-
	0-20	76(95.0)	1944(89.2)	2.30(0.83-6.34)	2.53(0.85-7.51)	0.096
BDS	0-9	1(1.3)	78(3.6)	1.00	1.00	-
	9-16	42(52.5)	900(41.3)	3.36(0.46-24.77)	2.06(0.23-18.58)	0.520
	17-28	37(46.3)	1200(55.1)	2.21 (0.30-16.33)	1.93(0.15-24.75)	0.615
FRAT	1	51(63.8)	810(37.2)	1.00	1.00	-
	2-3	29(36.3)	270(12.4)	1.71 (1.06-2.75)	2.26(1.34-3.85	0.003
	Missing	0(0.0)	1098(50.4)	0.43(0.40-0.45)	d	d
Institutionalization	Yes	79(98.7)	2026 (93.0)	1.00	1.00	-
	No	1 (1.3)	152 (7.0)	0.18 (0.02-1.28)	0.47 (0.06-3.53)	0.464
CCS	0-1	30 (37.5)	753 (34.8)	1.00	1.00	-
	2	23 (28.8)	672 (30.9)	0.86 (0.49-1.49)	0.96 (9.00-1.02)	0.145
	>3	27 (33.8)	753 (34.6)	0.90 (0.53-1.53)	0.52 (0.28-0.96)	0.026

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Smoking	No	74 (92.5)	2163 (99.3)	1.00	1.00	-
	Yes	6 (7.5)	15 (0.7)	11.67 (4.41-30.99)	33.90 (10.50-109.60)	<0.001
SSRI	No	70 (87.5)	2003 (92.0)	1.00	1.00	-
	Yes	10 (12.5)	175 (8.0)	1.64 (0.83-3.23)	1.45 (0.73-2.90)	0.288
Fracture history	No	59(73.8)	2016(92.6)	1.00	1.00	-
	Yes	21(26.3)	162(7.4)	4.43(2.63-7.47)	2.82 (1.60-4.99)	<0.001

Table 12: Characteristics of AD patients as function of the presence of hip fractures (group-cases) Abbreviations: CI, confidence interval; FRAT, Fracture Risk Assessment Tool; BMI, Body Mass Index; AD, Alzheimer's Disease; BDS, Blessed Dementia Scale; MMSE, Mini-Mental State Exam; CDR, Clinical Dementia Rating Scale; CCS, Charlson comorbidity score; SSRI, selective serotonin reuptake inhibitor. Results were adjusted to all confounders.

a- Values are *n* (%); percentages may not sum to 100% due to rounding.

b- Adjusted to the categorical parameters presented in this table.

c- Measured as weight in kilograms divided by the square of height in meters.

d- Not calculated due to insufficient data.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Characteristics		AChEI users (n=1771)	AChEI nonusers (n=487)	P
Age, y		82.15 (SD4.49)	83.49(SD4.16)	0.001a
Gender	Women	72(95%CI, 70-74)	69(CI95%, 65-73)	0.208b
	Men	25(22-28)	27(22-33)	0.208b
BMI c		26.0(SD3.5)	24.56(SD4.30)	0.001a
CDR		2.16(SD0.67)	2.59(SD0.58)	0.001a
BDS		15.72(SD3.48)	17.31(SD3.37)	0.001a
MMSE		15.66(SD3.98)	14.52(SD2.31)	0.001a
FRAT		1.33(SD0.55)	1.24(SD5.68)	0.025a
Smokers		1 (0-1)	1 (1-2)	0.415b
SSRI		19 (17-21)	26 (20-32)	0.030b
CCS		2.52 ± 1.57	2.38 ± 1.35	0.776a
Fracture history		15.97(13.34-18.61)	16.25(11.87-20.62)	0.300b

Table 13: Characteristics of AD patients as function of the presence of AChEI treatment. Abbreviations: CI, confidence interval; FRAT, Fracture Risk Assessment Tool; BMI, Body Mass Index; AD, Alzheimer's Disease; BDS, Blessed Dementia Scale; MMSE, Mini-Mental State Exam; CDR, Clinical Dementia Rating Scale. SSRI, selective serotonin reuptake inhibitor; CCS, Charlson comorbidity score.

a-Calculated with Student's *t*-test for independent samples.

b-Calculated with chi-square test.

c-Measured as weight in kilograms divided by the square of height in meters.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

4. Comparison between different AChEI treatments

Each specific AChEI had a different degree of association with the hip fracture risk. Patients treated with galantamine had similar risk of hip fracture as non users of AChEI [OR of 1.67 (0.63-4.45)] (p=0.305) (table 14). On the other hand, AD patients undergoing AChEIs treatment with donepezil, and rivastigmine were associated with a highly significant lower risk of hip fractures than nonusers, and this effect was dose dependent; OR of 0.39 (0.19-0.76) (p<0.002) and OR of 0.22 (0.10-0.45) (p<0.001) respectively (table 14). However, we could not observe a significant relationship between the duration of the treatment and the risk of hip fractures (table 15).

At α error = 0.05, we had 83% power for detecting risk of hip fracture in AD patients treated with AChEIs equal to 0.42 or lower. The overall relative risk for AD patients treated with AChEIs and for those not treated with AChEIs in relation to the general population was 0.97 (CI 95% 0.89-0.99) for those treated with AChEIs [0.48 (CI 95% 0.14-0.85) for rivastigmine 0.92 (CI 95% 0.46-1.0) for donepezil and 1.9 (CI 95% 0.98-3.1) for galantamine] and 3.2 (CI 95% 2.1-4.6) for patients not treated with AChEIs.

The dose and duration of AChEI treatments in AD patients was tested for association with risk of hip fracture (table 15). It was observed that patients receiving higher doses of rivastigmine were associated with a significantly lower risk of hip fractures than those treated with lower doses [crude OR 0.37 (CI 95% 0.12-1.10), adjusted OR 0.16 (CI 95% 0.03-0.79) (p =0.025]. The relationship between galantamine and donepezil dosage and the risk of hip fracture could not be assessed for statistical significance due to the small number of subjects collected, and the data missing from the patient's records [crude OR 0.12 (CI 95% 0.03-0.45), adjusted OR 0.02 (CI 95% 0.00-0.17) (p= not calculated) for donepezil and crude OR 0.83 (CI 95% 0.22-3.21), adjusted OR 0.51

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

(CI 95% 0.09-2.89) (p = not calculated due to insufficient data) for galantamine] (table 15).

The risk of hip fractures in patients that had been treated with either rivastigmine or donepezil for a longer period of time (>2 years) was not significantly different from those who had been treated for shorter periods of time (< 2 years) [crude OR 0.37 (CI 95% 0.12-1.10), adjusted OR 0.25 (CI 95% 0.06-1.04) (p=0.057) for rivastigmine, crude OR 0.83 (CI 95% 0.26-2.64), adjusted OR 0.90 (CI 95% 0.19-4.20) (p=0.879) for donepezil, and OR 0.83 (CI 95% 0.22-3.21), adjusted OR 0.51 (CI 95% 0.09-2.89) (p= not calculated due to insufficient data) for galantamine] (table 15).

Characteristics	Cases (n=80) No. (%) a	Controls (n=2178) No. (%) a	Crude Odds Ratio (95%CI)	Adjusted Odds Ratio (95%CI) b	P
Non users of AChEI	39(48.8)	450(20.7)	1.00	1.00	-
Users of AChEI	41(52.2)	1728(79.3)	0.30(0.19-0.48)	0.42(0.24-0.72)	0.002
Rivastigmine	16 (20.0)	900(41.3)	0.20(0.11-0.37)	0.22(0.10-0.45)	<0.001
Donepezil	15(18.8)	630(28.9)	0.29(0.16-0.54)	0.39(0.19-0.76)	<0.002
Galantamine	10 (12.5)	182(8.4)	0.68(0.33-1.39)	1.67(0.63-4.45)	0.305

Table 14: Use of AChEI and Fracture Risk (vs. Nonuse).

Abbreviations: CI, confidence interval; AChEI, Acetyl Cholinesterase.

a- Percentages may not sum to 100% due to rounding.

b- Adjusted to the categorical parameters in table 10.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

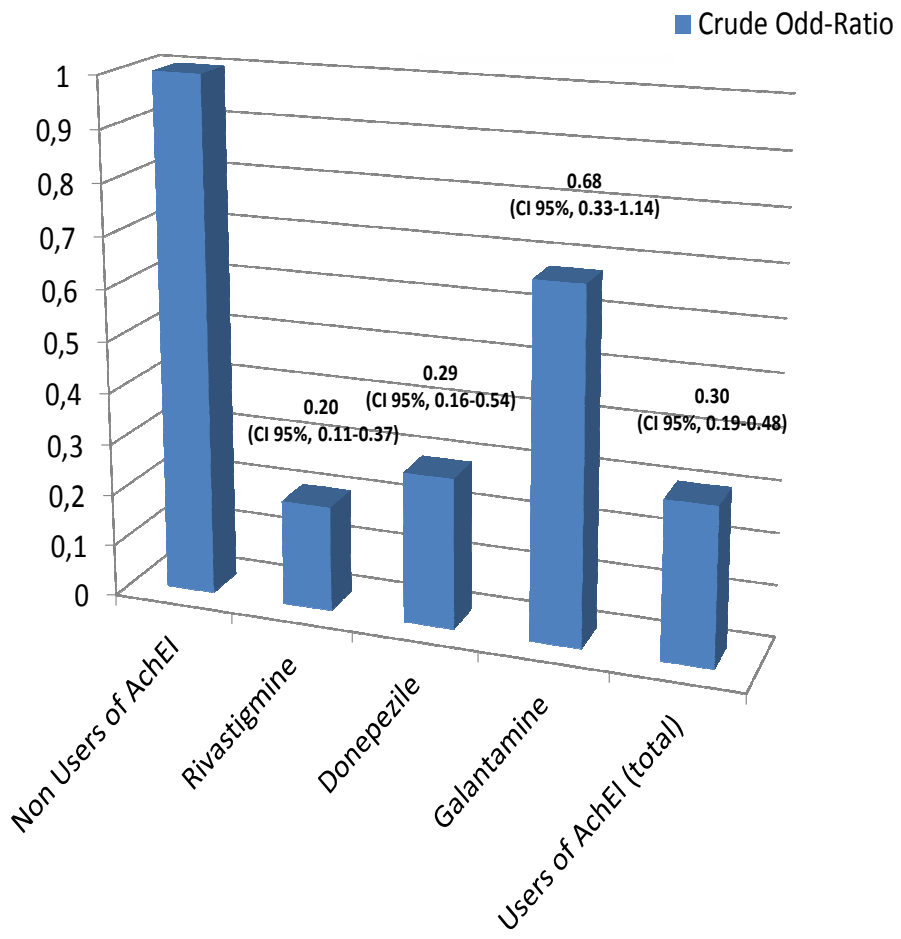


Figure 15: Use of AChEIs and fracture risk (vs. Nonuse); Odd Ratio. Abbreviations: CI, confidence interval.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Treatment characteristics		Cases (n=080) [a]	Controls (n=2178) [a]	Crude OR (95% CI)	Adjusted OR (95% CI) [b]	*p*
Rivastigmine	Duration, years 0.5-2	7	270	1.00	1.00	-
	>2	6	630	0.37 (0.12-1.10)	0.25 (0.06-1.04)	0.057
	Dose mg/day 3-9	7	270	1.00	1.00	-
	9.5-12	6	630	0.37 (0.12-1.10)	0.16 (0.03-0.79)	0.025
Donepezil	Duration, years 0.5-2	6	234	1.00	1.00	-
	>2	7	396	0.83 (0.26-2.64)	0.90 (0.19-4.20)	0.879
	Dose mg/day 5	3	18	1.00	1.00	-
	10	12	612	0.12 (0.03-0.45)	0.02 (0.00-0.17)	c
Galantamine	Duration, years 0.5-2	3	72	1.00	1.00	-
	>2	3	108	.67 (0.13-3.40)	c	c
	Dose mg/day 6-16	4	74	1.00	1.00	-
	17-24	5	108	0.83 (0.22-3.21)	0.51 (0.09-2.89)	c

Table 15: Treatment duration and dosage of AChEI and Fracture Risk (vs. Non users).

AChEI = acetylcholinesterase inhibitors; OR= odd ratio; CI= confidence interval.

[a] Values are number of cases or controls.

[b] Adjusted to the categorical parameters in table 10.

[c] Not calculated due to insufficient data.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Publicaciones y
Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Discussion

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

1. General aspects

Osteoporotic fractures such as hip, proximal humerus, distal radius and vertebral fractures are becoming a major health and economic problem in many countries. Worldwide, the total number of hip fractures is expected to reach 6 million per year by 2050. A total of 310,000 individuals were hospitalized with hip fractures in the US alone in 2003, accounting for 30% of all hospitalized patients. Approximately one third (101,800) of these patients underwent a hip replacement. The estimated cost for the treatment of hip fractures is approximately $10.3 to 15.2 billion a year in the US (Kanus et al, 1996) (Mark et al, 2012). Moreover, hip fractures substantially increase the risk of death and major morbidity in the elderly. One year mortality rates have ranged from 12% to 37% in these patients (Bentler et al, 2009) (Panula et al, 2011) (Wolinsky et al, 1997). On the other hand the annual budget spent in the treatment of vertebral compression fractures in the US alone is of $ 15 billion. It affects over 25% of the population aged more than 70 years and 50% of those above 80 years (Kanus et al, 1996)

The global costs associated to osteoporotic fractures will most likely increase in the near future as the world's population continues to grow older. Therefore, we highlight the need to prevent these fractures and to develop new lines of treatment that could eventually reduce the extremely expensive human and economic costs related to osteoporotic fractures (Lippuner, 2012). A better understanding of the cholinergic pathways of the ANS and their relation to bone remodeling could lead to an alternative approach in the treatment of osteoporosis and osteoporotic fractures.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Clinical conditions and drugs that alter the acetylcholine signaling system have been found to affect bone function (Alkondon et al, 2000) (En-Nosse et al, 2009) (Grimston et al, 2007) (Hollinger et al 1999) (Glowacki et al, 2008) (Walker et al, 2001) (Hoogduijn et al, 2009) (Warner et al, 2006). Botulinum neurotoxin inhibits the release of acetylcholine from motor neurons at the pre-synaptic level, and has been found to impair bone healing and induce a decrease in bone mineral content. Poliomyelitis destroys motor neurons that use acetylcholine neurotransmitters and appears to be directly related to osteoporosis. Smokers have high concentrations of nicotine in blood that interacts with the nicotinic nAChR causing a desensitization of these receptors (Mohammad et al. 2009).

The wide expression of cholinergic components in bone tissue points toward the important role they could play in bone remodeling. Bone cells have been shown to express all components of the cholinergic system (acetylcholine, cholinergic receptors and AChE) (Bajayo et al, 2012) (Shi et al, 2010) (Dani et al, 2007) (Liu et al, 2011) (En-Nosse et al, 2009) (Vogel-Hopker et al, 2012) (Grisaru et al, 1999) (Inkson et al, 2004) (Fujii et al, 2009). For instance, acetylcholine has been reported in osteoblasts (Sato et al, 2010). Other studies have shown that acetylcholine might regulate the migration of bone marrow mesenchymal stem cells (Tang et al, 2012). These are multipotent cells capable to differentiate into bone cells. AChE is expressed in bone cells such as bone marrow-derived monocytes, osteoclasts and osteoblasts (Bajayo et al, 2012) (Inkson et al, 2004) (Genever et al, 1999).

Recent research has also revealed the presence of a number of AChR subunits in bone cells. Nicotinic AChR subunits have been detected in human primary bone cells, MSCs and osteoblasts, while muscarinic receptors have been detected in MSCs and human

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

osteoblasts. It has also been found that osteoblasts express specific AChR and cholinergic components that may play a possible role in regulating the ALP activity as well as the proliferation and differentiation of osteoblasts (Walker et al, 2001) (Hoogduijn et al, 2009) (En-Nosse et al, 2009) (Alkondon et al, 2000) (Sato et al, 2010).

Cholinergic receptors, both nicoting and muscarinic, have shown to affect bone turnover. It appears that the only nicotinic receptor that shows an effect on the bone remodeling pattern is subtype α2 (Alkondon et al, 2000) (Kliemann et al, 2012) (Rothem et al, 2009) (Bajayo et al, 2012). Indeed, knockout nicotinic receptor subtype α2 mice are osteoporotic due to up-regulation of osteoclasts. Stimulation of nicotinic receptors in wild type mice has been shown to induce bone mass gain as a result of an increase in osteoclast apoptosis (Bajayo et al, 2012). On the other hand muscarinic receptors, more specifically M3 receptors, have been shown to influence bone remodeling favoring bone formation and decreasing bone resorption. Knockout M3 receptors mice are osteoporotic due to an increase in the number of osteoclasts and a decrease in the number of osteoblasts. Stimulation of muscarinic receptors with cholinergic agonists *in vitro* increases osteoblast proliferation, as previously illustrated in (figure 5) (Shi et al, 2010) (Kliemann et al, 2012) (Liu et al, 2011).

The activity of cholinergic receptors in bone is terminated by AChE which has been identified at sites of new bone formation, suggesting a role for AChE as a bone matrix protein. Indeed, it has been reported that AChE can regulate cell proliferations, differentiations, cell-cell contact as well as mesenchymal stem cells migration (Vogel et al, 2012) (Inkson et al, 2004) (Genever et al, 1999) (Fujii et al, 2009) .

Altogether, the available literature seems to indicate that the inhibition of the AChR at the bone level seems to cause a reduction in bone turnover. However, there are no

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

clinical studies that determine whether or not boosting up of the acetylcholine activity might have an anabolic effect on bone formation (Ma et al, 2010) (Klienmann et al, 2012) (Liu et al, 2011).

Recent studies have shown the role of the SNS in regulating bone turn over by inhibiting bone formation. Accordingly, beta blockers have been shown to increase bone density and reduce the incidence of bone fractures in osteoporotic patients. This effect could be explained by a decrease in the osteoclastogenic effect associated with β2 receptors inhibition. Mice deficient for the β2 adrenergic receptor have a lower bone resorption and increased bone formation, for this reason they appear to maintain greater trabecular bone microarchitecture as they grow older (Pierroz et al, 2006). On the other hand β-2 agonists used in obstructive lung disease patients have 2 x fold increased risk of hip fracture. In reflex sympathetic dystrophy (also known as Sudeck Syndrome), characterized by a hyperactivity of the adrenergic pathway, localized osteopenia is caused by an increase in bone resorption. Athletes that consume β2 agonists for their anabolic effect on muscle mass show bone loss and an increased risk of suffering fatigue fractures (De Vries et al, 2007) (Schwartzman et al, 2000). This osteopenic effect caused by an increased adrenergic signaling could be explained by the osteoclastogenic effect associated with β2 receptor stimulation.

The results presented in this study expand our understanding of the role the autonomous nervous system on bone remodeling, by confirming an anabolic effect of the cholinergic system that antagonizes the effect of the adrenergic SNS (Takeda et al, 2008, Schlienger et al, 2004).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

2. Methodological considerations

AD patients are known to be more susceptible to have falls than the general population, due to cognitive and behavioral disorders, visual and motor problems, gait and balance disturbances, and adverse effects of medications (Melton et al, 1994) (Visser, 1983) (Buchner et al, 1987) (Strubel et al, 2001). A cohort study in the early nineties already revealed that AD patients presented a much higher incidence of hip fractures than the regular population, with an overall relative risk ratio of 2.7 (95% CI, 1.8-4.2). A similar study done in the same period revealed that the rate of hip fractures in women with AD and vascular dementia was more than twice that found in the general population. In a more recent study performed between 1988 and 2007, AD patients had a hazard ratio for hip fractures of 2.8. However, in this studies the authors did not discriminate between AD patients receiving AChEIs and those who were not (Melton et al, 1994) (Johanson et al, 1996) (Baker et al, 2011). In our study the overall relative risk for AD patients treated with AChEIs and for those not treated with AChEIs in relation to the general population was 0.97 (CI 95% 0.89-0.99) [0.48 (CI 95% 0.14-0.85) for rivastigmine 0.92 (CI 95% 0.46-1.0) for donepezil and 1.9 (CI 95% 0.98-3.1) for galantamine] and 3.2 (CI 95% 2.1-4.6) respectively. We can appreciate from these figures that the relative risk for untreated AD patients found in our study are consistent with the results published in the English literature.

AChEIs were introduced for the treatment of AD in the mid nineties, and eventually became part of the standard treatment of AD patients. Unfortunately AChEIs can result only in marginal clinical improvement in measures of cognition and global assessment of dementia, and do not alter the natural course of the disease. In our study we observed that users of AChEIs had overall slightly better indices of mental status (MMES, CDR

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

and BDS) than nonusers, which may have been caused by the medication as reported elsewhere (Pepeu et al, 2009) (Rina et al, 2008) (Raschetti et al, 2005).

In our study we observed that AChEI users were associated with a higher risk of falling than nonusers. AChEI could have two competing effects on fall risk, on one hand, AChEI users are known to have a higher risk of bradycardia and consequently of syncope related falls, while on the other hand, AChEI can improve cognition and therefore prevent falls associated with mental issues. In our study we also observed that AChEI users had a higher FRAT than nonusers, indicating that probably the possible risks of falling due to bradycardia syncope might outweigh the cognitive benefits of AChEI (Strubel et al, 2001) (Hernandez et al, 2009).

Another interesting finding observed in our study was that BMI was significantly higher in AChEI users than in nonusers. The activity of the ANS has been shown to be a primary factor regulating body mass index. Sympathetic activity is an important factor limiting body weight gain. Indeed, recent studies have shown that the inhibition of sympathetic activity with beta blockers in patients treated for hypertension is associated with an increase in body mass index (Molfino et al, 2009) (Lee et al, 2011). On the other hand, parasympathetic activity may also play an important role in body weight regulation. Recent studies in animals have shown that enhanced parasympathetic activity is associated with increased fat deposition. Treatments with AChEI have been found to increase PSNS activity in the body, therefore, it is possible that the differences in BMI between users and nonusers of AChEIs is a result of the effect of the drugs on PSNS activity (Hernandez et al, 2009) (Balbo et al, 2007).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

3. Most relevant aspects

The annual incidence of hip fractures (per 1000 persons) in the general population of Malaga in the year 2008 appeared to increase with age following a linear pattern. The hip fracture incidence ranged from 1.4 x 10^{-3} (CI 95% 1.0-2.0) in the age group between 70-74 years (including both genders) and 17.3 x 10^{-3} (CI 95% 12.6-23.1) in patients ≥90 years (table 10) (figure 12). However this tendency was more obvious in the female population; the hip fracture incidence in female and male patients ≥90 years was 28.9 x 10^{-3} (CI 95% 20.5-39.4) and 4.9 x 10^{-3} (CI 95% 1.8-10.6) respectively, as previously illustrated in (table 10) and (figure 12). These results are similar to other studies published in the English literature (Sambrook et al, 2006) (De Laet et al, 1997).

In contrast to what was observed in the general population the average incidence of hip fractures (per 1000 persons) in AD patients in the period between 1/1/2004-31/12/2008 followed a negatively-skewed distribution, increasing in younger age groups and decreasing in older groups; ranging from 2.1 x 10^{-3} (CI 95% 0.1-11.9) in the age group between 70-74 years (including both genders), 8.0 x 10^{-3} (CI 95% 2.9-18.4) in the group between 75-79 years, and 1.6 x 10^{-3} (CI 95% 0.0-8.0) in patients ≥90 years (table 11) (figure 13). However the average hip fracture incidence for all the age groups remained higher in the female population; 6.4 x 10^{-3} (CI 95% 3.6-10.5) in females and 2.6 (CI 95% 0.5-7.6) in males, as previously illustrated in (table 11) and (figure 13). These results differ from the results that have been published in other studies where the hip fracture risk in AD patients followed a linear progression, however these studies did not discriminate between patients that were treated with AChEIs from those who didn't (Baker et al, 2011) (Melton et al, 1994) (Johanson et al, 1996).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

The progressive decrease in the hip fracture risk in AD patients that has been observed in our study, especially in the older age groups cannot be explained by the mobility status of the patients, as only 4.5% were bedridden, and an alternative explanation should be proposed. We believe that the prolonged pharmacological treatment with AChEI may explain this tendency.

We also observed that the risk of hip fracture varied dramatically among AD patients depending on the AChEI treatment received. The risk of hip fractures in patients treated with donepezil and rivastigmine was lower than in patients receiving no AChEIs [adjusted OR of 0.39 (0.19-0.76) ($p<0.002$) and of 0.22 (0.10-0.45) ($p<0.001$) respectively]. However, treatment with galantamine had no beneficial effect on the risk of hip fractures [adjusted OR of 1.67 (0.63-4.45)] ($p=0.305$). Galantamine has a similar, if not better, effect than donepezil and rivastigmine, on the cognitive abilities of AD patients, therefore the difference in hip fracture incidence observed is not justified by changes in patients AD conditions. On the other hand, the AChEIs evaluated in this study have different effects on AChRs subunits, and this may explain the variability of their association with hip fracture risk (Pepeu et al, 2009) (Raskind et al, 2000). For instance, galantamine is able to bind to all nAChRs regardless of their tissue or cell origin; but it cannot bind to mAChRs. Hence, the allosteric potentiating ligands (APL) action of galantamine produces a predominant nicotinic cholinergic enhancement. In contrast, the cholinergic enhancement by donepezil and rivastigmine is both, nicotinic and muscarinic, and is devoid of the nicotinic APL action (Maelicke et al, 2000) (Samochocki et al, 2003) (Basselin et al, 2009). Excessive nicotinic activity has been associated with reduced bone remodeling capacity. Therefore galantamine predominant action on nAChRs may have been the reason behind the significantly higher incidence of hip fractures in patients taking galantamine compared to those receiving donepezil or

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

rivastigmine. On the other hand, activity of muscarinic receptors has been found to favor bone mass accrual. Therefore the action rivastigmine and donepezil on mAChR may have been the reason behind a significant reduction of hip fracture risk in the patients receiving these treatments. This effect could be caused by the activation of M3 muscarinic receptors on osteoblasts and α2 nicotinic receptors on osteoclasts induced by higher levels of acetylcholine in the synaptic space (Kleinmann et al, 2012) (Shi et al, 2010) (Alkondon et al, 2000).

Nowadays the most commonly and successful drugs used for the prevention of bone fractures in elder osteoporotic patients are bisphosphonates, and PTH. In general terms, bisphosphonates have shown a protective RR in hip fractures relative to placebo of 0.66 (95% CI, 0.51–0.85). Moreover, the RRs of zoledronic acid, alendronate, and risedronate relative to placebo are 0.58 (95% CI, 0.41-0.82), 0.95 (95% CI, 0.54-1.68), and 0.73 (95% CI, 0.37-1.44), respectively (Shi et al, 2010) (Hodsman et al, 2002) (Jansen et al, 2011). On the other hand, PTH treatments have shown a reduction in the incidence of hip fractures with a RR relative to placebo of 0.46 (95% CI 0.25 - 0.86). In our case-control study, the OR shown by rivastigmine and donepezil vs. nonusers AChEIs was of 0.22 (95% CI 0.10-0.45) and 0.39 (95% CI, 0.19-0.76) respectively. Although these results cannot be directly compared with RR values from controlled trials, they still indicate that AChEIs stimulating mAChRs may have potential applications in the prevention of hip fractures in elder patients, especially in AD patients. However, future clinical and in vivo studies will have to be performed to further confirm this hypothesis (Neer et al, 2001).

Recent studies have shown the role of the adrenergic SNS in regulating bone turn over by inhibiting bone formation. Accordingly, β-blockers have been shown to increase bone density and reduce the incidence of bone fractures in osteoporotic patients. The

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

results presented in this study expands our understanding of the role the autonomous nervous system on bone remodeling, by confirming an anabolic effect of the cholinergic system that antagonizes the effect of the adrenergic SNS (Takeda et al, 2008) (Schlienger et al, 2004).

4. General conclusions

The main result observed in our study was that our working hypothesis was confirmed; AD users of AChEIs had a significantly lower risk of developing hip fractures than nonusers. These results were adjusted to cognitive factors related to the course of the disease, as well as to other factors affecting hip fractures previously shown in (table 12). Therefore, our findings are likely to be due to the effect of AChEIs on the cholinergic metabolic activity. The association that we observed between AChEI intake and BMI may also confirm the effect of AChEIs on cholinergic metabolic activity. The fact that the reduction in hip fracture risk in AChEIs users was dose dependent strengthens this possibility. Moreover, a recent retrospective cohort study carried out by our research team on female AD patients with hip fractures, has observed that the bone quality determined by the Singh index, and radiographic hip fracture consolidation capacity measured using the Hammer index, were both superior in patients treated with AChEIs compared to non users of AChEIs (Eimar, Perez Lara et al, 2013). These last findings add further evidence to our working hypothesis.

Nevertheless, the retrospective design of these studies does not allow us to determine a cause-effect relationship between AChEI intake, the ANS and bone metabolism; therefore, additional experimental controlled trials would need to be done in order to confirm these potentially important findings.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

The use of these medications is also associated with a series of side effects that can affect a significant percentage of the patients, such as nausea, vomiting, diarrhea, abdominal pain, loss of appetite, vertigo, dyspepsia, and headaches. All these factors should be taken in consideration when prescribing these drugs (Hoffmann et al, 2011) (Suh et al, 2011). Despite these generally bearable side effects, we believe that AD patients would benefit from the reduction in the hip fracture risk related to the use of AChEIs, and we encourage their prescription in this population group. However, their indication in healthy elderly groups or in osteoporotic patients will certainly need further evidence, but could become a real alternative option in the prevention of osteoporotic fractures in the future.

This study presents the first clinical evidence suggesting that stimulation of muscarinic cholinergic receptors may reduce the risk of hip fracture in a group of elderly patients (AD patients). These receptors are present in the CNS as well as in bone cells; therefore, it is not clear whether the effect of AChEIs on bone is a systemic or a local one. Recent studies have described the presence of cholinergic nerve fibers in bone that could be having a direct effect on the AChRs in bone. However it remains unclear whether these fibers are part of the sympathetic cholinergic system or the PSNS. On the other hand, AChEIs might be having a systemic effect on bone through either the SNS or humoral mechanisms (Shi et al, 2010) (Sisask et al, 1996) (Gajda et al, 2010). Therefore further investigations will be needed to unravel the exact mechanism by which the phenomenon works.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

5. Limitations

One limitation of this study was the reduced number of subjects that could be included, which lead to non significant statistical results regarding the effects of galantamine on the hip fracture risk and the relation between galantamine and donepezil dosage and the hip fracture risk. Another limitation of our case-control design was that we could not detect the competing risk of death on the different groups, and this might have an effect on the outcome of the study (Berry et al, 2010). AD leads to excess mortality (Larson et al, 2004); this renders comparison between AD patients and AD-free patients complicated, and for this reason we did not include patients free of AD in our study. However, mortality among AD patients themselves also varies depending on factors such as age of AD onset, as well as general and mental health of the patients. Median survival of AD is longer for patients with younger onset of the disease and shorter in patients with later onset (Larson et al, 2004). Accordingly, older AD patients have a much higher mortality than younger ones, and this could be contributing to the loss of the ‘‘normal’’ age distribution of fractures that is seen in this study. In a similar way, the association between the mortality of patients and their general and metal health conditions could also affect our results. However, even though our case-control design did not adjust for mortality, our risk estimates were adjusted to the strongest predictors of mortality in AD (age, MMSE, BDS, fall risk, and comorbidity) (Larson et al, 2004) and were still confirmatory of the protective effect of AChEIs on hip fracture. Confounding by indication could also be a limitation in this retrospective study. Despite the fact that AChEIs are strongly recommended for the treatment of AD, the criteria used by physicians to prescribe AChEIs are often not clear (Hoffmann et al, 2011). AChEIs do not have any significant effect on the mortality and survival of AD patients, regardless of their baseline comorbid medical conditions (Suh et al, 2011). Also, there is

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

no information in the literature correlating expected mortality of AD patients with physician's decisions to prescribe AChEIs. However, it is possible that AChEIs might be targeted to patients based on their expected residual life. Indeed, it has been found that clinicians are less likely to prescribe AChEIs to patients with worse overall clinical conditions and frailty (Hoffmann et al, 2011). In our study we observed that AD patients in an advanced stage of the disease were less likely to receive AChEIs, probably due to the terminal status of their condition. This issue could be a limitation in our study because it could drive an artificial risk reduction for hip fracture with AChEIs. However, risk estimates adjusted to the grade of AD and comorbidity were still confirmatory of the protective effect of AChEIs on hip fracture. Another limitation of this study was that our retrospective design does not allow us to determine a cause-effect relationship between AChEI intake and hip fracture. An additional limitation in our study was that the effect of AChEIs on bone was only examined in an indirect way by assessing risks of hip fractures, and we do not provide information on the physiological conditions of bone.

Other medications such as proton pump inhibitors that alter bone metabolism and act as potential confounders were not analyzed in this study, these could influence to a certain point our results. Compliance, which could act as a possible confounder, was not measured during the collection of data. For all these reasons, future large-scale prospective cohort studies will have to be performed in order to confirm our findings by increasing the sample size, determining cause-effect relationships, and eliminating the possibility of bias by competing risk of death. Moreover, in vivo and clinical studies assessing biochemical bone markers, bone density measurements, and bone microarchitecture (high-resolution [HR]-pQCT) will have to be undertaken to explore the effect of AChEIs on bone structure and mineral content.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Our research team is currently designing a large scale prospective cohort study that aims to analyze the hip fracture risk in AD patients in the United Kingdom. All the clinical information needed to complete this work will be acquired from the British health system computer database. A second line of research in collaboration with McGill University, University of Malaga and Carlos Haya Hospital is also in progress. It aims to study a possible syndrome of hypocholinergic activity which could include clinical conditions such as osteoporosis, AD, and several autonomic system dysfunctions. We believe that these studies will provide valuable information that could add further evidence to the results stated in this thesis

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Publicaciones y
Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Conclusions

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

- This is the first clinical evidence demonstrating that the administration of acetylcholinestarase inhibitors; donepezil and rivastigmine, is associated with a reduction in the risk of hip fracture in patients with Alzheimer disease.

- The administration galantamine an acetylcholinestarase inhibitor which also causes an allosteric stimulation of nicotinic receptors, does not reduce the risk of hip fracture is patients with Alzheimer disease.

- The decrease in the risk of hip fracture caused by the administration of donepezil and rivastigmine could be explained by the stimulation of type M3 muscarinic receptors on osteoblasts.

- Elderly patients with Alzheimer disease could benefit from the positive effects that acetylcholinestarase inhibitors may have on the hip fracture risk.

- It will be necessary to perform additional controlled clinical trials in order to fully confirm these findings.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

References

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

1. Abraham G, Paing WW, Kaminski J, Joseph A, Kohegyi E, Josiassen RC. Effects of elevated serum prolactin on bone mineral density and bone metabolism in female patients with schizophrenia: a prospective study. Am J Psychiatry. 2003 Sep;160(9):1618-20.
2. Agha-Hosseini F, Mirzaii-Dizgah I, Moosavi MS. Relationship of lumbar spine bone mineral density and oral dryness feeling in menopause. Menopause 2011; 18:625-8.
3. Ahlquist R.P. A Study of the Adenotrophic Receptors, Am. J. Physiol. 1948 153:586-600.
4. Albright F, Bloomberg E, Smith PH. Post-menopausal osteoporosis. Trans Assoc Am Phys 1940. 55:298–305.
5. Alkondon M, Pereira EF, Almeida LE, Randall WR, Albuquerque EX. Nicotine at concentrations found in cigarette smokers activates and desensitizes nicotinic acetylcholine receptors in CA1 interneurons of rat hippocampus. Neuropharmacology. 2000; 39(13):2726-39.
6. American Academy of Pediatrics Committee on School Health. Soft drinks in schools. Pediatrics 2004; 113 (1 Pt 1): 152–4.
7. Arends J, Wagner ML, Willms BL. Cholinergic muscarinic receptor blockade suppresses arginine-induced and exercise-induced growth-hormone secretion in type I diabetic subjects. J Clin Endocr Metab 1988; 66:389-94.
8. Aro H, Eerola E, Aho AJ, Penttinen R. Healing of experimental fractures in the denervated limbs of the rat. Clin Orthop Relat Res. 1981; 211-217.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

9. Asmus SE, Parsons S, Landis SC. Developmental changes in the transmitter properties of sympathetic neurons that innervate the periosteum. Journal of Neuroscience. 2000; 20, 1495-1504.

10. Athanasou NA. Cellular biology of bone-resorbing cells. J Bone Joint Surg Am. 1996 Jul; 78(7):1096-112.

11. Bacman S, Perez Leiros C, Sterin-Borda L, Hubscher O, Arana R, Borda E. Autoantibodies against lacrimal gland m3 muscarinic acetylcholine receptors in patients with primary sjogren's syndrome. Invest Ophthalmol Vis Sci 1998; 39:151-6.

12. Bajayo, A. Bar A, Denes A, Bachar M, Kram V, Attar-Namdar M, Zallone A, Kovács KJ, Yirmiya R, Bab I. Skeletal parasympathetic innervation communicates central IL-1 signals regulating bone mass accrual. Proc Natl Acad Sci USA 2012 Sep; 109, 15455-15460.

13. Baker NL, Cook MN, Arrighi HM, Bullock R. Hip fracture risk and subsequent mortality among Alzheimer's disease patients in the United Kingdom, 1988-2007.Age Ageing 2011 Jan;40(1):49-54

14. Balbo SL, Grassiolli S, Ribeiro RA, Bonfleur ML, Gravena C, Brito Mdo N, Andreazzi AE, Mathias PC, Torrezan R. Fat storage is partially dependent on vagal activity and insulin secretion of hypothalamic obese rat. Endocrine. 2007; 31(2):142-8.

15. Ballock RT, Reddi AH.Thyroxine is the serum factor that regulates morphogenesis of columnar cartilage from isolated chondrocytes in chemically defined medium. J Cell Biol. 1994 Sep; 126 (5):1311-8.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

16. Baloh RW, Honrubia V, Jacobson K. Benign positional vertigo: Clinical and oculographic features in 240 cases. Neurology 1987; 37:371-8.

17. Barnes GL, Kakar S, Vora S, Morgan EF, Gerstenfeld LC, Einhorn TA. Stimulation of fracturehealing with systemic intermittent parathyroid hormone treatment. J Bone Joint Surg Am. 2008 Feb; 90 Suppl 1:120-7.

18. Bart Clarke. Normal Bone Anatomy and Physiology. Clin J Am Soc Nephrol 2008; 3: S131–S13.

19. Basselin M, Nguyen HN, Chang L, Bell JM, Rapoport SI. Acute but not chronic donepezil increases muscarinic receptor-mediated signaling via arachidonic acid in unanesthetized rats. J Alzheimers Dis. 2009; 17(2):369-82.

20. Bassett JH, Williams GR. Critical role of the hypothalamic-pituitary-thyroid axis in bone. Bone. 2008 Sep; 43(3):418-26.

21. Bellido T, Jilka RL, Boyce BF, Girasole G, Broxmeyer H, Dalrymple SA, Murray R, Manolagas SC. Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the androgen receptor. J Clin Invest 1995 Jun; 95:2886–2895.

22. Bentler SE, Liu L, Obrizan M, Cook EA, Wright KB, Geweke JF, Chrischilles EA, Pavlik CE, Wallace RB, Ohsfeldt RL, Jones MP, Rosenthal GE, Wolinsky FD. The aftermath of hip fracture: discharge placement, functional status change, and mortality. Am J Epidemiol 2009; 170:1290.

23. Berry SD, Ngo L, Samelson EJ, Kiel DP. Competing risk of death: an important consideration in studies of older adults. J Am Geriatr Soc. 2010;58(4):783–7.

24. Bhattacharyya P, Paul R, Ghosh M, Dey R, Barooah N, Islam S, Acharya D, Nag S, Bardhan S. Prevalence of osteoporosis and osteopenia in advanced chronic obstructive pulmonary disease patients. Lung India 2011; 28:184-6.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

25. Bleuming SA, He XC, Kodach LL, Hardwick JC, Koopman FA, Ten Kate FJ, van Deventer SJ, Hommes DW, Peppelenbosch MP, Offerhaus GJ, Li L, van den Brink GR. Bone morphogenetic protein signaling suppresses tumorigenesis at gastric epithelial transition zones in mice. Cancer Research. 2007 Sep 1; 67 (17): 8149–55.

26. Bonnet N, Benhamou CL, Malaval L, Goncalves C, Vico L, Eder V, Pichon C, Courteix D. Low dose betablocker prevents ovariectomy-induced bone loss in rats without affecting heart functions J Cell Physiol 2008; Dec;217(3):819-27.

27. Bonnet N, Brunet-Imbault B, Arlettaz A, Horcajada MN, Collomp K, Benhamou CL, Courteix D .Alteration of trabecular bone under chronic beta-2 agonist's treatment. Med Sci Sports Exerc. 2005 Sep; 37(9):1493-501.

28. Bono CM, Einhorn TA. Overview of osteoporosis: pathophysiology and determinants of bone strength. Eur Spine J. Oct 2003; 12 Suppl 2:S90-6.

29. Brian KA, Koda-Kimble MA, Young LY, Kradjan WA, Guglielmo BJ (2009). Applied therapeutics: the clinical use of drugs. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins. pp. 101–3.

30. Buchner DM, Larson EB. Falls and fractures in patients with Alzheimer-type dementia. JAMA. 1987; 257(11):1492-5.

31. Bucholz RW, Heckman JD, Court-Brown CM, Tornetta P, Koval KJ, Wirth MA, BeatyJH, Kasser JR. Rockwood and Green's Fractures in Adults. 5th edition. Philadelphia, PA: Lippincott Williams & Wilkins; 2001:1635-1663.

32. Burford NT, Nahorski SR. Muscarinic M_1 receptor-stimulated adenylate cyclase activity in Chinese hamster ovary cells is mediated by Gs alpha and is not a

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

consequence of phosphoinositidase C activation. Biochem.J. 1996; 315 (3): 883–8.

33. Capeci CM, Tejwani NC. Bilateral low-energy simultaneous or sequential femoral fractures in patients on long-term alendronate therapy. J Bone Joint Surg Am. 2009 Nov; 91(11):2556-61.
34. Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol. Rev. 1998 Jun; 50 (2): 279–90.
35. Caulfield MP. Muscarinic receptors-characterization, coupling and function. Pharmacol Ther 1993;58:319-79.
36. Charlson ME, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chron Dis.* 1987; 40(5): 373– 83.
37. Chen-Izu Y, Xiao RP, Izu LT, Cheng H, Kuschel M, Spurgeon H, Lakatta EG. "G(i)-dependent localization of beta(2)-adrenergic receptor signaling to L-type Ca(2+) channels". Biophys. J. 2000; 79(5): 2547–56.
38. Chen JH, Liu C, You L, Simmons CA. Boning up on Wolff's Law: Mechanical regulation of the cells that make and maintain bone. Journal of Biomechanics. 2010 Jan 5; 43(1):108-18.
39. Chen Y, Guo Q, Pan X, Qin L, Zhang P. Smoking and impaired bone healing: Will activation of cholinergic anti-inflammatory pathway be the bridge? Int Orthop 2011; 35:1267-70.
40. Chen Y, Shohami E, Constantini S, Weinstock M.Rivastigmine, a brain-selective acetylcholinesterase inhibitor, ameliorates cognitive and motor deficits

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

induced by closed-head injury in the mouse. J Neurotrauma. 1998 Apr; 15(4):231-7.

41. Cheng JT, Kuo DY. "Both alpha1-adrenergic and D (1)-dopaminergic neurotransmissions are involved in phenylpropanolamine-mediated feeding suppression in mice". Neuroscience Letters 2003, 347 (2): 136–8.

42. Clayton BA, Hayashida K, Childers SR, Xiao R, Eisenach JC. Oral donepezil reduces hypersensitivity after nerve injury by a spinal muscarinic receptor mechanism. Anesthesiology. 2007 May; 106(5):1019-25.

43. Confederación Española de Familiares de Enfermos de Alzheimer y Otras Demencias (CEAFA) (2010).

44. Cummings SR, Melton LJ. Epidemiology and outcomes of osteoporotic fractures. Lancet. May 18 2002; 359(9319):1761-7.

45. Dani JA, Bertrand D. Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. Annu Rev Pharmacol.2007; 47, 699-729.

46. De Laet CE, van Hout BA, Burger H, Hofman A, Pols HA. Bone density and risk of hip fracture in men and women: cross sectional analysis. BMJ. 1997 Jul 26; 315(7102):221-5.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

47. De M, Sanford TR, Wood GW. Interleukin-1, interleukin-6, and tumor necrosis factor alpha are produced in the mouse uterus during the estrous cycle and are induced by estrogen and progesterone. Dev Biol. 1992 May; 151(1):297-305.

48. De Pedro-Cuesta J, Virués-Ortega J, Vega S. Seijo-Martínez M, Saz P, Rodríguez F, Rodríguez-Laso A, Reñé R, De las Heras SP, Mateos R, Martínez-Martín P,Manubens JM, Mahillo-Fernandez I, López-Pousa S, Lobo A, Reglà JL, Gascón J, García FJ, Fernández-Martínez M, Boix R, Bermejo-Pareja F, Bergareche A,Benito-León J, De Arce A, Del Barrio JL. Prevalence of dementia and major dementia subtypes in Spanish populations: A reanalysis of dementia prevalence surveys, 1990- 2008. BMC Neurol. 2009 Oct 19; 9:55.

49. De Vries F, Pouwels S, Bracke M, Leufkens HG, Cooper C, Lammers JW, van Staa TP. Use of beta-2 agonists and risk of hip/femur fracture: a populationbased case-control study. Pharmacoepidemiol Drug Saf 2007; 16:612-29.

50. Dell'Acqua ML, Carroll RC, Peralta EG. Transfected m2 muscarinic acetylcholine receptors couple to G alpha i2 and G alpha i3 in Chinese hamster ovary cells. Activation and desensitization of the phospholipase C signaling pathway. J. Biol. Chem. 1993 Mar; 268 (8): 5676–85.

51. Dennison EM, Syddall HE, Sayer AA, Gilbody HJ, Cooper C. Birth weight and weight at 1 year are independent determinants of bone mass in the seventh decade: the Hertfordshire cohort study. Pediatr Res. Apr 2005; 57(4):582-6.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

52. Duncan RL, Turner CH. Mechanotransduction and the functional response of bone to mechanical strain. Calcified Tissue International. 1995 Nov; 57 (5): 344–358.

53. Eimar H, Perez Lara A, Tamimi I, Márquez Sánchez P, Gormaz Talavera I, Rojas Tomba F, García de la Oliva T, Tamimi F. Acetylcholinesterase inhibitors and healing of hip fracture in Alzheimer's disease patients: a retrospective cohort study. J Musculoskelet Neuronal Interact. 2013 Dec;13(4):454-63.

54. Eglen RM. Muscarinic receptor subtypes in neuronal and non-neuronal cholinergic function. Auton Autacoid Pharmacol. 2006 Jul; 26 (3): 219–33.

55. Elefteriou F, Ahn JD, Takeda S, Starbuck M, Yang X, Liu X, Kondo H, Richards WG, Bannon TW, Noda M, Clement K, Vaisse C, Karsenty G. Leptin regulation of bone resorption by the sympathetic nervous system and CART. Nature 2005; 434:514-20.

56. Elefteriou F. Regulation of bone remodeling by the central and peripheral nervous system. Arch Biochem Biophys. 2008 May 15; 473(2):231-6.

57. Elliott J. "Alpha-adrenoceptors in equine digital veins: evidence for the presence of both α_1- and α_2-receptors mediating vasoconstriction". J. Vet. Pharmacol. Ther. 1997; 20 (4): 308–17.

58. En-Nosse M, Hartmann S, Trinkaus K, Alt V, Stigler B, Heiss C, Kilian O, Schnettler R, Lips KS. Expression of non-neuronal cholinergic system in osteoblast-like cells and its involvement in osteogenesis. Cell Tissue Res. 2009; 338(2):203-15.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

59. Fall C, Hindmarsh P, Dennison E, Kellingray S, Barker D, Cooper C. Programming of growth hormone secretion and bone mineral density in elderly men: a hypothesis. J Clin Endocrinol Metab. Jan 1998; 83(1):135-9.

60. Feng XH, Derynck R. Review Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol. 2005; 21:659-93.

61. Ferrer-Lorente R, Cabot C, Fernández-López JA, Alemany M (September 2005). "Combined effects of oleoyl-estrone and a β_3-adrenergic agonist (CL316, 243) on lipid stores of diet-induced overweight male Wistar rats". Life Sciences 77 (16): 2051-8.

62. Filmore D. It's a GPCR world. Modern Drug Discovery (American Chemical Society) 2004 Nov: 24–28.

63. Folstein MF, Folstein SE, McHugh PRT. 'Mini-Mental State': a practical method for grading the cognitive state patients for the clinician. J Psychiatr Res 1975; 12: 189-198.

64. Frost HM. Skeletal structural adaptations to mechanical usage (SATMU): 3. The hyaline cartilage modeling problem. Anat Rec 1990 Apr; 226:423–432.

65. Frost HM. Wolff's Law and bone's structural adaptations to mechanical usage: an overview for clinicians. The Angle Orthodontist 1994; 64 (3): 175–188.

66. Frymoyer JW. Pope M.H. Fracture healing in the sciatically denervated rat. J Trauma 1977; 17, 355-361.

67. Fujii T, Masai M, Misawa H, Okuda T, Takada-Takatori Y, Moriwaki Y, Haga T, Kawashima K. Acetylcholine synthesis and release in NIH3T3 cells coexpressing the high-affinity choline transporter and choline acetyltransferase. J Neurosci Res 2009; 87, 3024-3032.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

68. Furness JB. Types of neurons in the enteric nervous system. J Auton Nerv Syst 2000; 81:87-96.

69. Gage BF, Birman-Deych E, Radford MJ, Nilasena DS, Binder EF. Risk of osteoporotic fracture in elderly patients taking warfarin: results from the National Registry of Atrial Fibrillation 2. Arch. Intern. Med. 2006; 166 (2): 241–6.

70. Gajda M, Litwin JA, Tabarowski Z, Zagólski O, Cichocki T, Timmermans JP,Adriaensen D. Development of rat tibia innervation: colocalization of autonomic nerve fiber markers with growth-associated protein 43. Cells Tissues Organs. 2010; 191(6):489-99.

71. Garimella R, Tague SE, Zhang J, Belibi F, Nahar N, Sun BH, Insogna K, Wang J, Anderson HC. Expression and Synthesis of Bone Morphogenetic Proteins by Osteoclasts: A Possible Path to Anabolic Bone Remodeling. J Histochem Cytochem. 2008 June; 56(6): 569–577.

72. Gautam D, Han SJ, Duttaroy A, Mears D, Hamdan FF, Li JH, Cui Y, Jeon J, Wess J. Role of the m3 muscarinic acetylcholine receptor in beta-cell function and glucose homeostasis. Diabetes Obes Metab 2007; 9 Suppl 2:158-69.

73. Genant HK, Halse J, Briney WG, Xie L, Glass EV, Krege JH.The effects of teriparatide on the incidence of back pain in postmenopausal women with osteoporosis. Curr Med Res Opin. 2005 Jul; 21(7):1027-34.

74. Genever, P. G., Birch, M. A., Brown, E. & Skerry, T. M. Osteoblast-derived acetylcholinesterase: A novel mediator of cell-matrix interactions in bone? Bone 1999; 24, 297-303.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

75. Gispen WH, Biessels GJ. Cognition and synaptic plasticity in diabetes mellitus. Trends Neurosci 2000; 23:542-9.

76. Glowacki J, Schulten AJ, Perrott D, Kaban LB. Nicotine impairs distraction osteogenesis in the rat mandible. J Oral Maxillofacial Surg. 2008; 37 (2):156-61.

77. Gocht H. Ätiologie, Pathogenese und Therapie der Deformitäten im allgemeinen. In Hoffa Orthopädisch Chirurgie. Edited by Gocht H. Stuttgart: Ferdinand Enke; 1920:8–74.

78. Gourlay M, Franceschini N, Sheyn Y. Prevention and treatment strategies for glucocorticoid-induced osteoporotic fractures. Clin Rheumatol. 2007; 26 (2): 144–53.

79. Gray H. Anatomy of the human body, 20th edition, 1918 Philadelphia and New York, Lea & Febiger.

80. Grimsrud CD, Romano PR, D'Souza M, Puzas JE, Reynolds PR, Rosier RN, O'Keefe RJ. BMP-6 is an autocrine stimulator of chondrocyte differentiation. J Bone Miner Res. 1999 Apr; 14(4):475-82.

81. Grimston SK, Silva MJ, Civitelli R. Bone loss after temporarily induced muscle paralysis by Botox is not fully recovered after 12 weeks. Ann N Y Acad Sci. 2007; 1116:444-60.

82. Grisaru D, Lev-Lehman E, Shapira M, Chaikin E, LessingLessing JB, Eldor A, Eckstein F, Soreq H. Human osteogenesis involves differentiation-dependent increases in the morphogenically active 3' alternative splicing variant of acetylcholinesterase. Mol Cell Biol 1999; 19:788-95.

83. Groeneveld EH, Burger EH. Review Bone morphogenetic proteins in human bone regeneration. Eur J Endocrinol. 2000 Jan; 142(1):9-21.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

84. Hadjidakis DJ, Androulakis II. Bone remodeling. Ann N Y Acad Sci. 2006 Dec; 1092:385-96.

85. Hall JE, Hildebrandt DA, Kuo J. Obesity hypertension: role of leptin and sympathetic nervous system. Am J Hypertens.. 2001 Jun; 14(6 Pt 2):103S-115S.

86. Hall JE. Guyton and Hall Text book of medical physiology. ELSEVIER 2010. ISBN-10: 1416045740.

87. Hambleton P. Clostridium-botulinum toxins - a generalreview of involvement in disease, structure, mode of action and preparation for clinical use. J Neurol 1992; 239:16-20.

88. Hamrick MW, Ferrari SL. Leptin and the sympathetic connection of fat to bone. Osteoporos Int. 2008 Jul; 19 (7): 905–912.

89. Hernandez RK, Farwell W, Cantor MD, Lawler EV. Cholinesterase inhibitors and incidence of bradycardia in patients with dementia in the veterans affairs New England healthcare system. J Am Geriatr Soc. 2009; 57(11):1997-2003.

90. Hegde SS, Choppin A, Bonhaus D, Briaud S, Loeb M, Moy TM, Loury D, Eglen RM. Functional role of m2 and m3 muscarinic receptors in the urinary bladder of rats in vitro and in vivo. Br J Pharmacol 1997; 120:1409-18.

91. Hegsted M, Schuette SA, Zemel MB and Linkswiler HM. Urinary calcium and calcium balance in young men as affected by level of protein and phosphorus intake. The Journal of nutrition 1981 Mar; 111(3):553-62.

92. Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, Lubahn DB. A role for estrogens in the male reproductive system. Nature .1997, 390 (6659): 509–12.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

93. Hodsman AB, Hanley DA, Josse R. Do bisphosphonates reduce the risk of osteoporotic fractures? An evaluation of the evidence to date. CMAJ. 2002; 166(11):1426-30.

94. Hogan BL. Review Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. 1996 Jul 1; 10(13):1580-94.

95. Hoffmann F, van den Bussche H, Wiese B, Scho¨n G, Koller D, Eisele M, Glaeske G, Scherer M, Kaduszkiewicz H. Impact of geriatric comorbidity and polypharmacy on cholinesterase inhibitors prescribing in dementia. BMC Psychiatry. 2011; 11:190.

96. Hollenberg AN, Monden T, Flynn TR, Boers ME, Cohen O, Wondisford FE. The human thyrotropin-releasing hormone gene is regulated by thyroid hormone through two distinct classes of negative thyroid hormone response elements. Mol Endocrinol. 1995 May; 9(5):540-50.

97. Hollinger JO, Schmitt JM, Hwang K, Soleymani P, Buck D. Impact of nicotine on bone healing. J Biomed Matr Res. 1999; 45(4):294-301. Erratum in: J Biomed Mat Res 1999; 5; 46(3):438-9.

98. Holt EM, Evans RA, Hindley CJ, Metcalfe JW. 1000 femoral neck fractures: the effect of pre-injury mobility and surgical experience on outcome. Injury. 1994 Mar; 25(2):91-5.

99. Hong SJ, Chang CC. Nicotinic actions of oxotremorine on murine skeletal-muscle - evidence against muscarinic modulation of acetylcholine-release. Brain Res 1990; 534:142-48.Hong SJ, Chang CC. Nicotinic actions of oxotremorine on murine skeletal-muscle - evidence against muscarinic modulation of acetylcholine-release. Brain Res 1990; 534:142-48.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

100. Hoogduijn MJ, Cheng A, Genever PG. Functional nicotinic and muscarinic receptors on mesenchymal stem cells. Stem Cells Dev. 2009; 18(1):103-12.

101. Huang C, Ogawa R. Mechanotransduction in bone repair and regeneration. The FASEB Journal. 2010 Oct 24 (10):3625-32.

102. Huges CP, Berg L, Danziger L, et al. A new clinical scale for the staging of dementia. Br J Psychiatry 1982; 140:566–72.

103. Inkson CA, Brabbs AC, Grewal TS, Skerry TM, Genever PG. Characterization of acetylcholinesterase expression and secretion during osteoblast differentiation. Bone 2004 Oct;35, 819-827.

104. Ishii M, Kurachi Y. Muscarinic acetylcholine receptors". Curr. Pharm. Des. 2006; 12 (28): 3573–81.

105. Itier V, Bertrand D. Neuronal nicotinic receptors: from protein structure to function. FEBS Letters. 2001 Aug; 504 (3): 118–25.

106. Jain KK. Evaluation of memantine for neuroprotection in dementia. Expert Opin Investig Drugs. 2000; 9:1397–1406.

107. Jansen JP, Bergman GJ, Huels J, Olson M. The Efficacy of Bisphosphonates in the Prevention of Vertebral, Hip, and Nonvertebral-Nonhip Fractures in Osteoporosis: A Network Meta-Analysis. Semin Arthritis Rheum. 2011 Feb; 40(4):275-84.e1-2.

108. Jay R. Leiberman .AAOS Comprehensive Orthopaedic Review. Published by American Academy of Orthopaedic Surgeons, Rosemont IL. Copyright 2009.

109. Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, et al. Increased osteoclast development after estrogen loss: mediation by interleukin-6. Science. Jul 3 1992; 257(5066):88-91.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

110. Jimenez Caballero PE, Alfonso V, Gonzalez-Adalid M. Current treatments of Alzheimer disease: are main caregivers satisfied with the drug treatments received by their patients? Dement Geriatr Cogn Disord. 2009; 28:196–205.

111. Johanson C, Skoog I. A population – based study on the association between dementia and hip fractures in 85-year olds. Aging (Milano). 1996; 8 (3):189-96.

112. Johnson G. PDQ Pharmacology. 2002. Second edition. Hamilton, Ontario: BC Decker Inc. pp. 311 pages.

113. Kannus P, Parkkari J, Sievänen H, et al. Epidemiology of hip fractures. Bone 1996; 18:57S.

114. Kasperk CH, Wergedal JE, Farley JR, Linkhart TA, Turner RT, Baylink DJ. Androgens directly stimulate proliferation of bone cells in vitro. Endocrinology. 1989, 124:1576–1578.

115. Katz S, Weinerman S. Osteoporosis and gastrointestinal disease. Gastroenterol Hepatol (N Y) 2010; 6:506-17.

116. Kellenberger S, Muller K, Richener H, Bilbe G.Formoterol and isoproterenol induce c-fos gene expression in osteoblast-like cells by activating beta2-adrenergic receptors. Bone 1998; 22:471-8.

117. Kesler BS, Canning BJ. Regulation of baseline cholinergic tone in guinea-pig airway smooth muscle. J Physiol- London 1999; 518:843-55.

118. King N, Hittinger CT, Carroll SB. Evolution of key cell signaling and adhesion protein families predates animal origins. Science. 2003; 301 (5631): 361–3.

119. Kliemann K, Kneffel M, Bergen I, Kampschulte M, Langheinrich AC, Dürselen L, Ignatius A, Kilian O, Schnettler R, Lips KS. Quantitative

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

analyses of bone composition in acetylcholine receptor M3R and alpha7 knockout mice. Life Sci. 2012 Nov 27; 91(21-22):997-1002.

120. Koenen M, Peter C, Villarroel A, Witzemann V, Sakmann B. Acetylcholine receptor channel subtype directs the innervations pattern of skeletal muscle. EMBO Rep 2005; 6:570-6.

121. Kondo H, Takeuchi S, Togari A. β-Adrenergic signaling stimulates osteoclastogenesis via reactive oxygen species. Am J Physiol Endocrinol Metab. 2013 Mar 1; 304(5):E507-15.

122. Lapatsanis P, Kavadias, Vretos K. Juvenile Osteoporosis. Arch Dis Child. 1971 February; 46(245): 66–71.

123. Larson EB, Shadlen MF, Wang L, McCormick WC, Bowen JD, Teri L, Kukull WA. Survival after initial diagnosis of Alzheimer disease. AnnIntern Med. 2004; 140(7):501–9.

124. Lecka-Czernik. Marrow fat metabolism is linked to the systemic energy metabolism. Bone. 2012 Feb; 50(2):534-9.

125. Leder BZ, LeBlanc KM, Schoenfeld DA, Eastell R, Finkelstein JS. Differential Effects of Androgens and Estrogens on Bone Turnover in Normal Men. J Clin Endocrinol Metab. 2003 Jan; 88 (1): 204.

126. Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, Dacquin R, Mee PJ, McKee MD, Jung DY, Zhang Z, Kim JK, Mauvais-Jarvis F, Ducy P, Karsenty G. "Endocrine regulation of energy metabolism by the skeleton". Aug 2007; Cell 130 (3): 456–69.

127. Lee P, Kengne AP, Greenfield JR, Day RO, Chalmers J, Ho KK. Metabolic sequelae of β-blocker therapy: weighing in on the obesity epidemic? Int J Obes (Lond). 2011 Nov; 35(11):1395-403.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

128. Levitan IB, Kaczmarek LK. The Neuron: Cell and Molecular Biology .2002, Third edition pg. 285. Oxford University Press.

129. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, Harris SE, Wu D (May 2005). "Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling". *J. Biol. Chem.*280 (20): 19883-7.

130. Lippuner K. The future of osteoporosis treatment – a research updates Swiss Med Wkly. 2012 Jul 19; 142:w13624.

131. Liu PS, Chen YY, Feng CK, Lin YH, Yu TC. Muscarinic acetylchline receptors present in human osteoblast and bone tissue. Eur J Pharmacol. 2011 Jan 10; 650 (1):34-40.

132. Liu EY, Wactawski-Wende J, Donahue RP, Dmochowski J, Hovey KM, Quattrin T. Does low bone mineral density start in post-teenage years in women with type 1 diabetes? Diabetes Care 2003; 26:2365-69.

133. Louvet J, Harman S, Ross G. Effects of human chorionic gonadotropin, human interstitial cell stimulating hormone and human follicle-stimulating hormone on ovarian weights in estrogen-primed hypophysectomized immature female rats. Endocrinology. 1975. 96 (5): 1179–86.

134. Ma L, Zheng LW, Sham MH, Cheung LK. Uncoupled angiogenesis and osteogenesis in nicotine-compromised bone healing. J Bone Miner Res. 2010; 25(6):1305-13.

135. Maelicke A, Albuquerque EX. Allosteric modulation of nicotinic acetylcholine receptors as a treatment strategy for Alzheimer's disease. Eur J Pharmacol. 2000; 393(1-3):165-70.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

136. Marie PJ, Debiais F, Haÿ E. Regulation of human cranial osteoblast phenotype by FGF-2, FGFR-2 and BMP-2 signaling. Histol. Histopathol. 2002, 17 (3): 877–85.

137. Mark D, Miller MD, Stephen R. Review of Orthopaedics sixth edition, ELSEVIER, Philadelphia, 2012.

138. Martin RB. Targeted bone remodeling involves BMU steering as well as activation. Bone. 2007 Jun; 40(6):1574-80.

139. Martin-Du Pan RC. Estrogens and male sexuality: efficiency of antiestrogens in case of hypothalamic hypogonadism and late onset hypogonadism. Rev Med Suisse. 2011 Mar 23; 7(287):644-7.

140. Masaaki Sawa, Hiroshi Harada (2006). "Recent Developments in the Design of Orally Bioavailable β3-Adrenergic Receptor Agonists". Current Medicinal Chemistry 13 (1): 25–37.

141. Maselli RA, Wollmann R, Roos R. Function and ultrastructure of the neuromuscular junction in post-polio syndrome. Ann N Y Acad Sci 1995; 753:129-37.

142. Massoud F, Gauthier S. Update on the pharmacological treatment of Alzheimer's disease. Curr Neuropharmacol. 2010; 8(1): 69-80.

143. Matsuoka I, Domino EF. Cholinergic mechanisms in the cat vestibular system. Neuropharmacology 1975; 14:201-10.

144. Mau H. Specification of corresponding growth laws of Hueter-Volkmann and Pauwels (growth deformities) and their relation to deformities caused by loading. Z Orthop Ihre Grenzgeb. 1984 May-June; 122:293–298.

145. McDonald F. Electrical effects at the bone surface. Eur J Orthod. 1993 Jun; 15 (3): 175-83.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

146. Mehlman CT, Araghi A, Roy DR. Hyphenated history: the Hueter-Volkmann law. Am J Orthop (Belle Mead NJ) 1997 Nov; 26:798–800.

147. Melton LJ, Beard CM, Kokmen E, Atkinson EJ, O'Fallon WM. Fracture risk in patients with Alzheimer's disease. J Am Geriatr Soc. 1994; 42(6):614-9.

148. Melton LJ. Epidemiology worldwide. Endocrinol. Metab. Clin. North Am. 2003; 32 (1): 1–13.

149. Mirakhur RK, Clarke RS, Elliott J, Dundee JW. Atropine and glycopyrronium premedication. A comparison of the effects on cardiac rate and rhythm during induction of anaesthesia. Anaesthesia 1978; 33:906-12.

150. Miyazono K, Maeda S, Imamura T. BMP receptor signaling: Transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev. 2005 Jun; 16(3):251-63.

151. Mohammad AF, Khan KA, Galvin L, Hardiman O, O'Connell PG. High Incidence of Osteoporosis and Fractures in an Aging Post-Polio Population. Eur Neurol. 2009; 62(6):369-374.

152. Molfino A, Fiorentini A, Tubani L, Martuscelli M, Rossi Fanelli F, Laviano A. Body mass index is related to autonomic nervous system activity as measured by heart rate variability. Eur J Clin Nutr. 2009; 63(10):1263-5.

153. Moore RE, Smith CK, Bailey CS, Voelkel EF, Tashjian AH Jr. Characterization of beta-adrenergic receptors on rat and human osteoblast-like cells and demonstration that beta-receptor agonists can stimulate bone resorption in organ culture. Bone Miner 1993; 23:301-15.

154. Morris JC. The clinical dementia rating (CDR): current version and scoring rules. Neurology 1993; 43:2412–14.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

155. Moss SE, Klein R, Klein BEK. Prevalence of and risk factors for dry eye syndrome. Arch Ophthalmol-Chic 2000; 118:1264-68.

156. Murphy CE, Rodgers PT. Effects of thiazolidinediones on bone loss and fracture. Ann Pharmacother. 2007; 41 (12): 2014–8.

157. Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH. Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. N Engl J Med. 2001 May 10; 344(19):1434-41.

158. Nelson LR, Bulun SE. Estrogen production and action. J Am Acad Dermatol. 2001 Sep; 45(3 Suppl):S116-24.

159. Netter FH. Musculoskeletal system: anatomy, physiology, and metabolic disorders. 1987. Summit, New Jersey: Ciba-Geigy Corporation.

160. Nielsen HK, Laurberg P, Brixen K, Mosekilde L. Relations between diurnal-variations in serum osteocalcin, cortisol, parathyroid-hormone, and ionized calcium in normal individuals. Acta Endocrinol-Cop 1991; 124:391-98.

161. Nijweidi PJ, Burger EH. Feyen M. Cells of Bone: Proliferation, Differentiation, and Hormonal Regulation. Physiological Reviews. 1986 Oct; 66 (4): 855–886.

162. Nikolov R, Kuhl H, Golbs S. Estrogen replacement therapy and alzheimer's disease. Drugs Today (Barc) 1998; 34:927-33.

163. No authors. Effects of hormone therapy on bone mineral density: Results from the postmenopausal estrogen/progestin interventions (PEPI) trial. The writing group for the pepi. Jama 1996; 276:1389-96.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

164. Ojo F, Al Snih S, Ray LA, Raji MA, Markides KS. History of fractures as predictor of subsequent hip and nonhip fractures among older Mexican Americans. Journal of the National Medical Association. 2007; 99 (4): 412–8.

165. Olney RC. Regulation of bone mass by growth hormone. Med Pediatr Oncol. 2003 Sep; 41(3):228-34.

166. Onishi T, Ishidou Y, Nagamine T, Yone K, Imamura T, Kato M, Sampath TK, ten Dijke P, Sakou T. Distinct and overlapping patterns of localization of bone morphogenetic protein (BMP) family members and a BMP type II receptor during fracture healing in rats. Bone. 1998 Jun; 22(6):605-12.

167. Orwoll ES. Osteoporosis in men. Endocrinol Metab Clin North Am. 1998, 27:349–367.

168. Overington JP, Al-Lazikani B, Hopkins AL. How many drug targets are there?. Nat Rev Drug Discov. 2066 Dec; 5 (12): 993–6.

169. Panula J, Pihlajamäki H, Mattila VM, Jaatinen P, Vahlberg T, Aarnio P, Kivelä SL. Mortality and cause of death in hip fracture patients aged 65 or older: a population-based study. BMC Musculoskelet Disord 2011; 12:105.

170. Park K, Haberberger RV, Gordon TP, Jackson MW. Antibodies interfering with the type 3 muscarinic receptor pathway inhibit gastrointestinal motility and cholinergic neurotransmission in Sjogren's syndrome. Arthritis Rheum-Us 2011; 63:1426-34.

171. Parker K, Brunton L; Goodman LS, Lazo, JS, Gilman A. Goodman & Gilman's the pharmacological basis of therapeutics 2006, (11th Ed.). New York: McGraw-Hill.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

172. Pazirandeh A, Xue Y, Prestegaard T, Jondal M, Okret S. Effects of altered glucocorticoid sensitivity in the T cell lineage on thymocyte and T cell homeostasis. FASEB J. 2002 May; 16 (7): 727–9.

173. Pederson L, Kremer M, Judd J, Pascoe D, Spelsberg TC, Riggs BL, Oursler MJ. Androgens regulate bone resorption activity of isolated osteoclasts in vitro. Proc Natl Acad Sci USA 1999, 96:505–510.

174. Pepeu G, Giovannini MG. Cholinesterase inhibitors and beyond. Curr Alzheimer Res. 2009; 6(2):86-96.

175. Petty SJ, O'Brien TJ, Wark JD. Anti-epileptic medication and bone health. Osteoporosis international 2007; 18 (2): 129–42.

176. Pierroz DD, Baldock P, Bouxsein ML, Ferrari SL. Low cortical bone mass in mice lacking beta 1 and beta 2 adrenergic receptors is associated with low bone formation and circulating IGF-1. J Bone Miner Res 2006;21(Suppl.1):S277.

177. Pierroz DD, Bonnet N, Bianchi EN, Bouxsein ML, Baldock PA, Rizzoli R, Ferrari SL. Deletion of β-adrenergic receptor 1, 2, or both leads to different bone phenotypes and response to mechanical stimulation. J Bone Miner Res. 2012 Jun; 27(6):1252-62.

178. Poole KE, Compston JE. Osteoporosis and its management. BMJ 2006 Dec; 333 (7581): 1251–6.

179. Pouwels S, de Boer A, Javaid MK, Hilton-Jones D, Verschuuren J, Cooper C, Leufkens HG, de Vries F. Fracture rate in patients with myasthenia gravis: The general practice research database. Osteoporos Int 2013; 24(2):467-76.

180. Pressman MR, Fry JM. Relationship of autonomic nervous system activity to daytime sleepiness and prior sleep. Sleep 1989; 12:239-45.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

181. Purves D, Augustine GJ, Fitzpatrick D, Hall A, Lamantia AS, White LE . Neuroscience. Sinauer Associates Inc.,U.S.; Edición: 5th edition. 2012.

182. Raikhinstein M, Zohar M, Hanukoglu I. cDNA cloning and sequence analysis of the bovine adrenocorticotropic hormone (ACTH) receptor. Biochim. Biophys. Acta. 1994 Feb; 1220 (3): 329–32.

183. Raina P, Santaguida P, Ismaila A, Patterson C, Cowan D, Levine M, Booker L, Oremus M. Effectiveness Q5 of cholinesterase inhibitors and memantine for treating dementia: evidence review for a clinical practice guideline. Ann Intern Med. 2008; 148(5):379–97.

184. Raisz L. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest 2005; 115(12): 3318–25.

185. Ramachandran M. Basic Orthopaedics Sciences. The Stanmore Guide. Hodder Arnold, Stanmore 2007.

186. Rang, H. P. (2003). Pharmacology. Edinburgh: Churchill Livingstone. ISBN 0-443-07145-4. Page 163.

187. Raschetti R, Maggini M, Sorrentino GC, Martini N, Caffari B, Vanacore N. A cohort study of effectiveness of acetylcholinesterase inhibitors in Alzheimer's disease. Eur J Clin Pharmacol. 2005; 61(5-6):361-8.

188. Raskind MA, Peskind ER, Wessel T, Yuan W. Galantamine in AD: A 6-month randomized, placebo-controlled trial with a 6-month extension. The Galantamine USA-1 Study Group. Neurology. 2000; 54(12):2261-8.

189. Rauch F. Bone growth in length and width: the Yin and Yang of bone stability. J Musculoskelet Neuronal Interact 2005 Jul-Sep; 5:194–201.

190. Reddi AH. Cell biology and biochemistry of endochondral bone development. Collagen Rel Res 1981; 1:209–26.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

191. Reddi H. Bone morphogenetic proteins. Adv Dent Res. 1995 Nov; 9(3 Suppl):13.

192. Reddi AH, Reddi A. Bone morphogenetic proteins (BMPs): from morphogens to metabologens. Cytokine & growth factor reviews. 2009 Oct-Dec; 20 (5-6): 341–2.

193. Rina P, Santaguida, Ismaila A, Patterson C, Cowan D, Levine M, Booker L, Oremus M.. Effectiveness of cholinesterase inhibitors and memenatine for treating dementia: evidence review for a clinical practice guideline. Ann intern Med. 2008; 148 (5) :379-97.

194. Ripamonti U, Duneas N. Review tissue morphogenesis and regeneration by bone morphogenetic proteins. Plast Reconstr Surg. 1998 Jan; 101(1):227-39.

195. Rothem DE, Rothem L, Soudry M, Dahan A, Eliakim R. Nicotine modulates bone metabolism-associated gene expression in osteoblast cells. J Bone Miner Metab 2009;27 (5)55-561.

196. Rosen CJ, Tenenhouse A. Biochemical markers of bone turnover. A look at laboratory tests that reflect bone status. Postgrad Med. Oct 1998; 104(4):101-2, 107-10.

197. Rubenstein LA, Zauhar RJ, Lanzara RG. "Molecular dynamics of a biophysical model for β_2-adrenergic and G protein-coupled receptor activation".J. Mol. Graph. Model. 2006; 25 (4): 396–409.

198. Ruuskanen JO, Xhaard H, Marjamäki A, Salaneck E, Salminen T, Yan YL, Postlethwait JH, Johnson MS, Larhammar D, Scheinin M. "Identification of duplicated fourth alpha2-adrenergic receptor subtype by cloning and mapping of five receptor genes in zebrafish". Molecular Biology and Evolution 2004; 21 (1): 14–28.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

199. Sacher, Ronald; Richard A. McPherson. Wildmann's Clinical Interpretation of Laboratory Tests, 2000, 11th ed. F.A. Davis Company.

200. Sagrada A, Fargeas MJ, Bueno L."Involvement of α_1 and α_2 adrenoceptors in the postlaparotomy intestinal motor disturbances in the rat". Gut 1987; 28 (8): 955–9.

201. Sahota O. Reducing the risk of fractures with calcium and vitamin D. BMJ. 2010 Jan 12; 340:b5492.

202. Sambrook P, Cooper C. Osteoporosis. Lancet. 2006 Jun 17; 367(9527):2010-8.

203. Samochocki M, Höffle A, Fehrenbacher A, Jostock R, Ludwig J, Christner C, Radina M, Zerlin M, Ullmer C, Pereira EF, Lübbert H, Albuquerque EX, Maelicke A. Galantamine is an allosterically potentiating ligand of neuronal nicotinic but not of muscarinic acetylcholine receptors. J Pharmacol Exp Ther. 2003; 305(3):1024-36.

204. Santos J, Saunders PR, Hanssen NP, Yang PC, Yates D, Groot JA, Perdue MH .Corticotropin-releasing hormone mimics stress-induced colonic epithelial pathophysiology in the rat. Am. J. Physiol. 1999 Aug; 277 (2 Pt 1): G391–9.

205. Sato T, Abe T, Chida D, Nakamoto N, Hori N, Kokabu S, Sakata Y, Tomaru Y, Iwata T, Usui M, Aiko K, Yoda T. Functional role of acetylcholine and the expression of cholinergic receptors and components in osteoblasts. FEBS Lett. 2010; 584(4):817-24.

206. Satoh N, Ogawa Y, Katsuura G, Numata Y, Tsuji T, Hayase M, Ebihara K, Masuzaki H, Hosoda K, Yoshimasa Y, Nakao K. Sympathetic activation of leptin via the ventromedial hypothalamus: leptin-induced increase in catecholamine secretion. Diabetes. 1999; 48:1787–1793.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

207. Schaak S, Cayla C, Lymperopoulos A, Flordellis C, Cussac D, Denis C, Paris H.Transcriptional down-regulation of the human alpha2C-adrenergic receptor by cAMP. Mol Pharmacol. 2000 Oct; 58(4):821-7.

208. Schlienger RG, Kraenzlin ME, Jick SS, Meier CR. Use of beta-blockers and risk of fractures. JAMA. 2004; 292(11):1326-32.

209. Schmitz JM, Graham RM, Sagalowsky A, Pettinger WA. "Renal α_1 and α_2 adrenergic receptors: biochemical and pharmacological correlations". J. Pharmacol. Exp. Ther. 1981; 219 (2): 400–6.

210. Schwartzman RJ. New treatments for reflex sympathetic dystrophy. N Engl J Med 2000; 343:1811-2.

211. Shapses SA, Riedt CS. Bone, body weight, and weight reduction: what are the concerns? .J. Nutr. 2006 Jun; 136 (6): 1453–6.

212. Shi Y, Oury F, Yadav VK, Wess J, Liu XS, Guo XE, Murshed M, Karsenty G. Signaling through the m3 muscarinic receptor favors bone mass accrual by decreasing sympathetic activity. Cell Metab 2010; 11:231-38.

213. Simon MI, Strathmann MP, Gautam N. Diversity of G proteins in signal transduction. Science. 1991; 252 (5007): 802–8.

214. Simonelli, C. ICSI Health Care Guideline: Diagnosis and Treatment of Osteoporosis, 5th edition. 2006 Jul. Institute for Clinical Systems Improvement.

215. Sisask, G., Bjurholm, A., Ahmed, M., Kreicbergs, A. The development of autonomic innervation in bone and joints of the rat. J Auton Nerv Syst. 1996; 59, 27-33.

216. Smith DW, Gardiner BS, Dunstan C. Bone balance within a cortical BMU: local controls of bone resorption and formation. PLoS One. 2012;7(7):e40268. Epub 2012 Jul 23.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

217. Song D, Jiang X, Zhu S, Li W, Khadka A, Hu J. Denervation impairs bone regeneration during distraction osteogenesis in rabbit tibia lengthening. Acta Orthop 2012; 83, 406-410.

218. Stein MS, Thomas CD, Feik SA, Wark JD, Clement JG. Bone size and mechanics at the femoral diaphysis across age and sex. J Biomech. 1998 Dec; 31(12):1101 10.

219. Stokes IA. Mechanical effects on skeletal growth. J Musculoskelet Neuronal Interact 2002 Mar; 2:277–280.

220. Strubel D, Jacquot JM, Martin-Hunyadi C. Dementia and falls. Ann Readapt Med Phys. 2001; 44(1): 4-12.

221. Suh GH, Ryu SH, Lee DW, Han C, Ju YS, Kee BS, Lee JN, Bae JN, Choi JH, Kim DJ, Lee NJ, Lee JY, Go HJ, Yi JS, Cho SJ, Jeon YW. Cholinesterase inhibitors for Alzheimer disease: do they provide more than symptomatic benefits?. Am J Geriatr Psychiatry. 2011; 19(3):266–273.

222. Takeda S, Elefteriou F, Levasseur R, Liu X, Zhao L, Parker KL, Armstrong D, Ducy P, Karsenty G. Leptin regulates bone formation via the sympathetic nervous system. Cell. 2002; 111:305–317.

223. Takeda S, Karsenty G. Molecular bases of the sympathetic regulation of bone mass. Bone. 2008; 42(5):837-40.

224. Takeda S, Yoshizawa T, Nagai Y, Yamato H, Fukumoto S, Sekine K, Kato S, Matsumoto T, Fujita T. Stimulation of osteoclast formation by 1,25-dihydroxyvitamin D requires its binding to vitamin D receptor (VDR) in osteoblastic cells: studies using VDR knockout mice. Endocrinology. 1999 Feb; 140(2):1005-8.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

225. Weycker D, Taneja C, Edelsberg J, Erder MH, Schmitt FA, Setyawan J, Oster G. Cost-effectiveness of memantine in moderate-to-severe Alzheimer's disease patients receiving donepezil. Curr Med Res Opin. 2007 May; 23(5):1187-97.

226. Tang JM, Yuan J, Li Q, Wang JN, Kong X, Zheng F, Zhang L, Chen L, Guo LY, Huang YH, Yang JY, Chen SY. Acetylcholine induces mesenchymal stem cell migration via Ca^{2+}/PKC/ERK1/2 signal pathway. J Cell Biochem. 2012 Aug; 113, 2704-2713.

227. Tien D, Ohara PT, Larson AA, Jasmin L. Vagal afferents are necessary for the establishment but not the maintenance of kainic acid-induced hyperalgesia in mice. Pain 2003 Mar; 102, 39-49.

228. Togari A. Adrenergic regulation of bone metabolism: possible involvement of sympathetic innervation of osteoblastic and osteoclastic cells. Microsc Res Tech 2002; 58:77-84.

229. Togari A, Arai M, Mizutani S, Mizutani S, Koshihara Y, Nagatsu T. Expression of mRNAs for neuropeptide receptors and beta-adrenergic receptors in human osteoblasts and human osteogenic sarcoma cells. Neurosci Lett 1997; 233:125-8.

230. Tuominen JT, Impivaara O, Puukka P, Ronnemaa T. Bone mineral density in patients with type 1 and type 2 diabetes. Diabetes Care 1999; 22:1196-200.

231. Turner CH, Forwood MR, Otter MW . Mechanotransduction in bone: do bone cells act as sensors of fluid flow? The FASEB journal. 1994 Aug; 8 (11): 875-8.

232. Van Driel M, Pols HA, van Leeuwen JP. Osteoblast differentiation and control by vitamin D and vitamin D metabolites. Curr Pharm Des. 2004; 10(21):2535-55.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

233. Vignaux G, Besnard S, Ndong J, Philoxène B, Denise P, Elefteriou F. Bone remodeling is regulated by inner ear vestibular signals. J Bone Miner Res. 2013 Oct; 28(10):2136-44.

234. Visser H. Gait and balance in senile dementia of Alzheimer's type. Age Ageing. 1983; 12(4): 296-301.

235. Vogel-Hopker A, Sperling L E, Layer PG. Co-opting Functions of Cholinesterases in Neural, Limb and Stem Cell Development. Protein Peptide Lett, 2012; 19, 155-164.

236. Wakata N, Nemoto H, Sugimoto H, Nomoto N, Konno S, Hayashi N, Araki Y, Nakazato A. Bone density in myasthenia gravis patients receiving long-term prednisolone therapy. Clin Neurol Neurosurg 2004; 106:139-41.

237. Walker LM, Preston MR, Magnay JL, Thomas PB, El Haj AJ. Nicotinic regulation of c-fos and osteopontin expression in human-derived osteoblast-like cells and human trabecular bone organ culture. Bone. 2001; 28(6):603-8.

238. Wang Z, Shi H, Wang H. Functional m3 muscarinic acetylcholine receptors in mammalian hearts. Br J Pharmacol 2004; 142:395-408.

239. Warner SE, Sanford DA, Becker BA, Bain SD, Srinivasan S, Gross TS. Botox induced muscle paralysis rapidly degrades bone. Bone. 2006; 38(2):257-64.

240. Watson JT, Moed BR. Ipsilateral femoral neck and shaft fractures: complications and their treatment. Clin Orthop Relat Res. 2002 Jun; (399):78-86.

241. Weycker D, Taneja C, Edelsberg J, Erder MH, Schmitt FA, Setyawan J, Oster G. Cost-effectiveness of memantine in moderate-to-severe Alzheimer's disease patients receiving donepezil. Curr Med Res Opin. 2007; 23:1187–1197.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

242. WHO. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. Report of a WHO Study Group. World Health Organization technical report series. 1994; 843: 1–129.

243. WHO Scientific Group on the Prevention and Management of Osteoporosis (2000: Geneva, Switzerland) (2003).

244. Williams PA, Saha S. The electrical and dielectric properties of human bone tissue and their relationship with density and bone mineral content. Ann Biomed Eng. 1996 Mar-Apr; 24(2):222-33.

245. Wolff J. The Law of Bone Remodeling. Berlin Heidelberg New York: Springer, 1986 (translation of the German 1892 edition).

246. Wolinsky FD, Fitzgerald JF, Stump TE. The effect of hip fracture on mortality, hospitalization, and functional status: a prospective study. Am J Public Health 1997; 87:398.

247. Wong PK, Christie JJ, Wark JD. The effects of smoking on bone health. Clin Sci (Lond) 2007; 113:233-41.

248. Woodman OL, Vatner SF. "Coronary vasoconstriction mediated by α_1- and α_2-adrenoceptors in conscious dogs". Am. J. Physiol. 1987; 253 (2 Pt 2): H388–93.

249. Wozney JM, Rosen V. Review Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. Clin Orthop Relat Res. 1998 Jan; (346):26-37.

250. Xiong G, Doraiswamy PM. Combination drug therapy for Alzheimer's disease: what is evidence-based, and what is not? Geriatrics. 2005; 60:22–26.

251. Yang YX, Lewis JD, Epstein S, Metz DC. Long-term proton pump inhibitor therapy and risk of hip fracture. JAMA. 2006 Dec; 27; 296(24):2947-53.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

252. Yaturu S, Humphrey S, Landry C, Jain SK. Decreased bone mineral density in men with metabolic syndrome alone and with type 2 diabetes. Med Sci Monitor. 2009 Jan; 15 (1):Cr5-9.

253. Zaidi M, Blair HC, Moonga BS, Abe E, Huang CL. Osteoclastogenesis, bone resorption, and osteoclast-based therapeutics. J Bone Miner Res. 2003 Apr; 18(4):599-609.

254. Zamah AM, Delahunty M, Luttrell LM, Lefkowitz RJ. "Protein kinase A-mediated phosphorylation of the beta 2-adrenergic receptor regulates its coupling to Gs and Gi. Demonstration in a reconstituted system". J. Biol. Chem. 2002; 277 (34): 31249–56.

255. Zhang J, Watanabe Y, Yamada S, Urayama A, Kimura R. Neuroprotective effect and brain receptor binding of taltirelin, a novel thyrotropin-releasing hormone (TRH) analogue, in transient forebrain ischemia of C57BL/6J mice. Life Sci. 2001; 72 (4–5): 601–7.

256. Zheng LW, Ma L, Cheung LK. Changes in blood perfusion and bone healing induced by nicotine during distraction osteogenesis. Bone 2008; 43:355-61.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Article I

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

J Musculoskelet Neuronal Interact 2013; 13(2):...-...

Review Article

Hylonome

Cholinergic regulation of bone

H. Eimar[1], I. Tamimi[2], M. Murshed[1,3], F. Tamimi[1]

[1]Faculty of Dentistry, McGill University, Montreal, Quebec, H3A 0C7, Canada;
[2]Traumatology and Orthopedic Surgery Department, Hospital Regional Universitario Carlos Haya, Malaga, 29017, Spain;
[3]Faculty of Medicine at McGill University, Montreal, Quebec, H3G 1Y6, Canada

Abstract

Bone remodeling is regulated by the two branches of the autonomic nervous system: the adrenergic and the cholinergic branches. Adrenergic activity favors bone loss, whereas cholinergic activity has been recently shown to favor bone mass accrual. *In vitro* studies have reported that cholinergic activity induces proliferation and differentiation of bone cells. *In vivo* studies have shown that the inhibition of cholinergic activity favors bone loss, whereas its stimulation favors bone mass accrual. Clinical studies have shown that bone density is associated with the function of many cholinergic-regulated tissues such as the hypothalamus, salivary glands, lacrimal glands and langerhans cells, suggesting a common mechanism of control. Altogether, these observations and linked findings are of great significance since they improve our understanding of bone physiology. These discoveries have been successfully used recently to investigate new promising therapies for bone diseases based on cholinergic stimulation. Here, we review the current understanding of the cholinergic activity and its association with bone health.

Keywords: Cholinergic, Parasympathetic Nervous System, Bone, Acetylcholine, Osteoporosis

Introduction

Bone remodeling involves bone resorption by osteoclasts and subsequent formation of new bone by osteoblasts[1-3]. This process regulates the biomechanical features of bone and maintains the homeostasis of essential mineral ions in the body[1]. Alteration in the bone remodeling process results in diseases such as osteoporosis that can often cause life-threatening complications[3]. The mechanisms regulating bone remodeling are not fully understood, and unveiling them should eventually allow development of new therapeutic approaches for osteoporosis and other bone related diseases[3].

The process of bone formation and resorption is well-regulated, at least, at two different levels: locally and centrally[1]. Locally, bone remodeling is regulated through direct interaction between osteoblasts and osteoclasts, and by local interactions between cells of the immune system and bone cells[1]. Centrally, bone remodeling has been shown to be regulated at three axis: 1) the hypothalamic-pituitary-thyroid axis; 2) the co-regulation of bone, adipose tissue and energy metabolism mediated by the sympathetic nervous system (SNS) and 3) the IL 1- parasympathetic nervous system (PSNS)- bone axis[4-11]. The last two axes are mediated by adrenergic and cholinergic activities, the functional arms of autonomic nervous system[4-11].

The adrenergic activity of the SNS has been shown to be a negative regulator of bone mass; adrenergic signaling inhibits osteoblast proliferation and promotes osteoclastogenesis[4-6,12]. Moreover, SNS signaling induces the release of the osteoblast-derived soluble factor receptor activator of NF-κB ligand (RANKL), which in turn augments osteoclastogenesis and bone resorption[6]. SNS signaling are well controlled by the action of two key proteins: osteocalcin and leptin. Osteocalcin, produced by mature osteoblasts and osteocytes, regulates the production of leptin, an adipocyte-derived hormone. Leptin, in-turn, activates its receptors in the hypothalamus and promotes SNS activity[4-6].

Cholinergic activity has been shown to favor bone mass by increasing osteoblast proliferation via suppression of the SNS and promoting the apoptosis of osteoclasts[10,11]. The present article reviews the current understanding of the cholinergic effect on bone remodeling based on a variety of *in vitro, in vivo* and clinical studies, and suggests new ways for preventing bone fractures and osteoporosis by cholinergic stimulation.

The authors have no conflict of interest.

Corresponding author: Faleh Tamimi, Faculty of Dentistry, McGill University, 3640 University Street, Montreal, Quebec H3A 0C7, Canada
E-mail: faleh.tamimimarino@mcgill.ca

Edited by: .....
Accepted 18 April 2013

Publicaciones y Divulgación Científica
UMA UNIVERSIDAD DE MÁLAGA

Cholinergic system

Cholinergic system regulates the body's metabolic activity through the use of acetylcholine as a signal transmitter[13]. Acetylcholine is biosynthesized by choline acetyl transferase, and it is stored in small synaptic vesicles through the action of vesicular acetylcholine transporter enzyme to be released via exocytosis into the synapse space[14]. The secreted acetylcholine targets either nicotinic or muscarinic receptors[15,16]. Nicotinic receptors are compromised of different subunits α, β, γ, δ and ε that assemble to form ionic channels[17]. Muscarinic receptors are guanine nucleotide protein coupled receptors, and are classified into five subtypes: m1, m2, m3, m4 and m5[16,18]. Acetylcholine signal is terminated by its degradation through the enzyme acetylcholinesterase (AChE)[19].

Many neuronal cells such as all pre- and post-ganglionic nerves of the PSNS, preganglionic and some postganglionic nerves of the SNS, motor neurons and neurons within the central nervous system express the components of the cholinergic system (transmitter, enzymes and receptors)[10,11,20-22]. Moreover, many non-neuronal cells such as embryonic stem cells, epithelial cells and bone cells have been shown to express components of the cholinergic system as well[13,23,24].

Expression of cholinergic components in bone

Bone cells have been shown to express several components of the cholinergic system (transmitter, enzymes and receptors)[10,11,15,16,25-30]. For instance, acetylcholine has been suggested to be produced by osteosblasts due to the presence of the vesicular acetylcholine transporter enzyme in these cells[30]. Cholinergic receptors, both nicotinic and muscarinic, have been identified on the membranes of human primary bone cells, mesenchymal stem cells, osteoblasts and osteoclasts[10,11,25,26,31-34]. The mRNA of several nicotinic receptors subtypes (α_1, α_4, b_1 and g) are also present in osteocytes[35]. AChE is expressed in bone cells such as bone marrow-derived monocytes, osteoclasts and osteoblasts[11,29,36].

Possible role of cholinergic components in bone

The wide expression of cholinergic components in bone tissue points toward the important role they could play in bone remodeling[23]. Previous studies have shown that acetylcholine might regulate the migration of bone marrow-derived multiprogenitor mesenchymal stem cell[37] capable of differentiating into bone cells. Both families of cholinergic receptors, nicotinic and muscarinic, have been shown to affect bone turnover. Indeed, nicotinic stimulation in mice induces bone mass gain as a result of an increase in osteoclasts apoptosis[11], whereas muscarinic stimulation *in vitro* increases osteoblasts proliferation[25,30]. Among the cholinergic receptors, the nicotinic subtype-α_2 receptor and muscarinic-3 receptor appear important in bone physiology[10,11,38-40]. Indeed, mice with knockout nicotinic subtype-α_2 receptors are osteoporotic due to the up-regulation of osteoclasts[11], whereas mice with knockout muscarinic-3 receptors are osteoporotic due to a decrease of osteoblast numbers and an increase of osteoclast numbers[10].

Beside its capability to break down acetylcholine, AChE is thought to play significant roles during remodeling of bone. For instance, AChE has been detected at the sites of new bone formation[36], suggesting a role for AChE as a bone matrix protein[29,36]. Moreover, it has been reported that AChE can regulate bone cells proliferations, differentiations, cell-cell contact as well as mesenchymal stem cells migration[27,29,36,41,42].

Cholinergic innervation of bone

It has been shown that bone tissues are innervated by cholinergic fibers of both the PSNS and the SNS[11,43]. Cholinergic fibers transmit neuronal signaling from the PSNS nucleus within the CNS to cholinergic receptors located in bone[11]. Accordingly, it would be logical to expect that disruption in the function of these cholinergic fibers would affect bone. Indeed, it has been shown that mice subjected to subdiaphragmatic sectioning of the vagus nerve, a cranial nerve that caries cholinergic fibers of the PSNS, suffer from low bone mass in their lumbar vertebra[44]. Cholinergic fibers of the SNS have been shown to innervate the periosteum, a connective tissue that covers the bone and contains progenitor cells that develop into osteoblasts, indicating a possible role of these neuronal fibers in regulating bone formation and remodeling[45]. Indeed, denervation of the periosteum results in poor bone healing in animal models, indicating that periosteal nerves are required for bone formation and fracture healing[46-49].

Central nervous system nuclei

Hypothalamus

Previous studies have shown that the hypothalamus, a brain structure that encloses cholinergic (muscarinic and nicotinic) and adrenergic components, can affect bone remodeling[50,51]. It has been shown that Alzheimer's disease (AD) patients suffering from cholinergic degradation of the hypothalamus[52,53], are more prone to develop osteoporosis and suffer from a high incidence of fractures[54-57]. In fact, the association between bone mineral density and cholinergic degradation of the hypothalamus is so strong[58,59] that decrease in bone mineral density has been suggested as a predictor of AD[60]. These observations highlight the importance of cholinergic nucleus of the hypothalamus in regulating bone mass accrual.

One mechanism by which the hypothalamus might regulate bone remodeling is by controlling body weight[4], a known positive regulator of bone mass[61,62]. The hypothalamus encloses two structures that regulate body weight: the ventromedial and the lateral hypothalamic nuclei. The lateral hypothalamic nucleus is concerned in hunger, and any damage to this area can reduce body weight[63]. Lateral hypothalamic nucleus is known to enclose cholinergic neurons and receptors, such as muscarinic receptors[10,64]. Accordingly, suppression of cholinergic neurons activity in the lateral hypothalamic nucleus could explain why AD patients usually suffer from weight loss, hence low bone mass accrual[65]. However, future research has to be done to test these hypotheses.

It is well known that the hypothalamus regulate bone mass also through a neurohormonal pathway mediated by the pitu-

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Body tissue that expresses cholinergic receptors	Function of local cholinergic receptors	Effect of inhibition of local cholinergic receptors	Association between local inhibition of cholinergic receptors and bone mineral density (BMD)
Salivary glands[70]	Saliva secretion[78]	Dry mouth[78]	Patients with dry mouth are associated with low BMD[81]
Lacrimal glands[72]	Lacrimal secretion[114]	Dry eyes[79]	Patients with dry eyes are associated with low BMD[82]
Pancreas[73]	Insulin secretion[115]	Diabetes type[180,116]	Patients with diabetes type 1 are associated with low BMD[83,84,117,118]
Respiratory system1[19]	Smooth muscle contraction and epithelial regulation[119,120]	Obstructive pulmonary disease[88]	Patients with lung diseases are associated with low BMD[85]
Gastrointestinal system[74]	Smooth muscle contraction[74]	Decrease muscle tone[121]	Patients with gastrointestinal diseases are associated with low BMD[89]
Vestibular organ within ear[76]	Regulate balance and orientation[122]	Unbalance and disorientation (Vertigo)	Patients with vertigo are associated with low BMD[92]

Table 1. The relationship between cholinergic receptors in body tissues with bone turnover in various clinical conditions.

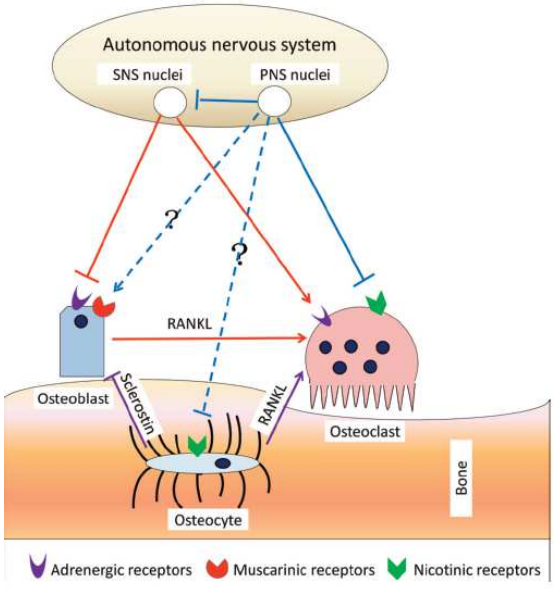


Figure 1. Model of the autonomic nervous system-mediated regulation of bone mass accrual. The SNS favors bone loss by inhibiting osteoblast proliferation, directly by activating the adrenergic receptors. Also, the SNS favors bone loss by promoting osteoclast proliferation, directly through activating the adrenergic receptors on osteoclasts and indirectly through stimulating RANKL secretion from osteoblasts. The PNS favors bone mass indirectly by suppressing the SNS signaling and directly by promoting apoptosis in osteoclasts through activating the nicotinic receptors. The two blue-dashed arrows refer to possible effects of the PNS on osteoblasts and osteocytes.

itary-thyroid-bone axis[2,8]. It has been reported that the expression of muscarinic receptors centrally might be regulated by the level of calcitonin[66], a hormone that plays a significant role in the pituitary-thyroid- bone axis. However, it has not been investigated whether this association between muscarinic receptors and calcitonin would affect bone mass.

Locus coeruleus

Muscarinic receptors, more specifically muscarinic-3 receptors, are expressed in non-adrenergic neurons in the locus coeruleus nucleus, a brain structure necessary for the SNS[10]. It has been shown that mice with knockout neural muscarinic-3 receptors are osteoporotic due to an increase in SNS signaling, indicating that muscarinic-3 receptors in the locus coeruleus nucleus down-regulate SNS signaling and favor bone mass indirectly[10].

Central IL-1

Central IL-1 is a proinflammatory cytokine produced by brain neurons that are known to regulate learning, memory and sleep patterns[67,68]. Central IL-1 can also affect bone metabolism through nicotinic nerve fibers of the PSNS[11]. Indeed, mice with knockout central IL-1 express very low levels of skeletal acetylcholine[11], and these mice are osteoporotic due to the decrease of apoptosis in osteoclasts[11,69].

Pathways by which cholinergic system regulates bone remodeling

Despite the substantial evidences we presented regarding the association between cholinergic activities at bone tissues-, neurons- and nuclei-level with bone remodeling, the pathways by which the cholinergic system affects bone remodeling are not well understood. It is still not known whether the cholinergic activity affects bone cells locally, systemically or both. In Figure 1, we suggest possible mechanisms for cholinergic regulation of bone. Recent studies have shown that cholinergic activity might regulate

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

bone mass systemically through neuronal pathways mediated parasympathetic activity[10,11].

Clinical observations

Cholinergic-regulated tissues

Cholinergic activity regulates many organs in which cholinergic receptors have been identified such as: salivary cells, lacrimal cells, langerhans cells, the respiratory system, the gastrointestinal system and the vestibular organ within the ear (Table 1)[24,70-77]. Decrease cholinergic activity in salivary glands function results in a dry mouth (xerostomia)[78]. Similarly, suppression of cholinergic activity in lacrimal glands results in dry eyes[79]. Damage to muscarinic-3 receptors in langerhans cells result in diabetes type 1[80]. Surprisingly, several articles in the literature have reported strong association of an unknown etiology between these medical conditions (xerostomia, dry eyes and diabetes type 1) and bone loss[81-84]. However, the evidence we present in this study seems to indicate that bone loss in these medical conditions could be due to alterations in cholinergic activity.

Suppression of cholinergic activity is also known to cause the onset of serious pathologies such as obstructive pulmonary diseases, disruption in the body's circadian rhythm and learning memory deficits[85-88]. Interestingly, all these conditions have as a common skeletal feature, a reduction in bone mineral density[85,89,90]. Another interesting condition which is associated with cholinergic activity is vertigo. Vertigo patients are characterized by dizziness and being off-balanced as a consequence of a damage to the vestibular end organ in the ear[91], a cholinergic-regulated organ[76]. Patients suffering from vertigo are associated with higher risk of osteoporosis[92].

Circadian rhythm

An interesting phenomenon that links cholinergic activity with bone remodeling is the circadian rhythm. The autonomic nervous system follows a circadian pattern that matches the bone remodeling cycle. Sympathetic activity is dominant during day hours when bone resorption activity reaches its peak, while the parasympathetic activity is intense during night hours when bone formation is more active[93,94].

Menopause

Another observation that links cholinergic activity to bone is the estrogen replacement therapy, a hormonal treatment for osteoporosis. Patients under estrogen replacement therapy are well known to have high bone mass and low fractures risk[95]. Surprisingly, these patients express an increase in cholinergic activity[96].

Smokers

Clinical studies have shown that heavy smoking is associated with a decreased bone mass and diminished fracture healing capacity[31,97,98,99]. Even though many hypotheses have been postulated to explain this phenomena, it has been found that the association between smoking and bone is strongly related to nicotine levels in the body[100]. Even though low concentration of nicotine up-regulates osteoblasts through activation of the nicotinic receptors[32,39], excesive levels down-regulate osteoblasts through desentistizating their nicotinic receptors[38]. Also excesive nicotinic levels up-regulate osteoclasts through activation of the niotinic receptors[11].

Poliomyelitis, Botox injection and Myasthenia gravis

Cholinergic neurons innervate muscle fibers[101], and their activity can affect bone mass through mechanical stress[102]. Indeed, it has been argued whether the muscle activity, rather than the neuronal activity, is the main regulator of bone mass. However, the following observations argue strongly against a model whereby the muscle activity is more important than neuronal activity in regulating bone mass. Underneath, we discuss how three conditions, known to affect neuro-muscular synapses: Poliomyelitis, Botox injections and Myasthenia gravis, affect bone health status

Poliomyelitis is a viral disease that destroys motor neurons that utilize acetylcholine neurotransmitter resulting in muscle paralysis[103]. Patients diagnosed with poliomyelitis are known to suffer from impaired bone growth affected by nerve depletion[104]. Interestingly, it has been found that after recovery of muscle activity, polio patients tend to develop osteoporosis in a much larger proportion than the rest of the population, indicating that the effect of polio virus on bone tissue is unrelated to muscle function[104].

Botox, known as botulinum neurotoxin, prevents the release of acetylcholine from its membrane vesicles at the terminal ends of cholinergic neurons resulting in muscle paralysis[105]. Botulinum neurotoxin causes bone loss that does not improve following the recovery of muscle function[106,107]. Moreover, bone loss due to botulinum neurotoxin has been associated with an increase in bone resorption due to osteoclasts up-regulation[106], a phenomena that is recently linked to the inhibited activity of cholinergic fibers of the PSNS[11]. Accordingly, these findings indicate that the effect of botulinum neurotoxin on bone tissue is unrelated to muscle function.

Myasthenia gravis is a disorder that is characterized with suppressed muscular activity due to autoantibodies blocking the cholinergic receptors in muscle fibers. Surprisingly, despite the low muscular activity, patients diagnosed with Myasthenia graves are not associated with the risk of developing bone diseases[108-110]. In contrast, Myasthenia gravis patients are more resistant to develop osteoporosis compared to general population[109]. Myasthenia gravis only affects acetylcholine receptors in muscles leaving cholinergic signaling in bone intact, which might explain why bone is not affected in these patients.

The linking findings described for the above three conditions indicated that cholinergic innervation is necessary and might be more important than muscle activity for healthy bone remodeling. However, future studies will have to be performed in order to confirm the role of cholinergic activity in defining the bone phenotype of patients with Poliomyelitis, Myasthenia graves or following Botox injections.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Future therapies for osteoporosis

The available literature indicates that the inhibition of cholinergic activity at the bone level and in the central nervous system reduces bone mass[10,11,40]. This has lead researchers to investigate whether boosting acetylcholine activity might have an anabolic effect on bone formation.

One way of stimulating cholinergic receptors is by the administration of cholinergic agonists such as acetylcholinesterase inhibitors (AChEIs). AChEIs are a group of drugs that cause stimulation of cholinergic receptors (nicotinic and/or muscarinic) by inhibiting the action of AChE and increasing the levels of acetylcholine in the synaptic space. A recent study has revealed that the use of pyridostigmine, a peripherally acting AChEI that stimulates nicotinic receptors, favors bone mass in animal models by stimulating osteoclast apoptosis[11]. Another recent clinical study reported that treatment with centrally acting AChEI that stimulates both nicotinic and muscarinic receptors such as donepezil and rivastigmine was associated with lower risk of hip fracture in Alzheimer's disease patients[111]. In the same study, it was shown that centrally acting AChEI that stimulates nicotinic receptors only such as galantamine had no beneficial effect in lowering the risk of hip fracture[111]. Accordingly, it seems that stimulating cholinergic receptors through pharmacological approaches could favor bone mass. These findings open the window for a new therapeutic approach to treat osteoporosis through stimulation of the cholinergic system.

Future studies

Even though there is substantial evidence in the literature indicating the importance of cholinergic activity on bone, there is however still much to learn about the complicated relationships between the cholinergic system and bone. For instance, future studies will have to be performed in order to investigate whether the cholinergic activity affects bone mass by other mechanism rather than the IL 1-PSNS-bone and Locus coeruleus-SNS-bone pathways. The interaction between the cholinergic system with endocrine system, and the effects of this interaction on bone mass need to be investigated.

Another area that needs investigation is the possible involvement of nicotinic receptors expressed by osteocytes. It is well known that osteocytes can suppress osteoblast proliferation through the secretion of sclerostin, an inhibitor of the Wnt signaling pathway in osteoblasts, and promote osteoclast proliferation directly through RANKL secretion[112,113]. Accordingly, nicotinic receptors in osteocytes could be mediating the interactions between these cells with osteoblasts and osteoclasts, although future studies will have to be performed to address this hypothesis.

Conclusion

In this review we have summarized several observations demonstrating that the cholinergic signaling is a positive regulator of bone mass. *In vitro* studies have suggested that acetylcholine might regulate proliferation and differentiation of bone cells. *In vivo* studies have shown that altering cholinergic activity regulates bone mass accrual. Clinical studies have shown that diseases caused by disruption of cholinergic activity seem to be associated with bone disorders. Even though we here presented substantial evidence from the literature indicating the importance of cholinergic activity on bone, however there is still much to learn about the complicated relationships between the cholinergic system and bone.

Acknowledgement

This work was supported by operating grants from the Institute of Musculoskeletal Health and Arthritis (IMHA) bridge funding, Canadian Institutes of Health Research (CIHR), the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Fondation de l'Ordre des Dentistes du Québec (FODQ) Funds to FT. HE receives studentships from the FRQS Réseau de Recherche en Santé Buccodentaire et Osseuse (RSBO).

References

1. Schett G, David JP. The multiple faces of autoimmune-mediated bone loss. Nat Rev Endocrinol 2010;6:698-706.
2. Teitelbaum SL, Ross FP. Genetic regulation of osteoclast development and function. Nat Rev Genet 2003;4:638-49.
3. Sommerfeldt DW, Rubin CT. Biology of bone and how it orchestrates the form and function of the skeleton. Eur Spine J 2001;10:S86-S95.
4. Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, Shen JH, Vinson C, Rueger JM, Karsenty G. Leptin inhibits bone formation through a hypothalamic relay: A central control of bone mass. Cell 2000;100:197-207.
5. Takeda S, Elefteriou F, Levasseur R, Liu X, Zhao L, Parker KL, Armstrong D, Ducy P, Karsenty G. Leptin regulates bone formation via the sympathetic nervous system. Cell 2002;111:305-17.
6. Elefteriou F, Ahn JD, Takeda S, Starbuck M, Yang X, Liu X, Kondo H, Richards WG, Bannon TW, Noda M, Clement K, Vaisse C, Karsenty G. Leptin regulation of bone resorption by the sympathetic nervous system and cart. Nature 2005;434:514-20.
7. Katayama Y, Battista M, Kao WM, Hidalgo A, Peired AJ, Thomas SA, Frenette PS. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell 2006;124:407-21.
8. Bassett JH, Williams GR. Critical role of the hypothalamic-pituitary-thyroid axis in bone. Bone 2008;43:418-26.
9. Rosen CJ. Bone remodeling, energy metabolism, and the molecular clock. Cell Metab 2008;7:7-10.
10. Shi Y, Oury F, Yadav VK, Wess J, Liu XS, Guo XE, Murshed M, Karsenty G. Signaling through the m3 muscarinic receptor favors bone mass accrual by decreasing sympathetic activity. Cell Metab 2010;11:231-38.
11. Bajayo A, Bar A, Denes A, Bachar M, Kram V, Attar-Namdar M, Zallone A, Kovacs KJ, Yirmiya R, Bab I.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Skeletal parasympathetic innervation communicates central il-1 signals regulating bone mass accrual. Proc Natl Acad Sci U S A 2012;109:15455-60.
12. Kondo H, Takeuchi S, Togari A. Beta-adrenergic signaling stimulates osteoclastogenesis via reactive oxygen species. Am J Physiol Endocrinol Metab 2013;304:E507-15.
13. Loewi O. Humoral transferability of the heart nerve effect. I. Announcement. Pflug Arch Ges Phys 1921; 189:239-42.
14. Nachmansohn D, John HM. On the formation of acetylcholine in the nerve axon. Science 1945;102:250-51.
15. Dani JA, Bertrand D. Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. Annu Rev Pharmacol 2007;47:699-729.
16. Caulfield MP, Birdsall NJM. International union of pharmacology. Xvii. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 1998;50:279-90.
17. Johnson RH, Spaulding JM. Disorders of the autonomic nervous system. Chapter 3. The nervous control of the circulation and its investigation. Contemp Neurol Ser 1974:33-58.
18. Caulfield MP. Muscarinic receptors - characterization, coupling and function. Pharmacol Ther 1993;58:319-79
19. Taylor P, Radic Z. The cholinesterases - from genes to proteins. Annu Rev Pharmacol 1994;34:281-320.
20. Clarke PB, Schwartz RD, Paul SM, Pert CB, Pert A. Nicotinic binding in rat brain: Autoradiographic comparison of [3h]acetylcholine, [3h]nicotine, and [125i]-alpha-bungarotoxin. J Neurosci 1985;5:1307-15.
21. Mandelzys A, De Koninck P, Cooper E. Agonist and toxin sensitivities of ach-evoked currents on neurons expressing multiple nicotinic ach receptor subunits. J Neurophysiol 1995;74:1212-21.
22. Bernard V, Normand E, Bloch B. Phenotypical characterization of the rat striatal neurons expressing muscarinic receptor genes. J Neurosci 1992;12:3591-600.
23. Wessler I, Kirkpatrick CJ. Acetylcholine beyond neurons: The non-neuronal cholinergic system in humans. Br J Pharmacol 2008;154:1558-71.
24. Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, Spindel ER. Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. Endocrinology 2004;145:2498-506.
25. Liu PS, Chen YY, Feng CK, Lin YH, Yu TC. Muscarinic acetylcholine receptors present in human osteoblast and bone tissue. Eur J Pharmacol 2011;650:34-40.
26. En-Nosse M, Hartmann S, Trinkaus K, Alt V, Stigler B, Heiss C, Kilian O, Schnettler R, Lips KS. Expression of non-neuronal cholinergic system in osteoblast-like cells and its involvement in osteogenesis. Cell Tissue Res 2009;338:203-15.
27. Vogel-Hopker A, Sperling LE, Layer PG. Co-opting functions of cholinesterases in neural, limb and stem cell development. Protein Peptide Lett 2012;19:155-64.
28. Grisaru D, Lev-Lehman E, Shapira M, Chaikin E, Lessing JB, Eldor A, Eckstein F, Soreq H. Human osteogenesis involves differentiation-dependent increases in the morphogenically active 3' alternative splicing variant of acetylcholinesterase. Mol Cell Biol 1999;19:788-95.
29. Inkson CA, Brabbs AC, Grewal TS, Skerry TM, Genever PG. Characterization of acetylcholinesterase expression and secretion during osteoblast differentiation. Bone 2004;35:819-27.
30. Sato T, Abe T, Chida D, Nakamoto N, Hori N, Kokabu S, Sakata Y, Tomaru Y, Iwata T, Usui M, Aiko K, Yoda T. Functional role of acetylcholine and the expression of cholinergic receptors and components in osteoblasts. FEBS Lett 2010;584:817-24.
31. Luo L, Bennett T, Jung HH, Ryan AF. Developmental expression of alpha 9 acetylcholine receptor mrna in the rat cochlea and vestibular inner ear. J Comp Neurol 1998;393:320-31.
32. Walker LM, Preston MR, Magnay JL, Thomas PB, El Haj AJ. Nicotinic regulation of c-fos and osteopontin expression in human-derived osteoblast-like cells and human trabecular bone organ culture. Bone 2001;28:603-8.
33. Romano SJ, Pugh PC, McIntosh JM, Berg DK. Neuronal-type acetylcholine receptors and regulation of alpha 7 gene expression in vertebrate skeletal muscle. J Neurobiol 1997;32:69-80.
34. Ono Y, Nagata N, Kimura N. Effects of adrenergic and cholinergic stimulations on cyclic nucleotides metabolism in bone of young rat. Endocrinologia Japonica 1978; 25:477-84.
35. Paic F, Igwe JC, Nori R, Kronenberg MS, Franceschetti T, Harrington P, Kuo L, Shin DG, Rowe DW, Harris SE, Kalajzic I. Identification of differentially expressed genes between osteoblasts and osteocytes. Bone 2009;45:682-92.
36. Genever PG, Birch MA, Brown E, Skerry TM. Osteoblast-derived acetylcholinesterase: A novel mediator of cell-matrix interactions in bone? Bone 1999;24:297-303.
37. Tang J-M, Yuan J, Li Q, Wang J-N, Kong X, Zheng F, Zhang L, Chen L, Guo L-Y, Huang Y-H, Yang J-Y, Chen S-Y. Acetylcholine induces mesenchymal stem cell migration via ca2+/pkc/erk1/2 signal pathway. J Cell Biochem 2012;113:2704-13.
38. Alkondon M, Pereira EFR, Almeida LEF, Randall WR, Albuquerque EX. Nicotine at concentrations found in cigarette smokers activates and desensitizes nicotinic acetylcholine receptors in ca1 interneurons of rat hippocampus. Neuropharmacology 2000;39:2726-39.
39. Rothem DE, Rothem L, Soudry M, Dahan A, Eliakim R. Nicotine modulates bone metabolism-associated gene expression in osteoblast cells. J Bone Miner Metab 2009;27:555-61.
40. Kliemann K, Kneffel M, Bergen I, Kampschulte M, Langheinrich AC, Duerselen L, Ignatius A, Kilian O, Schnettler R, Lips KS. Quantitative analyses of bone composition in acetylcholine receptor m3r and alpha7 knockout mice. Life Sci 2012;91:997-1002.
41. Fujii T, Masai M, Misawa H, Okuda T, Takada-Takatori

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Y, Moriwaki Y, Haga T, Kawashima K. Acetylcholine synthesis and release in nih3t3 cells coexpressing the high-affinity choline transporter and choline acetyltransferase. J Neurosci Res 2009;87:3024-32.
42. Galea E, Estrada C. Periendothelial acetylcholine synthesis and release in bovine cerebral cortex capillaries. J Cereb Blood Flow Metab 1991;11:868-74.
43. Sisask G, Bjurholm A, Ahmed M, Kreicbergs A. The development of autonomic innervation in bone and joints of the rat. J Auton Nerv Syst 1996;59:27-33.
44. Tien D, Ohara PT, Larson AA, Jasmin L. Vagal afferents are necessary for the establishment but not the maintenance of kainic acid-induced hyperalgesia in mice. Pain 2003;102:39-49.
45. Asmus SE, Parsons S, Landis SC. Developmental changes in the transmitter properties of sympathetic neurons that innervate the periosteum. J Neurosci 2000; 20:1495-504.
46. Song D, Jiang X, Zhu S, Li W, Khadka A, Hu J. Denervation impairs bone regeneration during distraction osteogenesis in rabbit tibia lengthening. Acta Orthop 2012; 83:406-10.
47. Frymoyer JW, Pope MH. Fracture healing in the sciatically denervated rat. J Trauma 1977;17:355-61.
48. Aro H, Eerola E, Aho AJ, Penttinen R. Healing of experimental fractures in the denervated limbs of the rat. Clin Orthop Relat Res 1981:211-7.
49. Madsen JE, Hukkanen M, Aune AK, Basran I, Moller JF, Polak JM, Nordsletten L. Fracture healing and callus innervation after peripheral nerve resection in rats. Clin Orthop Relat Res 1998:230-40.
50. Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. Nature 2003;423:349-55.
51. Zaidi M. Skeletal remodeling in health and disease. Nat Med 2007;13:791-801.
52. Callen DJA, Black SE, Gao F, Caldwell CB, Szalai JP. Beyond the hippocampus - mri volumetry confirms widespread limbic atrophy in ad. Neurology 2001;57:1669-74.
53. Raskind MA, Peskind ER, Holmes C, Goldstein DS. Patterns of cerebrospinal fluid catechols support increased central noradrenergic responsiveness in aging and alzheimer's disease. Biol Psychiat 1999;46:756-65.
54. Buchner DM, Larson EB. Falls and fractures in patients with alzheimer-type dementia. Jama 1987;257:1492-5.
55. Weller I, Schatzker J. Hip fractures and alzheimer's disease in elderly institutionalized canadians. Ann Epidemiol 2004;14:319-24.
56. Weller IMR, Schatzker J. The relation between hip fracture and alzheimer's disease in the canadian national population health survey health institutions data, 1994-1995 - a cross-sectional analysis. J Bone Miner Res 2000; 15:S414-S14.
57. Sato Y, Honda Y, Hayashida N, Iwamoto J, Kanoko T, Satoh K. Vitamin k deficiency and osteopenia in elderly women with alzheimer's disease. Arch Phys Med Rehab 2005;86:576-81.
58. Loskutova N, Honea RA, Brooks WM, Burns JM. Reduced limbic and hypothalamic volumes correlate with bone density in early alzheimer's disease. J Alzheimers Dis 2010;20:313-22.
59. Loskutova N, Honea RA, Vidoni ED, Brooks WM, Burns JM. Bone density and brain atrophy in early alzheimer's disease. J Alzheimers Dis 2009;18:777-85.
60. Tan ZS, Seshadri S, Beiser A, Zhang Y, Felson D, Hannan MT, Au R, Wolf PA, Kiel DP. Bone mineral density and the risk of alzheimer disease. Arch Neurol 2005;62:107-11.
61. Rauch F, Schoenau E. The developing bone: Slave or master of its cells and molecules? Pediatr Res 2001; 50:309-14.
62. Turner CH, Forwood MR, Rho JY, Yoshikawa T. Mechanical loading thresholds for lamellar and woven bone formation. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 1994;9:87-97.
63. Stellar E. The physiology of motivation. 1954. Psychol Rev 1994;101:301-11.
64. Buckley NJ, Bonner TI, Brann MR. Localization of a family of muscarinic receptor mrnas in rat brain. J Neurosci 1988;8:4646-52.
65. Geula C, Mesulam MM. Cholinesterases and the pathology of alzheimer disease. Alzheimer Dis Assoc Disord 1995;9(Suppl.2):23-8.
66. Arnaiz GRD, Schneider PG. Calcitonin modifies ligand binding to muscarinic receptor in cns membranes. Regul Peptides 2000;88:21-26.
67. Krueger JM, Fang JD, Ping TS, Chen ZT, Kushikata T, Gardi J. Sleep - a physiologic role for il-1 beta and tnf-alpha. Ann N Y Acad Sci 1998;856:148-59.
68. Avital A, Goshen I, Kamsler A, Segal M, Iverfeldt K, Richter-Levin G, Yirmiya R. Impaired interleukin-1 signaling is associated with deficits in hippocampal memory processes and neural plasticity. Hippocampus 2003; 13:826-34.
69. Bajayo A, Goshen I, Feldman S, Csernus V, Iverfeldt K, Shohami E, Yirmiya R, Bab I. Central il-1 receptor signaling regulates bone growth and mass. Proc Natl Acad Sci U S A 2005;102:12956-61.
70. Caulfield MP. Muscarinic receptors-characterization, coupling and function. Pharmacol Ther 1993;58:319-79
71. Hegde SS, Choppin A, Bonhaus D, Briaud S, Loeb M, Moy TM, Loury D, Eglen RM. Functional role of m2 and m3 muscarinic receptors in the urinary bladder of rats *in vitro* and *in vivo*. Br J Pharmacol 1997;120:1409-18.
72. Bacman S, Perez Leiros C, Sterin-Borda L, Hubscher O, Arana R, Borda E. Autoantibodies against lacrimal gland m3 muscarinic acetylcholine receptors in patients with primary sjogren's syndrome. Invest Ophthalmol Vis Sci 1998;39:151-6.
73. Gautam D, Han SJ, Duttaroy A, Mears D, Hamdan FF, Li JH, Cui Y, Jeon J, Wess J. Role of the m3 muscarinic acetylcholine receptor in beta-cell function and glucose homeostasis. Diabetes Obes Metab 2007;9 Suppl 2:158-69.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

74. Furness JB. Types of neurons in the enteric nervous system. J Auton Nerv Syst 2000;81:87-96.
75. Hong SJ, Chang CC. Nicotinic actions of oxotremorine on murine skeletal-muscle - evidence against muscarinic modulation of acetylcholine-release. Brain Res 1990; 534:142-48.
76. Matsuoka I, Domino EF. Cholinergic mechanisms in the cat vestibular system. Neuropharmacology 1975;14:201-10.
77. Wang Z, Shi H, Wang H. Functional m3 muscarinic acetylcholine receptors in mammalian hearts. Br J Pharmacol 2004;142:395-408.
78. Gautam D, Heard TS, Cui Y, Miller G, Bloodworth L, Wess J. Cholinergic stimulation of salivary secretion studied with m1 and m3 muscarinic receptor single- and double-knockout mice. Mol Pharmacol 2004;66:260-7.
79. Park K, Haberberger RV, Gordon TP, Jackson MW. Antibodies interfering with the type 3 muscarinic receptor pathway inhibit gastrointestinal motility and cholinergic neurotransmission in sjogren's syndrome. Arthritis Rheum-Us 2011;63:1426-34.
80. Arends J, Wagner ML, Willms BL. Cholinergic muscarinic receptor blockade suppresses arginine-induced and exercise-induced growth-hormone secretion in type-i diabetic subjects. J Clin Endocr Metab 1988;66:389-94.
81. Agha-Hosseini F, Mirzaii-Dizgah I, Moosavi MS. Relationship of lumbar spine bone mineral density and oral dryness feeling in menopause. Menopause 2011;18:625-8.
82. Moss SE, Klein R, Klein BEK. Prevalence of and risk factors for dry eye syndrome. Arch Ophthalmol-Chic 2000;118:1264-68.
83. Liu EY, Wactawski-Wende J, Donahue RP, Dmochowski J, Hovey KM, Quattrin T. Does low bone mineral density start in post-teenage years in women with type 1 diabetes? Diabetes Care 2003;26:2365-69.
84. Tuominen JT, Impivaara O, Puukka P, Ronnemaa T. Bone mineral density in patients with type 1 and type 2 diabetes. Diabetes Care 1999;22:1196-200.
85. Bhattacharyya P, Paul R, Ghosh M, Dey R, Barooah N, Islam S, Acharya D, Nag S, Bardhan S. Prevalence of osteoporosis and osteopenia in advanced chronic obstructive pulmonary disease patients. Lung India 2011; 28:184-6.
86. Gispen WH, Biessels GJ. Cognition and synaptic plasticity in diabetes mellitus. Trends Neurosci 2000;23:542-9
87. Mirakhur RK, Clarke RS, Elliott J, Dundee JW. Atropine and glycopyrronium premedication. A comparison of the effects on cardiac rate and rhythm during induction of anaesthesia. Anaesthesia 1978;33:906-12.
88. Kesler BS, Canning BJ. Regulation of baseline cholinergic tone in guinea-pig airway smooth muscle. J Physiol-London 1999;518:843-55.
89. Katz S, Weinerman S. Osteoporosis and gastrointestinal disease. Gastroenterol Hepatol (N Y) 2010;6:506-17.
90. Yaturu S, Humphrey S, Landry C, Jain SK. Decreased bone mineral density in men with metabolic syndrome alone and with type 2 diabetes. Med Sci Monitor 2009; 15:Cr5-Cr9.
91. Baloh RW, Honrubia V, Jacobson K. Benign positional vertigo: Clinical and oculographic features in 240 cases. Neurology 1987;37:371-8.
92. Jeong SH, Choi SH, Kim JY, Koo JW, Kim HJ, Kim JS. Osteopenia and osteoporosis in idiopathic benign positional vertigo. Neurology 2009;72:1069-76.
93. Nielsen HK, Laurberg P, Brixen K, Mosekilde L. Relations between diurnal-variations in serum osteocalcin, cortisol, parathyroid-hormone, and ionized calcium in normal individuals. Acta Endocrinol-Cop 1991;124:391-98.
94. Pressman MR, Fry JM. Relationship of autonomic nervous system activity to daytime sleepiness and prior sleep. Sleep 1989;12:239-45.
95. Effects of hormone therapy on bone mineral density: Results from the postmenopausal estrogen/progestin interventions (pepi) trial. The writing group for the pepi. Jama 1996;276:1389-96.
96. Nikolov R, Kuhl H, Golbs S. Estrogen replacement therapy and alzheimer's disease. Drugs Today (Barc) 1998; 34:927-33.
97. Hollinger JO, Schmitt JM, Hwang K, Buck D. Impact of nicotine on bone healing. J Biomed Mater Res 1999; 45:294-301.
98. Wong PK, Christie JJ, Wark JD. The effects of smoking on bone health. Clin Sci (Lond) 2007;113:233-41.
99. Chen Y, Guo Q, Pan X, Qin L, Zhang P. Smoking and impaired bone healing: Will activation of cholinergic anti-inflammatory pathway be the bridge? Int Orthop 2011; 35:1267-70.
100. Zheng LW, Ma L, Cheung LK. Changes in blood perfusion and bone healing induced by nicotine during distraction osteogenesis. Bone 2008;43:355-61.
101. Koenen M, Peter C, Villarroel A, Witzemann V, Sakmann B. Acetylcholine receptor channel subtype directs the innervation pattern of skeletal muscle. EMBO Rep 2005; 6:570-6.
102. Martin RB. The importance of mechanical loading in bone biology and medicine. J Musculoskelet Neuronal Interact 2007;7:48-53.
103. Maselli RA, Wollmann R, Roos R. Function and ultrastructure of the neuromuscular junction in post-polio syndrome. Ann N Y Acad Sci 1995;753:129-37.
104. Mohammad AF, Khan KA, Galvin L, Hardiman O, O'-Connell PG. High incidence of osteoporosis and fractures in an aging post-polio population. Eur Neurol 2009; 62:369-74.
105. Hambleton P. Clostridium-botulinum toxins - a general-review of involvement in disease, structure, mode of action and preparation for clinical use. J Neurol 1992; 239:16-20.
106. Warner SE, Sanford DA, Becker BA, Bain SD, Srinivasan S, Gross TS. Botox induced muscle paralysis rapidly degrades bone. Bone 2006;38:257-64.
107. Grimston SK, Silva MJ, Civitelli R. Bone loss after temporarily induced muscle paralysis by botox is not fully

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

recovered after 12 weeks. Ann Ny Acad Sci 2007; 1116:444-60.
108. Pouwels S, de Boer A, Javaid MK, Hilton-Jones D, Verschuuren J, Cooper C, Leufkens HG, de Vries F. Fracture rate in patients with myasthenia gravis: The general practice research database. Osteoporos Int 2013;24(2):467-76.
109. Wakata N, Nemoto H, Sugimoto H, Nomoto N, Konno S, Hayashi N, Araki Y, Nakazato A. Bone density in myasthenia gravis patients receiving long-term prednisolone therapy. Clin Neurol Neurosurg 2004;106:139-41.
110. D'Amelio P, Grimaldi A, Bernabei P, Pescarmona GP, Isaia G. Immune system and bone metabolism: Does thymectomy influence postmenopausal bone loss in humans? Bone 2006;39:658-65.
111. Tamimi I, Ojea T, Sanchez-Siles JM, Rojas F, Martin I, Gormaz I, Perez A, Dawid-Milner MS, Mendez L, Tamimi F. Acetylcholinesterase inhibitors and the risk of hip fracture in alzheimer's disease patients: A case-control study. J Bone Miner Res 2012;27:1518-27.
112. Pavalko FM, Norvell SM, Burr DB, Turner CH, Duncan RL, Bidwell JP. A model for mechanotransduction in bone cells: The load-bearing mechanosomes. J Cell Biochem 2003;88:104-12.
113. Moriishi T, Fukuyama R, Ito M, Miyazaki T, Maeno T, Kawai Y, Komori H, Komori T. Osteocyte network; a negative regulatory system for bone mass augmented by the induction of rankl in osteoblasts and sost in osteocytes at unloading. PloS one 2012;7.
114. Nakamura M, Tada Y, Akaishi T, Nakata K. M3 muscarinic receptor mediates regulation of protein secretion in rabbit lacrimal gland. Curr Eye Res 1997;16:614-9.
115. Layden BT, Durai V, Lowe Jr WL. G-protein-coupled receptors, pancreatic islets, and diabetes. Nature Education 2010;9:13.
116. Pietschmann P, Schernthaner G, Luger A. Effect of cholinergic muscarinic receptor blockade on human growth-hormone (gh)-releasing hormone-(1-44)-induced gh secretion in acromegaly and type-i diabetes-mellitus. J Clin Endocr Metab 1986;63:389-93.
117. Munoz-Torres M, Jodar E, Escobar-Jimenez F, Lopez-Ibarra PJ, Luna JD. Bone mineral density measured by dual x-ray absorptiometry in spanish patients with insulin-dependent diabetes mellitus. Calcified Tissue Int 1996;58:316-9.
118. Valerio G, del Puente A, Esposito-del Puente A, Buono P, Mozzillo E, Franzese A. The lumbar bone mineral density is affected by long-term poor metabolic control in adolescents with type 1 diabetes mellitus. Horm Res 2002;58:266-72.
119. Proskocil BJ, Sekhon HS, Jia YB, Savchenko V, Blakely RD, Lindstrom J, Spindel ER. Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. Endocrinology 2004; 145:2498-506.
120. Song P, Sekhon HS, Lu A, Arredondo J, Sauer D, Gravett C, Mark GP, Grando SA, Spindel ER. M3 muscarinic receptor antagonists inhibit small cell lung carcinoma growth and mitogen-activated protein kinase phosphorylation induced by acetylcholine secretion. Cancer Res 2007;67:3936-44.
121. OBrien MD, Camilleri M, Thomforde GM, Wiste JA, Hanson RB, Zinsmeister AR. Effect of cholecystokinin octapeptide and atropine on human colonic motility, tone, and transit. Digest Dis Sci 1997;42:26-33.
122. Monzani D, Genovese E, Marrara A, Presutti L, Gherpelli C, Panzetti P, Forghieri M. Stimulation of the cholinergic neurotransmissions enhances the efficacy of vestibular rehabilitation. Acta Otorhinolaryngol Ital 2010;30:11-9.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Article II

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

ORIGINAL ARTICLE

JBMR

Acetylcholinesterase Inhibitors and the Risk of Hip Fracture in Alzheimer's Disease Patients: A Case-Control Study

Iskandar Tamimi,[1] Tomas Ojea,[1] Juan Manuel Sanchez-Siles,[1] Facundo Rojas,[1] Ignacio Martin,[1] Irene Gormaz,[1] Almudena Perez,[1] Marc Stefan Dawid-Milner,[2] Luis Mendez,[1] and Faleh Tamimi[3]

[1]Traumatology and Orthopedic Surgery Department, Hospital Regional Universitario Carlos Haya, Malaga, Spain
[2]Department of Neurosciences, Centro de Investigaciones Médico-Sanitarias (CIMES), Universidad de Malaga, Spain
[3]Faculty of Dentistry, McGill University, Montreal, Quebec, Canada

ABSTRACT

Recent studies have reported the presence of acetylcholine (ACh) receptor subtypes in bone tissue, and have demonstrated that inhibition of the ACh receptors has negative effects on bone mass and fracture healing capacity. However, little is known about the potential clinical effects that increased ACh signaling might have on bone. Accordingly, this study was designed to determine whether the use of acetylcholinesterase inhibitors (AChEIs), a group of drugs that stimulate ACh receptors and are used to treat Alzheimer's disease (AD), is associated with a decreased risk of hip fracture in AD patients. To accomplish this objective, a case-control analysis was performed using the AD population, aged above 75 years, based in the local health area of the Carlos Haya Hospital, in Malaga, Spain. The cases were 80 AD patients that suffered a hip fracture between January 2004 and December 2008. The controls were 2178 AD patients without hip fracture followed at our health care area during the same period of time. Compared with patients who did not use AChEIs, the hip fracture adjusted odds ratio (OR) for users of AChEIs was 0.42 (95% confidence interval [CI], 0.24–0.72), for users of rivastigmine was 0.22 (95% CI, 0.10–0.45), and for users of donepezil was 0.39 (95% CI, 0.19–0.76). Data were adjusted for the following parameters: body mass index, fall risk, smoking habits, cognition, dependence, degree of AD, comorbidity score, treatment with selective serotonin reuptake inhibitors, age, and gender. Our data suggests that use of AChEIs donepezil and rivastigmine is associated with a reduced risk of fractures in AD patients. Many elderly patients with AD disease who are at risk of developing osteoporosis may potentially benefit from therapy with the AChEIs donepezil and rivastigmine. © 2012 American Society for Bone and Mineral Research.

KEY WORDS: CHOLINERGIC; BONE; ACETYLCHOLINE; ACETYLCHOLINESTERASE; CASE-CONTROL STUDY; ADJUSTED ODDS RATIO; RISK OF HIP FRACTURE

Introduction

Clinical conditions and drugs that alter the acetylcholine (ACh) signaling system have been found to have interesting effects on bone function.[1–8] At the presynaptic level, botulinum neurotoxin inhibits the release of ACh from motor neurons, and has been found to impair bone healing and induce a decrease in bone mineral content.[4,5] However, bone density does not improve after the recovery of muscle function, indicating an effect of botulinum toxin on bone tissue, unrelated to muscle function.[5] Poliomyelitis destroys motor neurons that use ACh neurotransmitters. Patients suffering from poliomyelitis experience impaired bone growth in the limbs affected by nerve depletion. However, many years after recovery of muscle activity, poliomyelitis patients tend to develop osteoporosis in a much larger proportion than the rest of the population.[9]

At the postsynaptic level, smokers are well known to have impaired bone healing, in part due to the effect of nicotine.[6–8] Smokers have high concentrations of nicotine in blood that interacts with the nicotinic ACh receptor (nAChR), causing a desensitization of these receptors. All these observations suggest that ACh and its receptors in bone tissue may play certain roles in the process of bone remodeling.

Recent research has revealed the presence of a number of AChR subunits in bone cells.[1–3] nAChR subunits have been detected in human primary bone cells, mesenchymal stem cells (MSCs), and osteoblasts, whereas muscarinic receptors have been detected in MSCs.[2,3,6] AChR and cholinergic components

Received in original form January 24, 2012; revised form March 14, 2012; accepted March 19, 2012. Published online March 29, 2012.
Address correspondence to: Faleh Tamimi, BDS, PhD, Faculty of Dentistry, McGill University, 3640 University Street, Montreal, Quebec H3A 2B2, Canada. E-mail: faleh.tamimimarino@mcgill.ca
Journal of Bone and Mineral Research, Vol. 27, No. 7, July 2012, pp 1518–1527
DOI: 10.1002/jbmr.1616
© 2012 American Society for Bone and Mineral Research

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

expressed on osteoblasts may play a possible role in regulating alkaline phosphatase (ALP) activity as well as the proliferation and differentiation of osteoblasts.[10] Moreover, the enzyme responsible for metabolizing ACh at the AChR site, acetylcholinesterase (AChE) is highly expressed on bone cells, especially during the osteoblast differentiation stage.[11,12] Altogether, the available literature seems to indicate that the inhibition of the AChR seems to cause a reduction in bone turnover.[4–14] However, it is not clear whether boosting ACh activity would have an anabolic effect on bone formation.

Acetylcholinesterase inhibitors (AChEIs) are a group of drugs that cause AChR stimulation by inhibiting the action of AChE and increasing the levels of ACh in the synaptic space.[15] These drugs have been widely used in the treatment of Alzheimer's disease (AD) and other dementias since the mid-1990s[16]; however, their potential effect on bone biology has not previously been explored.

We hypothesize that the administration of AChEIs may have a beneficial effect on bone turnover that could translate into reduction of bone fracture risk. In order to test this hypothesis we have designed a retrospective case-control study in which we compare the incidence of hip fractures in a group of AD patients under AChEI treatment with AD patients receiving no AChEIs.

Patients and Methods

Approval from the ethical committee of "Carlos Haya Hospital" was obtained to carry out a retrospective case-control study on AD patients living within the local health area of the city of Malaga. Records of AD patients in our healthcare area were identified in the computerized database of the Department of Neurology, and the original hardcopy files were retrieved for manual examination. The overall study period was 5 years, between January 1, 2004 and December 31, 2008. All the hip fractures in AD patients that occurred within the study period were reviewed.

Definition and validation of cases

We identified all AD patients with an incident diagnosis of a hip fracture recorded in the computerized medical records between January 2004 and December 2008. Cases were identified without any exposure information. Potential cases had to be aged between 75 and 95 years at the date of the fracture (case index date), and they had to have been recorded in the database for at least 2 years prior to the index date. Case patients with a medical disorder known to substantially affect bone metabolism (ie, a history of osteoporosis diagnosed by dual-energy X-ray absorptiometry [DXA], osteomalacia, Paget's disease, vitamin D deficiency, hyperthyroidism, cancer [excluding non-melanoma skin cancer], or alcoholism) as well as patients who were on corticosteroids, antiepileptic drugs, or bisphosphonates (as treatment for osteoporosis or bone metastases) prior to the index date were excluded from the analysis. Data regarding the socioeconomic status of the patients was not registered because the Spanish public healthcare system provides universal free healthcare, and all of our patients have equal access to the drugs assessed in this study.

Controls

Controls included all AD patients in our local health area registered in the Neurology Department database between January 2004 and December 2008 who did not present any incidence of hip fracture prior to December 31, 2008 (control index date). Controls had to be alive on the index date and had to be recorded in the database for at least 2 years. The same exclusion criteria applied to case patients were also applied to controls.

Measurements

The patient-specific characteristics at the index date were registered. The following parameters were retrieved from the patients' files, computerized records, and standardized questionnaires: patient age, gender, mobility, institutionalization, Charlson comorbidity score (CCS),[17] treatment with selective serotonin reuptake inhibitors (SSRIs), history of hip fractures (including subcapital, transcervical, basicervical, pertrochanteric, and subtrochanteric femoral fractures), history of other osteoporosis-related fractures (including, vertebral, distal radius, proximal humeral, and previous hip fractures), body mass index (BMI), fall risk status according to the Falls Risk Assessment Tool (FRAT),[18] smoking, and familial osteoporosis. The grade of AD according to the clinical dementia rating scale (CDR)[19,20] and the cognitive state according to the Mini-Mental State Exam (MMSE) and Blessed Dementia Scale (BDS) were also retrieved.[21] Specific treatment for AD prior to index date (AChEIs [ie, donepezil hydrochloride, galantamine, rivastigmine, or no AChEI treatment] and/or memantine) including the dose and duration (0.5–2 years, >2 years) were registered.

Statistical analysis

We conducted logistic regression analyses using the software IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA). Risk estimates were presented as odds ratio (OR) with 95% confidence intervals (CIs). Odds ratio were adjusted for the potential confounders: age, gender, mobility, institutionalization, BDS (<9, 9–16.9, ≥17), CDR (score 1, 2, or 3), CCS (1, 2, >3), treatment with SSRIs, MMSE (score ≤20 or >20), FRAT (score 1 or >1), other fractures in the body, AChEI treatment received, BMI (<25, 25–29.9, ≥30), family history of osteoporosis, and smoking habits (smoker, nonsmoker). Missing values for BMI and FRAT were treated as a separate group in the analysis. Values of p were two-sided and were considered statistically significant if less than 0.05. Differences between users and nonusers of AChEIs were assessed with Student's t test (for continuous variables; ie, age, MMSE, CDR, BDS, BMI, CCS, and FRAT) and with chi square test (for nominal variables; ie, gender, other fractures, and smoking). Values of p were two-sided and were considered statistically significant if less than 0.05.

Results

A total of 2794 AD patients without hip fractures were identified in our health care area during the year 2008; among these patients 2178 fulfilled our control group inclusion criteria (Fig. 1).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

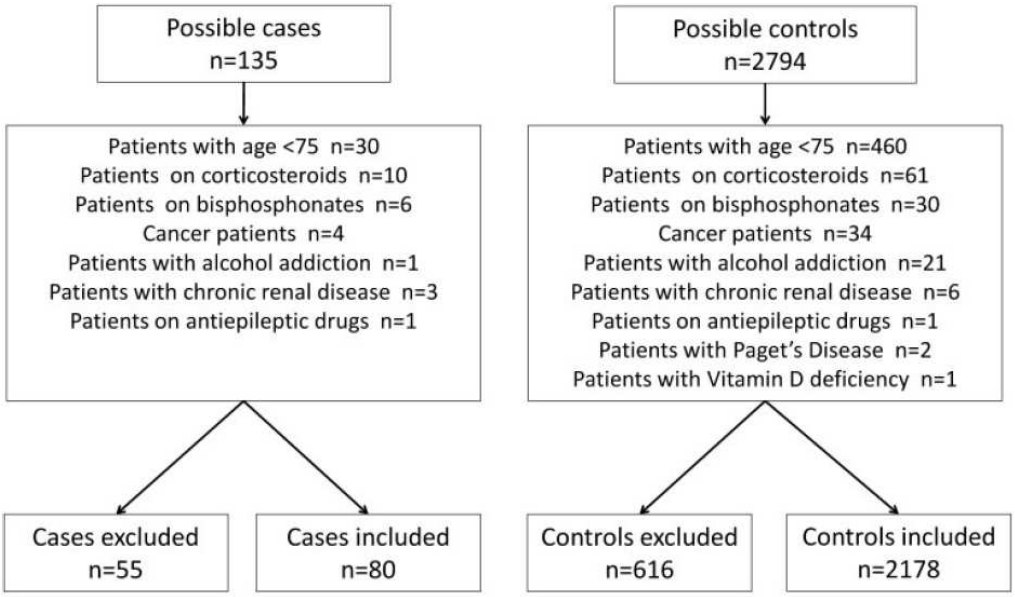


Fig. 1. Flowchart describing the selection process for case and control subjects.

We also identified 135 cases of hip fractures in AD patients between 2004 and 2008, among whom 80 patients fulfilled our case group inclusion criteria (Fig. 1). The distribution of hip fractures in the study group as a function of age, gender, BMI, MMSE, FRAT, CDR, BDS, CCS, institutionalization, smoking habits, treatment with SSRIs, and the presence of other fractures is shown in Table 1.

Smokers, patients with a FRAT score >1, and patients with BMI <25 showed a higher risk of hip fracture compared to nonsmokers, patients with FRAT score $=1$, and patients with BMI >25, respectively. Female patients had a slightly higher risk of hip fracture than male patients, although not significant, and patients between the age of 80 to 85 years had a lower risk of hip fracture than patients between the age of 75–79 and >85 years. On the other hand, the risk of hip fracture was not associated with CDR, BDS, institutionalization, MMSE, and SSRI treatment (Table 1). Patients with a CCS ≥ 3 had a lower risk of hip fracture than those with a CCS $=1$.

Patients using AChEIs had better mental indices (CDR, BDS, and MMSE) than nonusers (Table 2). BMI was also higher in AChEIs users than in nonusers. Smoking habits, CCS, institutionalization, treatment with SSRIs, and incidence of other fractures were not significantly different between users and nonusers of AChEIs. Interestingly enough, users of AChEIs were associated with a lower risk of hip fracture than nonusers (adjusted OR, 0.44; 95%CI, 0.24–0.72), even though they had a higher risk of falling (FRAT) than nonusers (Table 3). Each specific AChEI had a different degree of association with the risk of hip fracture. Patients treated with galantamine had a risk of hip fracture similar to nonusers of AChEIs. On the other hand, AD patients undergoing AChEI treatment with donepezil and rivastigmine were associated with a highly significant lower risk of hip fractures than nonusers, and this effect was dose-dependent. However, we could not observe a significant relationship between the duration of the treatment and the risk of hip fractures. Administration of rivastigmine and donepezil had a total protective adjusted OR of 0.22 (95% CI, 0.10–0.45) and 0.39 (95% CI, 0.19–0.76), respectively, compared to AD patients who were not users of AChEIs (Table 3). At α error $=0.05$, we had 83% power for detecting risk of hip fracture in AD patients treated with AChEIs equal to 0.42 or lower.

The dose and duration of AChEI treatments in AD patients was tested for association with risk of hip fracture (Table 4). It was observed that patients receiving higher doses of rivastigmine were associated with a significantly lower risk of hip fractures than those treated with lower doses. The relationship between galantamine and donepezil dosage and the risk of hip fracture could not be assessed for statistical significance due to the small number of subjects collected, and the data missing from the patients' files. The risk of hip fractures in patients that had been treated with either rivastigmine or donepezil for longer periods of time (>2 years) was not significantly different from those who had been treated for shorter periods of time (<2 years).

Discussion

Hip fractures in AD patients

AD patients have a higher risk of developing hip fractures than the general population.[22–30] A cohort study in the early 1990s revealed that AD patients had a relative risk ratio for hip fractures of 2.7 compared to the general population.[22] A similar study done in the same period revealed that the rate of hip fractures in

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Table 1. Characteristics of AD Patients as a Function of the Presence of Hip Fractures (Cases)

Characteristics	Cases ($n = 80$)[a]	Controls ($n = 2178$)[a]	Crude OR (95%CI)	Adjusted OR (95% CI)[b]	p
Age, years					
75–79	21 (26.3)	594 (27.3)	1.00	1.00	–
80–84	28 (35.0)	846 (38.8)	0.94 (0.53–1.66)	0.39 (0.18–0.85)	0.018
>85	31 (38.8)	738 (33.9)	1.19 (0.68–2.09)	1.73 (0.81–3.69)	0.159
Gender					
Women	66 (82.5)	1548 (71.1)	1.00	1.00	–
Men	14 (17.5)	630 (28.9)	0.52 (0.29–0.94)	0.41 (0.15–1.08)	0.072
BMI[c]					
<25	53 (66.3)	396 (18.2)	1.00	1.00	–
25–29.9	21 (26.3)	468 (21.5)	0.34 (0.20–0.57)	0.40 (0.21–0.74)	0.004
>30	6 (7.5)	216 (9.9)	0.21 (0.09–0.49)	0.16 (0.06–0.41)	<0.001
Missing	0 (0.0)	1097 (50.4)	0.27 (0.24–0.29)	0.47 (0.23–0.96)	0.039
CDR					
1	13 (16.3)	288 (13.2)	1.00	1.00	–
2	33 (41.3)	1062 (48.8)	0.69 (0.36–1.33)	0.59 (0.22–1.16)	0.304
3	34 (42.5)	828 (38.0)	0.91 (0.47–1.75)	0.40 (0.07–2.14)	0.282
BDS					
0–9	1 (1.3)	78 (3.6)	1.00	1.00	–
9–16	42 (52.5)	900 (41.3)	3.36 (0.46–24.77)	2.06 (0.23–18.58)	0.520
17–28	37 (46.3)	1206 (55.4)	2.21 (0.30–16.33)	1.93 (0.15–24.75)	0.615
MMSE					
21–30	4 (5.0)	234 (10.7)	1.00	1.00	–
0–20	76 (95.0)	1943 (89.2)	2.30 (0.83–6.34)	2.53 (0.85–7.51)	0.096
FRAT					
1	51 (63.8)	810 (37.2)	1.00	1.00	–
2–3	29 (36.3)	270 (12.4)	1.71 (1.06–2.75)	2.26 (1.34–3.85)	0.003
Missing	0 (0.0)	1098 (50.4)	0.43 (0.40–0.45)	[d]	[d]
Institutionalization					
No	79 (98.7)	2026 (93.0)	1.00	1.00	–
Yes	1 (1.3)	152 (7.5)	0.18 (0.02–1.28)	0.47 (0.06–3.53)	0.464
CCS					
0–1	30 (37.5)	753 (34.8)	1.00	1.00	–
2	23 (28.8)	672 (30.9)	0.86 (0.49–1.49)	0.96 (9.00–1.02)	0.145
>3	27 (33.8)	753 (34.6)	0.90 (0.53–1.53)	0.52 (0.28–0.96)	0.026
Smoking					
No	74 (92.5)	2163 (99.3)	1.00	1.00	–
Yes	6 (7.5)	15 (0.7)	11.67 (4.41–30.99)	33.90 (10.50–109.60)	<0.001
SSRI					
No	70 (87.5)	2003 (92.0)	1.00	1.00	–
Yes	10 (12.5)	175 (8.0)	1.64 (0.83–3.23)	1.45 (0.73–2.90	0.288
Fracture history					
No	59 (73.8)	2016 (92.6)	1.00	1.00	–
Yes	21 (26.3)	162 (7.4)	4.43 (2.63–7.47)	2.82 (1.60–4.99)	<0.001

AD = Alzheimer's disease; OR = odds ratio; CI = confidence interval; FRAT = Fall Risk Assessment Tool; BMI = body mass index; BDS = Blessed dementia scale; MMSE = Mini-Mental State Exam; CDR = clinical dementia rating scale; CCS = Charlson comorbidity score; SSRI = selective serotonin reuptake inhibitor.

[a]Values are n (%); percentages may not sum to 100% due to rounding.

[b]Adjusted to the categorical parameters presented in this table.

[c]Measured as weight in kilograms divided by the square of height in meters.

[d]Not calculated due to insufficient data.

women with AD and vascular dementia was more than twice that found in the general population.[26] In a more recent study performed between 1988 and 2007, AD patients had a hazard ratio for hip fractures of 2.8.

Even though AD onset is associated with the loss of balance and gait,[22–30] there is no correlation between the mental conditions of AD patients and their risk for hip fracture[24] (see Table 1), and the reasons behind the high risk of hip

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Table 2. Characteristics of AD Patients as a Function of the Presence of AChEIs Treatment

Characteristics	Users of AChEIs ($n = 1771$)	Nonusers of AChEIs ($n = 487$)	p
Age, years	82.2 ± 4.5	83.5 ± 4.2	0.001[a]
Gender			
Women	72 (70–74)	69 (65–73)	0.208[b]
Men	25 (22–28)	27 (22–33)	0.208[b]
BMI[c]	26.0 ± 3.5	24.6 ± 4.3	0.001[a]
CDR	2.16 ± 0.67	2.59 ± 0.58	0.001[a]
BDS	15.7 ± 3.5	17.3 ± 3.4	0.001[a]
MMSE	15.7 ± 4.0	14.5 ± 2.3	0.001[a]
FRAT	1.33 ± 0.55	1.24 ± 0.68	0.025[a]
Smokers	1 (0–1)	1 (1–2)	0.415[b]
SSRI	19 (17–21)	26 (20–32)	0.030[b]
CCS	2.52 ± 1.57	2.38 ± 1.35	0.776[a]
Fracture history	16.0 (13.3–18.6)	16.3 (11.9–20.6)	0.300[b]

Values are mean (95% confidence interval).

AD = Alzheimer's disease; AChEIs = acetylcholinesterase inhibitors; BMI = body mass index; CDR = clinical dementia rating scale; BDS = Blessed dementia scale; MMSE = Mini-Mental State Exam; FRAT = Fall Risk Assessment Tool; SSRI = selective serotonin reuptake inhibitor; CCS = Charlson comorbidity score.

[a]Calculated with Student's t-test for independent samples.

[b]Calculated with chi-square test.

[c]Measured as weight in kilograms divided by the square of height in meters.

fracture in AD remain largely unclear. Therefore, our case-control study brings new insight that would help understand and prevent hip fractures in AD patients by identifying some of the factors associated with this issue. Similar to previous reports on AD patients, our study revealed that AD patients with low BMI, high risk of falling (FRAT), and high comorbidity score (CCS) were associated with a higher risk of hip fracture.[24,27,28] We also confirmed that, as observed in the general population,[31] female and smoking AD patients are also associated with a higher risk of hip fracture than males and nonsmokers.

Another interesting result was the unexpected pattern of association between hip fracture risk and age. In the general population, the risk of hip fracture increases with age; however, in our study the risk of hip fracture did not increase beyond the age of 80 years. A similar result could be observed in a previous study in which it was shown that the incidence of hip fractures in female AD patients did not increase beyond the age of 80 years.[27] One possible explanation for this phenomenon could be worsening general and bone health conditions associated with early onset of AD that could be associated with increased risk of hip fracture. Indeed, previous studies have demonstrated that bone mineral density (BMD) is reduced in the earliest clinical stages of AD patients as a result of neurological deterioration of the hypothalamus and limbic system.[32,33] Moreover, in our study there were more patients with high CCS (>4) among the younger patients (75–79 years) than among the older ones (>80 years) (data not shown). This status also indicates that early onset of AD might be associated with a greater disease burden and a higher likelihood of fracture, although future studies will have to be done to confirm this possibility.

AChEIs

Most AChEIs able to cross the blood-brain barrier were introduced in the mid-1990s[15] and eventually became standard treatment for AD patients. Unfortunately, AChEIs can only result in marginal clinical improvement in measures of cognition and global assessment of dementia, and do not alter the natural course of the disease.[34] In our study we observed that users of AChEIs had overall slightly better indices of mental status (MMES, CDR, and BDS) than nonusers, which was probably caused by the beneficial effect of AChEIs on brain function.[35]

Table 3. Use of AChEIs and Fracture Risk (versus Nonuse)

Characteristics	Cases ($n = 80$)[a]	Controls ($n = 2178$)[a]	Crude OR (95% CI)	Adjusted OR (95% CI)[b]	p
Nonuse of AChEIs	37 (46.3)	450 (20.7)	1.00	1.00	–
Use of AChEIs	43 (53.8)	1728 (79.3)	0.30 (0.19–0.48)	0.42 (0.24–0.72)	0.002
Rivastigmine	11 (13.8)	900 (41.3)	0.20 (0.11–0.37)	0.22 (0.10–0.45)	<0.001
Donepezil	15 (18.8)	630 (28.9)	0.29 (0.16–0.54)	0.39 (0.19–0.76)	<0.002
Galantamine	9 (11.3)	182 (8.4)	0.68 (0.33–1.39)	1.67 (0.63–4.45)	0.305

AChEIs = acetylcholinesterase inhibitor; OR = odds ratio; CI = confidence interval.

[a]Values are n (%); percentages may not sum to 100% due to rounding.

[b]Adjusted to the categorical parameters in Table 1.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Table 4. Treatment Duration and Dosage of AChEIs and Fracture Risk (versus Nonuse)

Treatment characteristics	Cases ($n = 80$)[a]	Controls ($n = 2178$)[a]	Crude OR (95% CI)	Adjusted OR (95% CI)[b]	p
Rivastigmine					
Duration, years					
0.5–2	7	270	1.00	1.00	–
>2	6	630	0.37 (0.12–1.10)	0.25 (0.06–1.04)	0.057
Dose, mg/day					
3–9	7	270	1.00	1.00	–
9.5–12	6	630	0.37 (0.12–1.10)	0.16 (0.03–0.79)	0.025
Donepezil					
Duration, years					
0.5–2	6	234	1.00	1.00	–
>2	7	396	0.83 (0.26–2.64)	0.90 (0.19–4.20)	0.879
Dose, mg/day					
5	3	18	1.00	1.00	–
10	12	612	0.12 (0.03–0.45)	0.02 (0.00–0.17)	[c]
Galantamine					
Duration, years					
0.5–2	3	72	1.00	1.00	–
>2	3	108	0.67 (0.13–3.40)	[c]	[c]
Dose, mg/day					
6–16	4	74	1.00	1.00	–
17–24	5	108	0.83 (0.22–3.21)	0.51 (0.09–2.89)	[c]

AChEIs = acetylcholinesterase inhibitors; OR = odds ratio; CI = confidence interval.
[a]Values are number of cases or controls.
[b]Adjusted to the categorical parameters in Table 1.
[c]Not calculated due to insufficient data.

AChEIs could have two competing effects on fall risk: on the one hand, AChEIs users are known to have a higher risk of bradycardia and consequently of syncope-related falls[36]; while on the other hand, AChEIs can improve cognition and therefore prevent falls associated with mental issues.[25] In our study we observed that AChEIs users had higher risk of falling (FRAT) than nonusers; therefore, it could be speculated that risks of falling due to bradycardia syncope might outweigh the cognitive benefits of AChEIs.[36]

Another interesting finding observed in our study was that BMI was significantly higher in AChEIs users than in nonusers. The activity of the autonomic nervous system is a primary factor regulating BMI.[37] For instance, sympathetic activity is known to limit body weight gain,[38] and inhibition of sympathetic activity with beta-blockers is associated with an increase in BMI.[38] On the other hand, parasympathetic stimulation has been associated with increased fat deposition in animals.[39] Treatments with AChEIs have been found to increase the activity of the parasympathetic nervous system (PNS) in the body[36]; therefore, it is possible that the differences in BMI between users and nonusers of AChEIs is caused by the effect of the drugs on PNS activity.

AChEIs and hip fractures

Recent studies have shown that the sympathetic nervous system (SNS) regulates bone turnover by inhibiting bone formation.[40] Accordingly, beta-blockers, a group of drugs that inhibit sympathetic activity, have been shown to increase bone density and reduce the incidence of bone fractures in osteoporotic patients.[41] These observations raise the question of whether stimulation of the opposing arm of the autonomic nervous system, the PNS, with drugs such as AChEIs, would be able to increase bone accrual.

In our study, AChEIs users had a significantly lower risk of developing hip fractures than nonusers. These results were adjusted to cognitive factors related to the course of the disease, as well as to other factors affecting hip fractures (Table 3). Therefore, our findings are likely to be due to the effect of AChEIs on PNS metabolic activity. The fact that the reduction in hip fracture risk in AChEIs users was dose-dependent (Table 4) strengthens this possibility. Moreover, the association that we observed between intake of AChEIs and BMI may also confirm the effect of AChEIs on PNS metabolic activity. These results expand our understanding of the role of the autonomic nervous system on bone remodeling by confirming an anabolic effect of the cholinergic system that antagonizes the effect of the SNS.

Onset of AD creates a reduction in BMD that could be the reason behind the high incidence of fractures in these patients. This reduction in BMD is proportional to neuronal degradation in the hypothalamus and limbic system.[32,33] In fact, low BMD is so closely related to AD that it has been found to be an indicator associated with a higher risk of developing AD.[42] The main hypothesis behind this relationship between AD and BMD is based on the high degradation of cholinergic neurons that occurs in AD.[32,33] This could result in overactivity of the SNS and subsequently in loss of bone mass.[43,44] Therefore, it would be logical to expect that increasing cholinergic activity in the brain

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

through drugs such as AChEIs should improve the bone conditions in these patients, and this is exactly what we observed in our study. Accordingly, our study not only shows a potential treatment for preventing fractures in AD patients, but it also helps identify the etiology behind these fractures.

In a recently published meta-analysis, it was observed that AChEIs had no beneficial effect in preventing bone fractures in AD patients.[45] This study was based on eight randomized controlled clinical trials designed to effectively evaluate the therapeutic benefits of AChEIs on AD.[45–52] However, there are a series of issues in these studies that limit their capacity to identify correlation between hip fractures and AChEI treatment. None of the eight studies used in this meta-analysis clarified what type of bone fracture occurred in the patients. The etiology of bone fracture is usually site-dependent.[53] Some fractures are strongly associated with the fragility of bone (ie, hip, wrist), whereas other fractures are related to high-energy trauma (ie, ankle, elbow, face).[53] For this reason our study was only focused on hip fractures. Also, the randomized controlled trials used in the meta-analysis were mainly designed to investigate the effect of AChEIs on cognition, and did not take into account pathologic conditions (osteoporosis, Paget's disease) or pharmacologic treatments (bisphosphonates, SSRIs) that influence bone.[41] Moreover, patient BMI, a very important factor regulating bone accrual, is not reported in any of these studies.[41] Accordingly, our case-control study is the first clinical study specifically designed to assess the association between hip fracture risk and AChEI treatments.

Muscarinic activity of AChEIs

In the present study we observed that the risk of hip fracture varied among AD patients depending on the AChEI treatment received. Patients treated with rivastigmine and donepezil were associated with lower risk of hip fracture than AChEI nonusers, whereas galantamine-treated patients had the same risk of hip fractures as AChEI nonusers. Galantamine has a similar, if not better, effect than donepezil and rivastigmine on the cognitive abilities of AD patients[54]; therefore, the difference in hip fracture incidence observed is not likely to be due to changes in patients' AD conditions.

A major biochemical difference between rivastigmine and donepezil on one hand, and galantamine on the other, is their affinity to specific cholinergic receptors. Galantamine binds to all nAChRs,[15,55] but cannot bind to mAChRs.[56] Hence, the allosteric potentiating ligands (APL) action of galantamine produces a selective nicotinic cholinergic enhancement. In contrast, the cholinergic enhancement by donepezil and rivastigmine is both nicotinic and muscarinic, and is devoid of the nicotinic APL action.[56,57] Excessive nicotinic activity has been associated with reduced bone remodeling capacity,[6] whereas activity of muscarinic receptors has been found to favor bone mass accrual.[58] Therefore, the different mAChR activity procured by each AChEI could be the reason behind the significantly low risk of hip fracture associated with patients receiving rivastigmine and donepezil, but not those receiving galantamine. Accordingly, this study presents the first clinical evidence suggesting that stimulation of cholinergic receptors may reduce the risk of hip fracture in a group of elderly patients (AD patients).

Muscarinic receptors are present in the central nervous system as well as in bone cells; therefore, it is not clear whether the effect of AChEIs on bone is systemic or a local one. Recent studies have described the presence of parasympathetic nerve fibers in bone that could have a direct effect on the AChRs in bone.[59,60] On the other hand, AChEIs might be having a systemic effect on bone through humoral mechanisms or by inhibition of the SNS.[58] Therefore, further investigations will be needed to unravel the exact mechanism behind this phenomenon.

Potential therapy for osteoporosis

Nowadays the most successful and commonly used drugs for the prevention of bone fractures in elderly osteoporotic patients are bisphosphonates and parathyroid hormone. In general terms, bisphosphonates have shown a protective relative risk (RR) in hip fractures relative to placebo of 0.66 (95% CI, 0.51–0.85).[61] Moreover, the RRs of zoledronic acid, alendronate, and risedronate, relative to placebo, are 0.58 (95% CI, 0.41–0.82), 0.95 (95% CI, 0.54–1.68), and 0.73 (95% CI, 0.37–1.44), respectively.[62] On the other hand, parathyroid hormone treatments have shown a reduction in the incidence of hip fractures with a RR relative to placebo of 0.46 (95% CI, 0.25–0.86).[63] In our cohort study, the OR shown by rivastigmine and donepezil versus nonusers of AChEIs was 0.22 (95% CI, 0.10–0.45) and 0.39 (95% CI, 0.19–0.76), respectively. Although these results cannot be directly compared with RR values from controlled trials, they still indicate that AChEIs stimulating mAChRs may have potential applications in the prevention of hip fractures in elderly patients, especially in AD patients. However, future clinical and in vivo studies will have to be performed to further confirm this hypothesis. Moreover, AChEIs are known to have a series of side effects that limit their tolerance by a large spectrum of the population,[64,65] and this issue will have to be addressed before applying this family of drugs as treatments for osteoporosis.

Limitations and future directions

One limitation of this study was the reduced number of subjects that could be included. Another limitation of our case-control design was that we could not detect the competing risk of death on the different groups, and this might have an effect on the outcome of the study.[66] AD leads to excess mortality[67]; this renders comparison between AD patients and AD-free patients complicated, and for this reason we did not include patients free of AD in our study. However, mortality among AD patients themselves also varies depending on factors such as age of AD onset, as well as general and metal health of the patients. Median survival of AD is longer for patients with younger onset of the disease and shorter in patients with later onset.[67] Accordingly, older AD patients have a much higher mortality than younger ones, and this could be contributing to the loss of the "normal" age distribution of fractures that is seen in this study. In a similar way, the association between patients' mortality and their general and metal health conditions could also affect our results. However, even though our case-control design did not adjust for

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

mortality, our risk estimates were adjusted to the strongest predictors of mortality in AD (age, MMSE, BDS, fall risk, and comorbidity)[67] and were still confirmatory of the protective effect of AChEIs on hip fracture.

Confounding by indication could also be a limitation in this retrospective study. Despite the fact that AChEIs are strongly recommended for the treatment of AD, the criteria used by physicians to prescribe AChEIs are often not clear.[68] AChEIs do not have any significant effect on the mortality and survival of AD patients, regardless of their baseline comorbid medical conditions.[69] Also, there is no information in the literature correlating expected mortality of AD patients with physicians' decisions to prescribe them AChEIs. However, it is possible that AChEIs might be targeted to patients based on their expected residual life. Indeed, it has been found that clinicians are less likely to prescribe AChEIs to patients with worse overall clinical conditions and frailty.[68] In our study we observed that AD patients in an advanced stage of the disease were less likely to receive AChEIs, probably due to the terminal status of their condition. This issue could be a limitation in our study because it could drive an artificial risk reduction for hip fracture with AChEIs. However, risk estimates adjusted to the grade of AD and comorbidity were still confirmatory of the protective effect of AChEIs on hip fracture.

Another limitation of this study was that our retrospective design does not allow us to determine a cause-effect relationship of AChEIs and hip fracture. An additional limitation in our study was that the effect of AChEIs on bone was only examined in an indirect way by assessing risks of hip fractures, and we do not provide information on the physiological conditions of bone.

For these reasons, future large-scale prospective cohort studies will have to be performed in order to confirm our findings by increasing the sample size, determining cause-effect relationships, and eliminating the possibility of bias by competing risk of death. Moreover, in vivo and clinical studies assessing biochemical bone markers, bone density measurements (DXA), and bone microarchitecture (high-resolution [HR]-pQCT) will have to be undertaken to explore the effect of AChEIs on bone structure and mineral content.

Conclusions

To the best of our knowledge this is the first clinical evidence demonstrating that administration of AChEIs stimulating mAChRs, such as donepezil and rivastigmine, are associated with a reduced risk of hip fractures in AD patients. On the other hand, solely stimulating nAChRs through AChEI galantamine is not associated with a reduced risk of hip fractures. Although additional observational studies and controlled trials are needed to confirm these potentially important findings, many elderly patients with AD or other dementias that are at risk of developing osteoporosis and pathological fractures may benefit from treatments with AChEIs.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

We acknowledge Dr. Rubens Albuquerque and Dr. Zaher Jabbour for their excellent advice and critiques. We acknowledge financial support from the "COBO" MDEIE (PSR-SIIRI) grant, The Faculty of Dentistry at McGill University, The Fondation de l'Ordre des dentistes du Québec's (FODQ), and the "Harry Rosen" salary award (FT).

Authors' roles: IT had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: FT, IT. Acquisition of data: IT, TO, MSD, LM, JMS, FR, IM, IG, and AP. Analysis and interpretation of data: IT, FT. Drafting of the manuscript: IT, FT. Critical revision of the manuscript for important intellectual content: IT, FT. Statistical analysis: FT. Obtained funding: FT. Administrative, technical, or material support: TO, MSD, and LM.

References

1. Walker LM, Preston MR, Magnay JL, Thomas PB, El Haj AJ. Nicotinic regulation of c-fos and osteopontin expression in human-derived osteoblast-like cells and human trabecular bone organ culture. Bone. 2001;28(6):603–8.
2. Hoogduijn MJ, Cheng A, Genever PG. Functional nicotinic and muscarinic receptors on mesenchymal stem cells. Stem Cells Dev. 2009;18(1):103–12.
3. En-Nosse M, Hartmann S, Trinkaus K, Alt V, Stigler B, Heiss C, Kilian O, Schnettler R, Lips KS. Expression of non-neuronal cholinergic system in osteoblast-like cells and its involvement in osteogenesis. Cell Tissue Res. 2009;338(2):203–15.
4. Warner SE, Sanford DA, Becker BA, Bain SD, Srinivasan S, Gross TS. Botox induced muscle paralysis rapidly degrades bone. Bone. 2006; 38(2):257–64.
5. Grimston SK, Silva MJ, Civitelli R. Bone loss after temporarily induced muscle paralysis by Botox is not fully recovered after 12 weeks. Ann N Y Acad Sci. 2007;1116:444–60.
6. Alkondon M, Pereira EF, Almeida LE, Randall WR, Albuquerque EX. Nicotine at concentrations found in cigarette smokers activates and desensitizes nicotinic acetylcholine receptors in CA1 interneurons of rat hippocampus. Neuropharmacology. 2000;39(13):2726–39.
7. Hollinger JO, Schmitt JM, Hwang K, Soleymani P, Buck D. Impact of nicotine on bone healing. J Biomed Mater Res. 1999;45(4):294–301. [Erratum in: J Biomed Mat Res 1999; 5; 46(3) 438-9.]
8. Glowacki J, Schulten AJ, Perrott D, Kaban LB. Nicotine impairs distraction osteogenesis in the rat mandible. J Oral Maxillofacial Surg. 2008;37(2):156–61.
9. Mohammad AF, Khan KA, Galvin L, Hardiman O, O'Connell PG. High incidence of osteoporosis and fractures in an aging post-polio population. Eur Neurol. 2009;62(6):369–74.
10. Sato T, Abe T, Chida D, Nakamoto N, Hori N, Kokabu S, Sakata Y, Tomaru Y, Iwata T, Usui M, Aiko K, Yoda T. Functional role of acetylcholine and the expression of cholinergic receptors and components in osteoblasts. FEBS Lett. 2010;584(4):817–24.
11. Genever PG, Birch MA, Brown E, Skerry TM. Osteoblast-derived acetylcholinesterase: a novel mediator of cell-matrix interactions in bone?. Bone. 1999;24(4):297–303.
12. Inkson CA, Brabbs AC, Grewal TS, Skerry TM, Genever PG. Characterization of acetylcholinesterase expression and secretion during osteoblast differentiation. Bone. 2004;35(4):819–27.
13. Zhang D, Zhang P, Wang Y, Han N, Tang C, Jiang B. The influence of brain injury or peripheral nerve injury on calcitonin gene-related

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA
180

peptide concentration variation and fractures healing process. Artif Cells Blood Substit Immobil Biotechnol. 2009;37(2):85–91.

14. Ma L, Zheng LW, Sham MH, Cheung LK. Uncoupled angiogenesis and osteogenesis in nicotine-compromised bone healing. J Bone Miner Res. 2010;25(6):1305–13.
15. Pepeu G, Giovannini MG. Cholinesterase inhibitors and beyond. Curr Alzheimer Res. 2009;6(2):86–96.
16. Massoud F, Gauthier S. Update on the pharmacological treatment of Alzheimer's disease. Curr Neuropharmacol. 2010;8(1):69–80.
17. Nandy S, Parsons S, Cryer C, Underwood M, Rashbrook E, Carter Y, Eldridge S, Close J, Skelton D, Taylor S, Feder G; Falls Prevention Pilot Steering Group. Development and preliminary examination of the predictive validity of the Falls Risk Assessment Tool (FRAT) for use in primary care. J Public Health (Oxf). 2004;26(2):138–43.
18. Hughes CP, Berg L, Danziger WL, Coben LA, Martin RL. A new clinical scale for the staging of dementia. Br J Psychiatry. 1982; 140:566–72.
19. Morris JC. The clinical dementia rating (CDR): current version and scoring rules. Neurology. 1993;43:2412–4.
20. Folstein MF, Folstein SE, McHugh PRT. "Mini-Mental State": a practical method for grading the cognitive state patients for the clinician. J Psychiatr Res. 1975;12:189–98.
21. Charlson ME, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. J Chron Dis. 1987;40(5):373–83.
22. Melton LJ 3rd, Beard CM, Kokmen E, Atkinson EJ, O'Fallon WM. Fracture risk in patients with Alzheimer's disease. J Am Geriatr Soc. 1994;42(6):614–9.
23. Visser H. Gait and balance in senile dementia of Alzheimer's type. Age Ageing. 1983;12(4):296–301.
24. Buchner DM, Larson EB. Falls and fractures in patients with Alzheimer-type dementia. JAMA. 1987;257(11):1492–5.
25. Strubel D, Jacquot JM, Martin-Hunyadi C. Dementia and falls. Ann Readapt Med Phys. 2001;44(1):4–12.
26. Johanson C, Skoog I. A population-based study on the association between dementia and hip fractures in 85-year olds. Aging (Milano). 1996;8(3):189–96.
27. Baker NL, Cook MN, Arrighi HM, Bullock R. Hip fracture risk and subsequent mortality among Alzheimer's disease patients in the United Kingdom, 1988-2007. Age Ageing. 2011;40(1):49–54.
28. Sato Y, Kanoko T, Satoh K, Iwamoto J. Risk factors for hip fracture among elderly patients with Alzheimer's disease. J Neurol Sci. 2004; 223(2):107–12.
29. Weller I, Schatzker J. Hip fractures and Alzheimer's disease in elderly institutionalized Canadians. Ann Epidemiol. 2004;14(5):319–24.
30. Hussain A, Barer D. Fracture risk in Alzheimer's disease patients. J Am Geriatr Soc. 1995;43(4):454.
31. Høidrup S, Prescott E, Sørensen TI, Gottschau A, Lauritzen JB, Schroll M, Grønbaek M. Tobacco smoking and risk of hip fracture in men and women. Int J Epidemiol. 2000;29(2):253–259.
32. Loskutova N, Honea RA, Vidoni ED, Brooks WM, Burns JM. Bone density and brain atrophy in early Alzheimer's disease. J Alzheimers Dis. 2009;18(4):777–85.
33. Loskutova N, Honea RA, Brooks WM, Burns JM. Reduced limbic and hypothalamic volumes correlate with bone density in early Alzheimer's disease. J Alzheimers Dis. 2010;20(1):313–22.
34. Raina P, Santaguida P, Ismaila A, Patterson C, Cowan D, Levine M, Booker L, Oremus M. Effectiveness of cholinesterase inhibitors and memantine for treating dementia: evidence review for a clinical practice guideline. Ann Intern Med. 2008;148(5):379–97.
35. Raschetti R, Maggini M, Sorrentino GC, Martini N, Caffari B, Vanacore N. A cohort study of effectiveness of acetylcholinesterase inhibitors in Alzheimer's disease. Eur J Clin Pharmacol. 2005;61(5–6):361–8.
36. Hernandez RK, Farwell W, Cantor MD, Lawler EV. Cholinesterase inhibitors and incidence of bradycardia in patients with dementia in the Veterans Affairs New England Healthcare System. J Am Geriatr Soc. 2009;57(11):1997–2003.
37. Molfino A, Fiorentini A, Tubani L, Martuscelli M, Rossi Fanelli F, Laviano A. Body mass index is related to autonomic nervous system activity as measured by heart rate variability. Eur J Clin Nutr. 2009; 63(10):1263–5.
38. Lee P, Kengne AP, Greenfield JR, Day RO, Chalmers J, Ho KK. Metabolic sequelae of β-blocker therapy: weighing in on the obesity epidemic?. Int J Obes (Lond). 2011;35(11):1395–403.
39. Balbo SL, Grassiolli S, Ribeiro RA, Bonfleur ML, Gravena C, Brito Mdo N, Andreazzi AE, Mathias PC, Torrezan R. Fat storage is partially dependent on vagal activity and insulin secretion of hypothalamic obese rat. Endocrine. 2007;31(2):142–8.
40. Takeda S, Karsenty G. Molecular bases of the sympathetic regulation of bone mass. Bone. 2008;42(5):837–40.
41. Schlienger RG, Kraenzlin ME, Jick SS, Meier CR. Use of beta-blockers and risk of fractures. JAMA. 2004;292(11):1326–32.
42. Zhou R, Deng J, Zhang M, Zhou HD, Wang YJ. Association between bone mineral density and the risk of Alzheimer's disease. J Alzheimers Dis. 2011;24(1):101–8.
43. Pascualy M, Petrie EC, Brodkin K, Peskind ER, Wilkinson CW, Raskind MA. Hypothalamic pituitary adrenocortical and sympathetic nervous system responses to the cold pressor test in Alzheimer's disease. Biol Psychiatry. 2000;48:247–54.
44. Szot P, Leverenz JB, Peskind ER, Kiyasu E, Rohde K, Miller MA, Raskind MA. Tyrosine hydroxylase and norepinephrine transporter mRNA expression in the locus coeruleus in Alzheimer's disease. Brain Res Mol Brain Res. 2000;84:135–40.
45. Kim DH, Brown RT, Ding EL, Kiel DP, Berry SD. Dementia medications and risk of falls, syncope, and related adverse events: meta-analysis of randomized controlled trials. J Am Geriatr Soc. 2011;59(6):1019–31.
46. Rogers SL, Farlow MR, Doody RS, Mohs R, Friedhoff LT. A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. Donepezil Study Group. Neurology. 1998;50(1): 136–45.
47. Homma A, Takeda M, Imai Y, Udaka F, Hasegawa K, Kameyama M, Nishimura T. Clinical efficacy and safety of donepezil on cognitive and global function in patients with Alzheimer's disease. A 24-week, multicenter, double-blind, placebo-controlled study in Japan. E2020 Study Group. Dement Geriatr Cogn Disord. 2000;11(6):299–313.
48. Winblad B, Engedal K, Soininen H, Verhey F, Waldemar G, Wimo A, Wetterholm AL, Zhang R, Haglund A, Subbiah P; Donepezil Nordic Study Group. A 1-year, randomized, placebo-controlled study of donepezil in patients with mild to moderate AD. Neurology. 2001; 57(3):489–95.
49. Wilkinson D, Doody R, Helme R, Taubman K, Mintzer J, Kertesz A, Pratt RD; Donepezil 308 Study Group. Donepezil in vascular dementia: a randomized, placebo-controlled study. Neurology. 2003;61(4): 479–86.
50. Winblad B, Kilander L, Eriksson S, Minthon L, Båtsman S, Wetterholm AL, Jansson-Blixt C, Haglund A; Severe Alzheimer's Disease Study Group. Donepezil in patients with severe Alzheimer's disease: double-blind, parallel-group, placebo-controlled study. Lancet. 2006 Apr 1; 367(9516):1057–65. [Erratum in: Lancet. 2006; 368(9548):1650. Lancet. 2006; 367(9527) 1980.]
51. Howard RJ, Juszczak E, Ballard CG, Bentham P, Brown RG, Bullock R, Burns AS, Holmes C, Jacoby R, Johnson T, Knapp M, Lindesay J, O'Brien JT, Wilcock G, Katona C, Jones RW, DeCesare J, Rodger M; CALM-AD Trial Group. Donepezil for the treatment of agitation in Alzheimer's disease. N Engl J Med. 2007;357(14):1382–92.
52. Donepezil Hydrochloride (E2020) in Dementia Associated With Cerebrovascular Disease. Clinical Trial Identifier NCT00165737. A 24-week, multi-center, randomized, double-blind, placebo-controlled

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA
181

evaluation of the efficacy, safety, and tolerability of donepezil hydrochloride (E2020) in patients with dementia associated with cerebrovascular disease [Internet]. Bethesda, MD: National Institutes of Health; 2005 Sep 12 [updated 2011 Mar 31; cited 2012 Mar 27]. Available from http://clinicaltrials.gov/ct2/show/NCT00165737/.

53. Seeley DG, Browner WS, Nevitt MC, Genant HK, Scott JC, Cummings SR. Which fractures are associated with low appendicular bone mass in elderly women? The Study of Osteoporotic Fractures Research Group. Ann Intern Med. 1991;115(11):837–42.
54. Raskind MA, Peskind ER, Wessel T, Yuan W. Galantamine in AD: a 6-month randomized, placebo-controlled trial with a 6-month extension. The Galantamine USA-1 Study Group. Neurology. 2000; 54(12):2261–8.
55. Maelicke A, Albuquerque EX. Allosteric modulation of nicotinic acetylcholine receptors as a treatment strategy for Alzheimer's disease. Eur J Pharmacol. 2000;393(1–3):165–70.
56. Samochocki M, Höffle A, Fehrenbacher A, Jostock R, Ludwig J, Christner C, Radina M, Zerlin M, Ullmer C, Pereira EF, Lübbert H, Albuquerque EX, Maelicke A. Galantamine is an allosterically potentiating ligand of neuronal nicotinic but not of muscarinic acetylcholine receptors. J Pharmacol Exp Ther. 2003;305(3):1024–36.
57. Basselin M, Nguyen HN, Chang L, Bell JM, Rapoport SI. Acute but not chronic donepezil increases muscarinic receptor-mediated signaling via arachidonic acid in unanesthetized rats. J Alzheimers Dis. 2009; 17(2):369–82.
58. Shi Y, Oury F, Yadav VK, Wess J, Liu XS, Guo XE, Murshed M, Karsenty G. Signaling through the M(3) muscarinic receptor favors bone mass accrual by decreasing sympathetic activity. Cell Metab. 2010;11(3): 231–8.
59. Sisask G, Bjurholm A, Ahmed M, Kreicbergs A. The development of autonomic innervation in bone and joints of the rat. J Auton Nerv Syst. 1996;59(1–2):27–33.
60. Gajda M, Litwin JA, Tabarowski Z, Zagólski O, Cichocki T, Timmermans JP, Adriaensen D. Development of rat tibia innervation: colocalization of autonomic nerve fiber markers with growth-associated protein 43. Cells Tissues Organs. 2010;191(6):489–99.
61. Hodsman AB, Hanley DA, Josse R. Do bisphosphonates reduce the risk of osteoporotic fractures? An evaluation of the evidence to date. CMAJ. 2002;166(11):1426–30.
62. Jansen JP, Bergman GJ, Huels J, Olson M. The efficacy of bisphosphonates in the prevention of vertebral, hip, and nonvertebral-nonhip fractures in osteoporosis: a network meta-analysis. Semin Arthritis Rheum. 2011;40(4):275–84. e1-2.
63. Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH. Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. N Engl J Med. 2001;344(19):1434–41.
64. Auriacombe S, Pere JJ, Loria-Kanza Y, Vellas B. Efficacy and safety of rivastigmine in patients with Alzheimer's disease who failed to benefit from treatment with donepezil. Curr Med Res Opin. 2002; 18(3):129–38.
65. Kinnair D, Machili C, Prettyman R, Van Diepen E. Cardio-vascular safety of acetyl cholinesterase inhibitors in patients suffering from Alzheimer's disease; factors that predict poor tolerability. Int J Geriatr Psychiatry. 2011;26(3):328–9.
66. Berry SD, Ngo L, Samelson EJ, Kiel DP. Competing risk of death: an important consideration in studies of older adults. J Am Geriatr Soc. 2010;58(4):783–7.
67. Larson EB, Shadlen MF, Wang L, McCormick WC, Bowen JD, Teri L, Kukull WA. Survival after initial diagnosis of Alzheimer disease. Ann Intern Med. 2004;140(7):501–9.
68. Hoffmann F, van den Bussche H, Wiese B, Schön G, Koller D, Eisele M, Glaeske G, Scherer M, Kaduszkiewicz H. Impact of geriatric comorbidity and polypharmacy on cholinesterase inhibitors prescribing in dementia. BMC Psychiatry. 2011;11:190.
69. Suh GH, Ryu SH, Lee DW, Han C, Ju YS, Kee BS, Lee JN, Bae JN, Choi JH, Kim DJ, Lee NJ, Lee JY, Go HJ, Yi JS, Cho SJ, Jeon YW. Cholinesterase inhibitors for Alzheimer disease: do they provide more than symptomatic benefits?. Am J Geriatr Psychiatry. 2011; 19(3):266–273.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Publicaciones y
Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Resumen de Tesis Doctoral

La regularización colinérgica del remodelado óseo: el riesgo de fractura de cadera en pacientes diagnosticados de Alzheimer en tratamiento con inhibidores de acetilcolinesterasa.

Iskandar Ahmad Tamimi Mariño

Málaga, Mayo 2014

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Introducción

Las condiciones clínicas y fármacos que alteran el sistema de señalización cholinérgica parecen tener una serie de efectos sobre la fisiología ósea.

La toxina botulínica inhibe la liberación de acetilcolina de la neurona motora a nivel presináptico y ejerce un efecto negativo sobre la consolidación ósea así como una disminución de la mineralización del hueso. Sin embargo la mineralización ósea no se mejora con la recuperación de la actividad muscular. Esto indica que el efecto negativo que ejerce la toxina botulínica sobre el hueso no está relacionado con la actividad muscular. La poleomielitis destruye las neuronas motoras que dependen de la acetilcolina. Los pacientes que padecen esta enfermedad sufren alteraciones en el crecimiento de los miembros afectados y tienen una tendencia a desarrollar osteoporosis mucho más elevada que en la población general, incluso muchos años después de haber recuperado la actividad muscular.

La nicotina parece tener un efecto negativo sobre la consolidación ósea en los fumadores. Estos tienen una elevada concentración de nicotina en la sangre que interacciona con los receptores de acetilcolina de tipo nicotínico (nRACh) que conlleva a una desensibilización de los mismos.

Varios estudios recientes han identificado la presencia de un número de subunidades de RACh en las células que componen el tejido óseo. Se han detectado subunidades de receptores de tipo nRACh en los osteoblastos y en las células mesenquimales primarias del hueso (CMSs), en estas últimas también se han identificado receptores de tipo muscarínico (mRACh). Por otra parte, otros estudios han hallado una relación entre la

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

regulación de la actividad de la fosfatasa alcalina (FLP), la expresión de RAChs en los osteoblastos y la proliferación de los osteoblastos.

La información disponible en la literatura indica que la inhibición de los RACh podría conllevar una reducción en el metabolismo del tejido óseo. A pesar de lo anterior, no está claro si estimulación de estos receptores puede tener un efecto anabólico sobre la formación del hueso.

Los inhibidores del actilcolinestarasa (IAChEs) son un grupo de medicamentos que estimulan los RChA mediante la inhibición de la AChE y aumentan los niveles de acetilcolina en el espacio sináptico. Estos medicamentos se han utilizado en el tratamiento de la enfermedad de Alzheimer (EA) y otras demencias desde la década de los noventa.

Hipótesis

La literatura disponible parece indicar que la inhibición de los receptores AChR presentes en las células del tejido óseo puede causar una reducción en el remodelado óseo. Sin embargo, todavía no está claro si la estimulación de estos receptores puede tener un efecto anabólico sobre la formación de tejido óseo.

Consideramos que la administración de IAChEs puede tener un efecto beneficioso sobre el remodelado óseo que podría traducirse en una reducción en el riesgo de fracturas. Con el objetivo de probar esta hipótesis, hemos diseñado un estudio retrospectivo de casos y controles en el que se compara la incidencia de fracturas de cadera en un grupo de pacientes con EA en tratamiento IAChEs, con un segundo grupo de pacientes con EA que no reciben tratamiento con IAChEs.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Materiales y Métodos

Tras obtenerse la aprobación del comité de ética de nuestro centro hospitalario se lleva a cabo un estudio retrospectivo de casos y controles sobre los pacientes diagnosticados de EA residentes dentro de la zona sanitaria correspondiente al " Hospital Carlos Haya". Los datos demográficos y epidemiológicos se obtuvieron de los archivos informatizados de los servicios de Neurología y Cirugía Ortopédica, y de los registros estadísticos del "Hospital Carlos Haya". La duración del estudio fue de 5 años; entre el 1 de enero 2004 y el 31 de diciembre de 2008. Se revisaron todas las fracturas de cadera en pacientes con EA tratadas en nuestro centro durante dicho periodo.

A continuación se seleccionaron los pacientes que tenían una edad entre los 75 a 95 años en el momento de la fractura, y con al menos 2 años de seguimiento en el servicio de Cirugía Ortopédica. Se excluyeron los pacientes que padecían algún tipo de trastorno médico que pudiera afectar sustancialmente el metabolismo óseo (ej. Antecedentes de osteoporosis, osteomalacia, enfermedad de Paget, procesos malignos [excluyendo el carcinoma basocelular], o el alcoholismo), así como los pacientes que se estaban en tratamiento con corticosteroides , anticonvulsivos o bisfosfonatos (como tratamiento para la osteoporosis o metástasis ósea).

En el grupo de control se incluyeron a todos los pacientes diagnosticados de EA registrados en la base de datos del servicio de Neurología entre enero de 2004 y diciembre de 2008 y que no tenían antecedentes de haber sufrido una fractura de cadera antes del 31 de diciembre de 2008 (fecha de finalización del estudio). Los pacientes pertenecientes al grupo de control tenían que continuar con vida al final del estudio. Estos fueron sometidos a los mismos criterios de exclusión utilizados en el grupo de estudio.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

A continuación se revisaron las historias clínicas de los pacientes seleccionados y se completaron una serie de cuestionarios estandarizados. Los parámetros obtenidos fueron los siguientes: edad del paciente, sexo , movilidad, institucionalización, tipo de fractura de cadera (incluyendo fracturas subcapitales , transcervicales , basicervicales ,pertrocantericas y subtrocantericas), antecedentes de fracturas osteoporóticas (incluyendo, fracturas vertebrales , de radio distal, húmero proximal, y fracturas de cadera previas al estudio), índice de masa corporal (IMC), el riesgo de caída según la escala " FRAT ", consumo de tabaco, institucionalización, escala de comorbilidad de Charlson (CCS), el uso de inhibidores selectivos de la recaptación de serotonina (ISRS) y antecedentes de osteoporosis familiar . La severidad de la EA de acuerdo con la escala de "CDR" y el estado cognitivo según las escalas de valoración cognitiva "MMSE" y "BDS". También se revisó el tratamiento específico para la EA (IAChEs [ej. clorhidrato de donepezilo, galantamina, rivastigmina, o ningún IAChE] y / o memantina), incluyendo la dosis y la duración (0,5 a 2 años > 2 años).

Se realizaron análisis de regresión logística condicional utilizando el SPSS IBM Statistics 19TM (SPSS Inc., Chicago, IL). Se calculó la incidencia anual de fracturas de cadera en la población general y en la población de pacientes con EA con sus correspondientes intervalos de confianza (IC) del 95%. Las estimaciones de riesgo de fractura entre los distintos subgrupos de pacientes con EA según el tratamiento seguido se presentaron como odds ratio (OR) con IC del 95% . Los valores de P fueron de 2 - lados y se consideraron estadísticamente significativas si éstos eran inferiores a 0,05. Los OR se ajustaron a posibles factores de confusión: edad, género, movilidad, institucionalización, BDS (< 9,9-16.9 , ≥ 17), puntuación del CDR (1,2,3), puntuación del MMSE (≤ 20 , > 20), puntuación del FRAT (= 1 , > 1), antecedentes de fracturas osteoporóticas, tipo de IAChE utilizado, IMC (< 25,25-29.9 , ≥ 30), CCS

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

(0-1, 2, >3), el uso de ISRS y tabaquismo (fumador, no fumador). Las diferencias entre pacientes tratados con o sin IAChEs fueron evaluadas con el test t-Student (para variables continuas, es decir: la edad, MMSE, CDR, BDS, el IMC, CCS y FRAT), y con la prueba de Chi cuadrado (para variables nominales, es decir: sexo, otras fracturas, uso de ISRS y tabaquismo) los valores de P fueron de 2 caras y se consideraron estadísticamente significativas si éstos eran inferiores a 0,05.

Resultados

La población registrada en la zona sanitaria correspondiente al Hospital Universitario Carlos Haya el año 2008 fue de 607.217 habitantes, de los cuales un total de 40011 personas tenía una edad superior a los 75 años (Tabla 1). De estos últimos se identificaron un total de 2794 pacientes con EA, de los cuales 2178 pacientes cumplieron con los criterios de inclusión del grupo de control (Tabla 2).

El número total de fracturas de cadera tratadas en nuestro centro durante el año 2008 fue de 381 (Tabla 3). También se identificaron 135 casos de fracturas de cadera en pacientes con EA entre 2004 y 2008, de los cuales 80 pacientes cumplieron los criterios de inclusión de grupos de casos (Tabla 4).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Grupos de edad	Población masculina	Población femenina	Población femenina y masculina residentes en nuestra zona hospitalaria el año 2008
≤69	270054 (49,6%)	274993 (50,4%)	545047
70-74	9444 (42,6%)	12715 (57,4%)	22159
75-79	7322 (39,4%)	11266 (60,6%)	18588
80-84	4091 (34,0%)	7938 (66,0%)	12029
85-89	1465 (21,4%)	5379 (78,6)	6844
≥90	1233 (48,4%)	1317 (51,6%)	2550
Total	293609 (48,4%)	313608 (51,6%)	607217

Tabla 1: La población general registrada en la zona sanitaria del Hospital Carlos Haya de Málaga el año 2008.

Grupos de edad	Varones /Alzheimer población	Mujeres/ Alzheimer población	Población total de Alzheimer
70-74	158 (34,3%)	302 (65,7%)	460
75-79	162 (26,6%)	448 (73,4%)	610
80-84	286 (31,9%)	610 (68,1%)	896
85-89	277 (48,6%)	293 (51,4%)	570
≥90	111 (43,0%)	147 (57,0%)	258
Total	994 (35,6%)	1800 (64,4%)	2794

Tabla 2: La población de pacientes con Alzheimer (≥70 años) en la zona sanitaria del Hospital Carlos Haya de Málaga el año 2008.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Grupos de edad	Fracturas de cadera en varones	Fracturas de cadera en mujeres	Numero total de fracturas de cadera
≤69	36 (55,4%)	29 (44,6%)	65
70-74	7 (21,9%)	25 (78,1%)	32
75-79	21 (28,8%)	52 (71,2%)	73
80-84	27 (27,0%)	73 (73,0%)	100
85-89	11 (36,6%)	56 (63,4%)	67
≥90	6 (13,6%)	38 (86,4%)	44
Total	108 (28,3%)	273 (71,7%)	381

Tabla 3: El número de fracturas de cadera (en todos los grupos de edad) tratadas en la zona sanitaria del Hospital Carlos Haya de Málaga el año 2008 (por grupos de edad).

Grupos de edad	Fracturas de cadera en varones / Población Alzheimer	Fracturas de cadera en mujeres / Población Alzheimer	Fracturas de cadera /Total población Alzheimer
70-74	1 (20,0%)	4 (80,0%)	5
75-79	1 (4,5%)	21 (95,5%)	22
80-84	5 (17,9%)	23 (82,1%)	28
85-89	7 (28,0%)	18 (72,0%)	25
≥ 90	1 (20,0%)	4 (80,0%)	5
Total	15 (17,6%)	70 (82,4%)	85

Tabla 4: La distribución de las fracturas de cadera por edad y sexo en el grupo de estudio n=80 (pacientes con Alzheimer tratados en el periodo 1/1/2004- 31/12/2008, tras aplicar los criterios de inclusión-exclusión). El grupo de edad entre 70-74 años

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

también se incluye en esta tabla (este no forma parte del grupo de estudio, pero se utilizo para calcular la incidencia de fracturas en pacientes con EA).

La incidencia anual de fracturas de cadera en la población general aumenta con la edad. Esta tendencia fue más evidente en la población femenina (tabla 5). No obstante, la incidencia de fractura de cadera parece disminuir con la edad en los pacientes con EA, tanto en hombres como en mujeres, en contraste con lo observado en la población en general (Tabla 6) (Figuras 1, 2 y 3).

La distribución de las fracturas de cadera en el grupo de estudio según la edad, sexo, IMC, MMSE, FRAT, CDR, CCS, BDS, uso de ISRS, institucionalización y la presencia de otras fracturas se muestra en la (Tabla 7).

Los pacientes con una puntuación FRAT> 1 e IMC <25 mostraron un mayor riesgo de sufrir una fractura de cadera que los pacientes con una puntuación FRAT = 1 o con IMC> 25, respectivamente. Las mujeres diagnosticadas de EA tenían un riesgo ligeramente mayor de sufrir una fractura de cadera que los varones con EA, aunque esta diferencia no era significativa. Por otra parte los pacientes entre los 85 a 89 años tenían un menor riesgo que los pacientes con edades que oscilaban entre 75-84 años. No se apreció una asociación entre el riesgo de fractura de cadera y la puntuación obtenida en las escalas CDR, BDS y MMSE (Tabla 7). Los pacientes en tratamiento con IAChEs tuvieron un mejor rendimiento en los test cognitivos (CDR, BDS y MMSE) que los pacientes que tomaban IAChEs (Tabla 8). El uso de ISRS parecía ser mayor en los pacientes tratados con IAChEs que en los no tratados. No se apreciaron diferencias significativas con respecto al sexo, tabaquismo, y la puntuación en la escala CCS entre los pacientes tratados y no tratados con IAChEs. El IMC también fue más elevado en los pacientes en tratamiento con IAChEs que en los pacientes no tratados.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Curiosamente, los pacientes tratados con IAChEs tenían un menor riesgo de fractura de cadera que los que no tomaban estos medicamentos a pesar de que los primeros tenían un mayor riesgo de caída en la escala (FRAT) en comparación con los pacientes no tratados (Tablas 8 y 9).

Los distintos tipos de IAChEs tuvieron resultados dispares en cuanto a su efecto sobre el riesgo de fractura de cadera. Los pacientes tratados con galantamina tuvieron el mismo riesgo de sufrir una fractura de cadera que los pacientes que tomaban IAChEs. Pese a esto, los pacientes tratados con donepezilo, rivastigmina tuvieron un menor riesgo de sufrir fracturas de cadera en comparación con los pacientes que no tomaban IAChEs. Las diferencias entre estos últimos dos grupos eran muy significativas y se asociaban a la dosis administrada. No se observó una relación significativa entre la duración del tratamiento y el riesgo de fracturas de cadera. La administración de rivastigmina y donepezilo tenía una protección total OR ajustado de 0,22 (IC del 95% 0,10 a 0,45) y 0,39 (IC del 95% 0,19-0,76), respectivamente, en comparación con los pacientes no tratados con IAChEs (Tabla 9) (Figura 4). Con un error $\alpha = 0.05$, se obtuvo un potencia del 83% para detectar el riesgo de fractura de cadera en pacientes con EA tratados con IAChEs igual a 0,42 o menor.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Grupos de edad	Incidencia de fracturas de cadera en varones	Incidencia de fracturas de cadera en mujeres	Incidencia total de fracturas de cadera
70-74	0.74 (0.3-1.5)	2.0 (1.3-2.8)	1.4 (1.0- 2.0)
75-79	2.9 (1.8-4.4)	4.6 (3.5-6.0)	3.9 (3.1-4.9)
80-84	6.6 (4.4-9.6)	9.2 (7.2-11.6)	8.3 (6.8-10.1)
85-89	7.5 (3.8-13.4)	10.4 (7.9-13.5)	9.8 (7.6-12.4)
≥90	4.9 (1.8-10.6)	28.9 (20.5-39.4)	17.3 (12.6-23.1)
Total	3.1 (2.4-3.9)	6.3 (5.6-7.2)	5.1 (4.5-5.7)

Tabla 5: La incidencia anual (CI 95%) de fracturas de cadera en la población en general (por 1000 habitantes) el año 2008 (n=316). El grupo de edad entre 70-74 años también se incluye en esta tabla (este no forma parte del grupo de estudio, pero se utilizo para calcular la incidencia de fracturas en pacientes con EA).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Grupos de edad	Incidencia fracturas cadera / población EA-varones	Incidencia fracturas cadera / población EA-mujeres	Incidencia fracturas cadera / población EA-total
70-74	1.2 (0.2-2.2)	2.7 (0.1-12.2)	2.1 (0.1-11.9)
75-79	1.0 (0.1-.1.7)	11.6 (3.2-29.3)	8.0 (2.9-18.4)
80-84	3.4 (0.1-18.7)	8.3 (3.0-19.1)	6.5 (2.7-14.1)
85-89	6.8 (1.2-26.6)	5.8 (1.9-14.9)	4.8 (1.4-11.0)
≥90	0.7 (0.1-1.3)	2.4 (0.1-10.9)	1.6 (0.0-8.0)
Total	2.6 (0.5-7.6)	6.4 (3.6-10.5)	5.0 (3.0-8.0)

Tabla 6: La incidencia anual (CI 95%) de fracturas de cadera en pacientes diagnosticados de Alzheimer (por 1000 habitantes) durante el periodo 1/1/2004-31/12/2008 (n=80). El grupo de edad entre 70-74 años también se incluye en esta tabla (este no forma parte del grupo de estudio, pero se utilizo para calcular la incidencia de fracturas en pacientes con EA).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

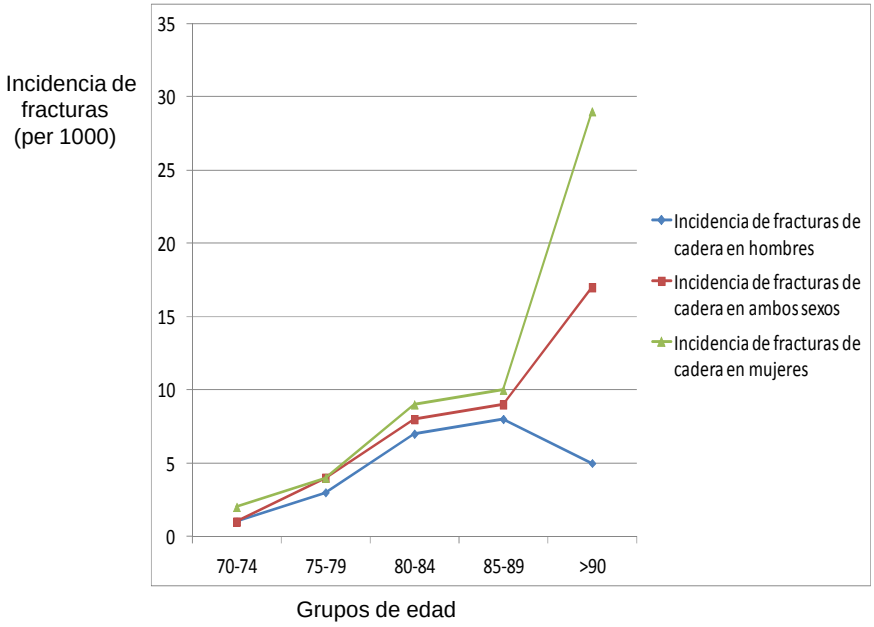


Figura 1: La incidencia anual de fracturas de cadera en la población en general (pacientes ≥70 años) por 1000 habitantes el año 2008, por edad y sexo.

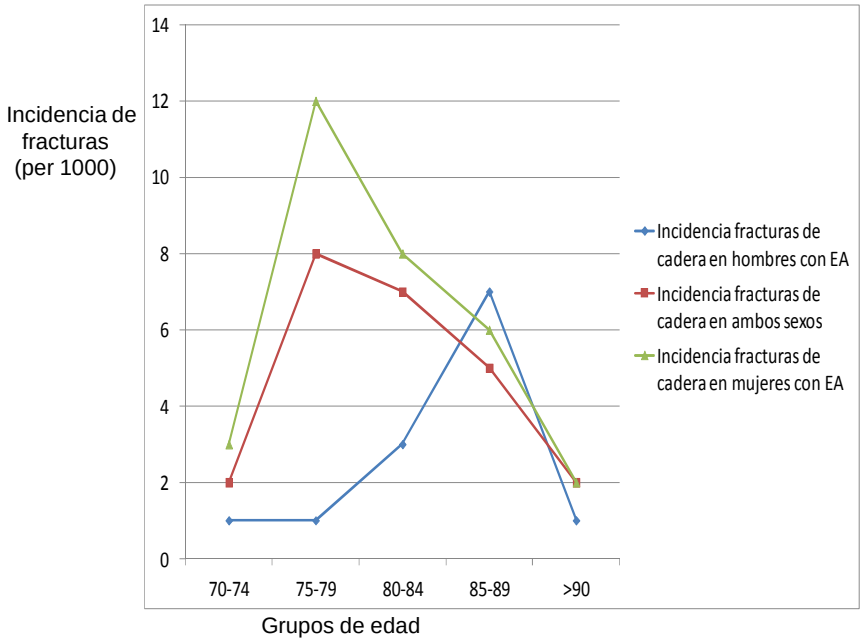


Figura 2: La incidencia anual de fracturas de cadera en pacientes diagnosticados de Alzheimer (pacientes ≥70 años) por 1000 habitantes durante el periodo 1/1/2014-31/12/2008. (pacientes ≥70 años) por 1000 habitantes el año 2008, por edad y sexo.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

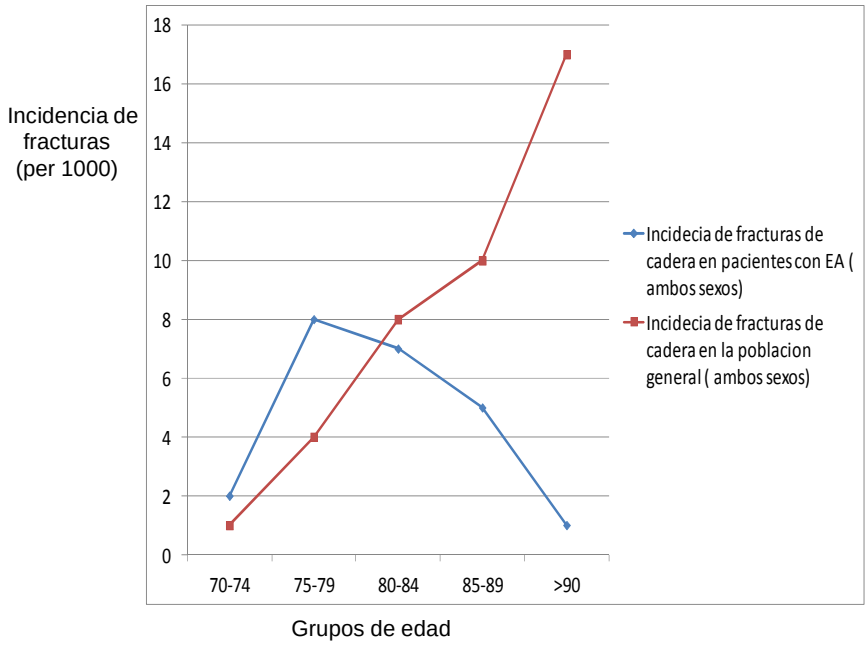


Figura 3: La incidencia anual media (por 1000 habitantes) de fracturas de cadera en pacientes diagnosticados de Alzheimer durante el periodo 1/1/2004-31/12/2008 comparada con la incidencia de fracturas de cadera en la población general el año 2008.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Characteristicas		Casos (n=80)a	Controles (n=2178)a	Odds Ratio (95%CI)	Odds Ratio Ajustado (95%CI) b	Valor -P
Edad, a	75-79	21(26.3)	594(27.3)	1.00	1.00	-
	80-84	28(35.0)	846(38.8)	0.94(0.53-1.7)	0.39(0.18-0.85)	0.018
	>85	31(38.8)	738(33.9)	1.19(0.68-2.09)	1.730.81-3.69)	0.159
	Women	66(82.5)	1548(71.1)	1.00	1.00	-
	Men	14(17.5)	630(28.9)	0.52(0.29-0.94)	0.41(0.15-1.08)	0.072
IMC c	<25	53(66.3)	396(18.2)	1.00	1.00	-
	25-29.9	21(26.3)	468(21.5)	0.34(0.20-0.57)	0.40(0.21-0.74)	0.004
	>30	6(7.5)	216(9.9)	0.21(0.09-0.49)	0.16(0.06-0.41)	<0.001
	Faltan	0(0.0)	1097(50.4)	0.27(0.24-0.29)	0.47(0.23-0.96)	0.039
CDR	1	13(16.3)	288(13.2)	1.00	1.00	-
	2	33(41.3)	1062(48.8)	0.69(0.36-1.33)	0.59(0.22-1.16)	0.304
	3	34(42.5)	828(38.0)	0.91(0.47-1.75)	0.40(0.07-2.14)	0,282
MMSE	21-30	4(5.0)	234(10.7)	1.00	1.00	-
	0-20	76(95.0)	1944(89.2)	2.30(0.83-6.34)	2.53(0.85-7.51)	0.096
BDS	0-9	1(1.3)	78(3.6)	1.00	1.00	-
	9-16	42(52.5)	900(41.3)	3.36(0.46-24.77)	2.06(0.23-18.58)	0.520
	17-28	37(46.3)	1200(55.1)	2.21 (0.30-16.33)	1.93(0.15-24.75)	0.615

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

FRAT	1	51(63.8)	810(37.2)	1.00	1.00	-
	2-3	29(36.3)	270(12.4)	1.71 (1.06-2.75)	2.26(1.34-3.85	0.003
	Falta	0(0.0)	1098(50.4)	0.43(0.40-0.45)	d	d
Institucionalización	Si	79(98.7)	2026 (93.0)	1.00	1.00	-
	No	1 (1.3)	152 (7.0)	0.18 (0.02-1.28)	0.47 (0.06-3.53)	0.464
CCS	0-1	30 (37.5)	753 (34.8)	1.00	1.00	-
	2	23 (28.8)	672 (30.9)	0.86 (0.49-1.49)	0.96 (9.00-1.02)	0.145
	>3	27 (33.8)	753 (34.6)	0.90 (0.53-1.53)	0.52 (0.28-0.96)	0.026
Fumador	No	74 (92.5)	2163 (99.3)	1.00	1.00	-
	Si	6 (7.5)	15 (0.7)	11.67 (4.41-30.99)	33.90 (10.50-109.60)	<0.001
ISRS	No	70 (87.5)	2003 (92.0)	1.00	1.00	-
	Si	10 (12.5)	175 (8.0)	1.64 (0.83-3.23)	1.45 (0.73-2.90)	0.288
Otras fracturas	No	59(73.8)	2016(92.6)	1.00	1.00	-
	si	21(26.3)	162(7.4)	4.43(2.63-7.47)	2.82 (1.60-4.99)	<0.001

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Tabla 7: Relación entre los factores de riesgo en pacientes con EA y la presencia de fracturas de cadera; comparación entre casos y controles.

Abreviaturas: IC, intervalo de confianza; FRAT, escala de evaluación de riesgo de fractura, IMC, índice de masa corporal, EA, Enfermedad de Alzheimer, Y, edad, BDS, escala "Blessed Dementia Score"; MMSE, "Mini-examen del Estado Mental", CDR, "Clinical Dementia Rating Scale"; CCS, escala de comorbilidad de Charlson; ISRS, inhibidores selectivos de la recaptación de serotonina. Los resultados han sido ajustados a todos los factores de confusión.

a- Los porcentajes no suman el 100% debido al redondeo.

b- Ajustado a otros parámetros categóricos en esta tabla.

c- Medido como el peso en kilogramos dividido por el cuadrado de la altura en metros.

d- No calculado por falta de datos.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Caracteristicas		Tratados con IAChEs (n=1771)	No tratados con IAChEs (n=487)	P
Edad, a		82.15 (SD4.49)	83.49(SD4.16)	0.001a
Sexo	Mujeres	72(95%CI, 70-74)	69(CI95%, 65-73)	0.208b
	Hombres	25(22-28)	27(22-33)	0.208b
IMC c		26.0(SD3.5)	24.56(SD4.30)	0.001a
CDR		2.16(SD0.67)	2.59(SD0.58)	0.001a
BDS		15.72(SD3.48)	17.31(SD3.37)	0.001a
MMSE		15.66(SD3.98)	14.52(SD2.31)	0.001a
FRAT		1.33(SD0.55)	1.24(SD5.68)	0.025a
Fumadores		1 (0-1)	1 (1-2)	0.415b
SSRI		19 (17-21)	26 (20-32)	0.030b
CCS		2.52 ± 1.57	2.38 ± 1.35	0.776a
Otras fracturas		15.97(13.34-18.61)	16.25(11.87-20.62)	0.300b

Tabla 8: Las características de los pacientes con Alzheimer en relación al tratamiento con IAChEs. Abreviaturas: IC, intervalo de confianza; FRAT, escala de evaluación el riesgo de fractura, IMC, índice de masa corporal, EA, la enfermedad de Alzheimer, y, edad, BDS, escala "Blessed Dementia"; MMSE, Mini Examen del Estado Mental, CDR, escala "Clinical Dementia Rating"; CCS, escala de comorbilidad de Charlson; ISRS, inhibidores selectivos de la recaptación de serotonina.

a- Calculado con la t de Student para muestras independientes.

b- Calculado con la prueba de Chi cuadrado.

c- Medido como el peso en kilogramos dividido por el cuadrado de la altura en metros.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Características	Casos (n=80) No. (%)a	Controles (n=2178) No. (%)a	Odds Ratio (95%CI)	Odds Ratio ajustado (95%CI) b	Valor-P
Tratados con IAChEs	39(48.8)	450(20.7)	1.00	1.00	-
No trarados con IAChEs	41(52.2)	1728(79.3)	0.30(0.19-0.48)	0.42(0.24-0.72)	0.002
Rivastigmina	16 (20.0)	900(41.3)	0.20(0.11-0.37)	0.22(0.10-0.45)	<0.001
Donezepilo	15(18.8)	630(28.9)	0.29(0.16-0.54)	0.39(0.19-0.76)	<0.002
Galantamina	10 (12.5)	182(8.4)	0.68(0.33-1.39)	1.67(0.63-4.45)	0.305

Tabla 9: Comparación entre el uso de IAChEs y riesgo de fractura.

Abreviaturas: IC, intervalo de confianza; IAChEs, inhibidores de acetilcolinesterasa.

a- Los porcentajes pueden no sumar el 100% debido al redondeo.

b- Ajustado para los parámetros categóricos en la tabla 10.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

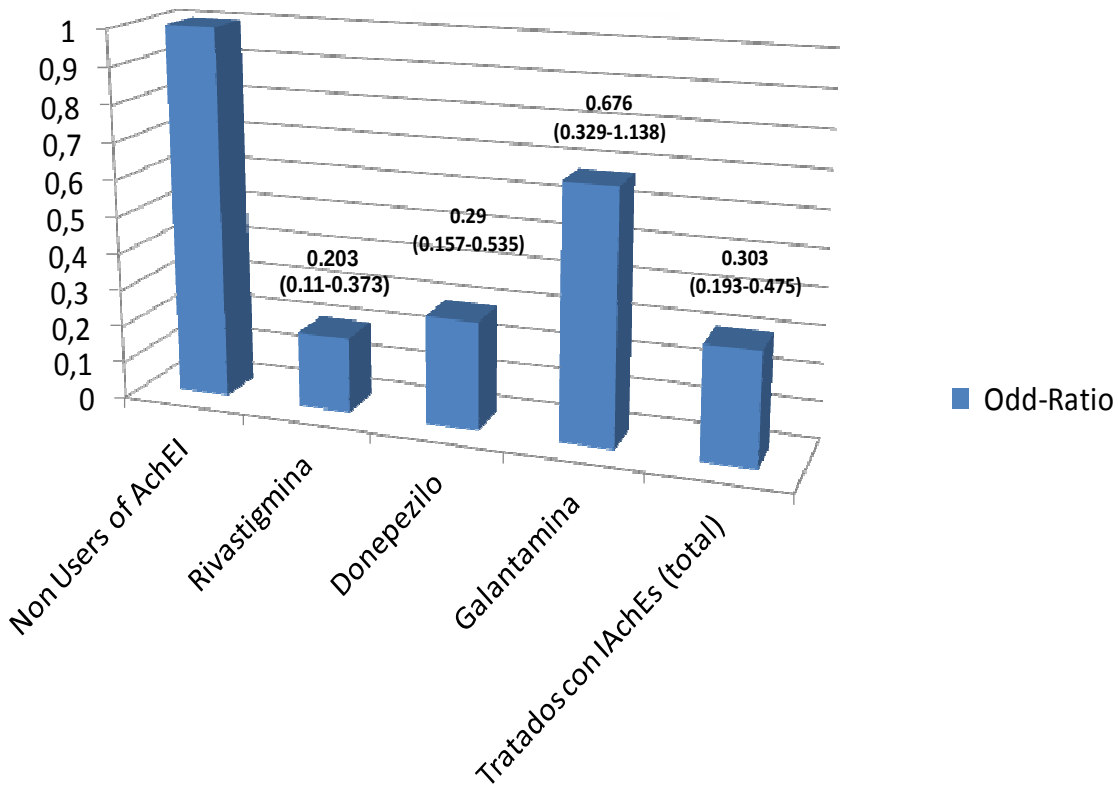


Figura 4: Relación entre el uso de IAChEs y el riesgo de fractura de cadera (en Odd Ratio).

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Tratamiento / caracteristicas		Casos (n=080) [a]	Controles (n=2178) [a]	OR (95% CI)	OR (95% CI) [b] ajustado	Valor-*p*
Rivastigmina	Duration, years 0.5-2	7	270	1.00	1.00	-
	>2	6	630	0.37 (0.12-1.10)	.25 (0.06-1.04)	0.057
	Dose mg/day 3-9	7	270	1.00	1.00	-
	9.5-12	6	630	0.37 (0.12-1.10)	0.16 (0.03-0.79)	0.025
Donepezilo	Duration, years 0.5-2	6	234	1.00	1.00	-
	>2	7	396	0.83 (0.26-2.64)	0.90 (0.19-4.20)	0.879
	Dose mg/day 5	3	18	1.00	1.00	-
	10	12	612	0.12 (0.03-0.45)	0.02 (0.00-0.17)	c
Galantamina	Duration, years 0.5-2	3	72	1.00	1.00	-
	>2	3	108	.67 (0.13-3.40)	c	c
	Dose mg/day 6-16	4	74	1.00	1.00	-
	17-24	5	108	0.83 (0.22-3.21)	0.51 (0.09-2.89)	c

Tabla 10: Relación entre la duración del tratamiento con IAChEs, la dosis utilizada y riesgo de fractura.

Abreviaturas: IAChEs, inhibidores de la acetilcolinesterasa; OR, Odd Ratio, IC, intervalo de confianza.

a-Número de casos o controles.

b- Valores justados a los parámetros categóricos en la tabla 7.

c- No calculado por falta de datos.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Discusión

Los pacientes con EA son más susceptibles de sufrir caídas accidentales que la población general, esto se debe a los problemas cognitivos y alteraciones sensitivas asociadas a la EA (ej. trastornos del comportamiento, de la marcha, del equilibrio, alteraciones visuales, y efectos secundarios asociados a los medicamentos). Un estudio de cohorte realizado en los años noventa reveló que los pacientes con EA presentan una incidencia mucho mayor de fracturas de cadera que la población general, con un riesgo relativo global de 2,7 (IC del 95 % 1.8 a 4.2). Un estudio similar realizado en el mismo período reveló que la tasa de fracturas de cadera en mujeres con EA y demencia vascular era dos veces mayor que la población general. Un estudio más reciente realizado entre 1988 y 2007, reveló que los pacientes con EA tenían un "hazard ratio" del 2,8. Aun así, en este último estudio, los autores no diferenciaron entre los pacientes tratados con IAChEs y los no tratados con estos medicamentos.

Los IAChEs se comenzaron a utilizar en el tratamiento de la EA a mediados de los años noventa, y posteriormente pasaron a formar parte del tratamiento estándar de estos pacientes. Desgraciadamente, estos medicamentos sólo causan una leve mejoría de los síntomas asociados a la EA y no alteran el curso natural de la enfermedad. En nuestro estudio observamos que los pacientes tratados con IAChEs mostraban en general unos índices de estado mental (MMES, CDR y BDS) levemente mejores que los pacientes no tratados con IAChEs. Esta diferencia entre los dos grupos podría deberse al efecto farmacológico de los IAChEs sobre el estado cognitivo de los pacientes. También se puede apreciar en nuestro estudio un mayor riesgo de caída en los pacientes tratados con IAChEs que en los no tratados. Esto se podría explicar por una mayor prevalencia de bradicardias y cuadros sincopales asociados al uso de estos fármacos. Por otra parte,

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

algunos estudios indican que los IAChEs pueden disminuir el riesgo de caída al mejorar el estado cognitivo de los pacientes. En nuestro estudio hemos observado que los pacientes tratados con IAChEs presentaron un mayor riesgo de caída según la escala (FRAT) en comparación con los pacientes no tratados. Esto se podría explicar por el predominio del efecto bradicárdico sobre los beneficios cognitivos de los IAChEs.

Otro hallazgo interesante observado en nuestro estudio fue el IMC significativamente mayor en los pacientes tratados con IAChEs que en los no tratados. El sistema nervioso autónomo desempeña un papel primordial en la regulación del IMC, ya que la actividad simpática limita el aumento de peso corporal, y su inhibición se asocia a un aumento del IMC. Por otro lado, algunos estudios realizados en animales han demostrado que el incremento de la actividad parasimpática se asocia a un aumento de la producción grasa en los modelos utilizados. Los IAChEs ejercen un efecto estimulador sobre el sistema nerviosos colinérgico en el cuerpo, por lo tanto, es posible que las diferencias en el IMC entre los pacientes tratados con IAChEs y los no tratados se deban a un aumento de la actividad colinérgica causada por estos fármacos.

El resultado más relevante de nuestro estudio fue la confirmación de la hipótesis de trabajo: los pacientes con EA tratados con IAChEs tuvieron un riesgo de desarrollar fracturas de cadera significativamente menor que los no tratados. Estos resultados se ajustaron a factores cognitivos relacionados con el curso de la enfermedad, así como con otros factores asociados a las fracturas de cadera (Tabla 7). En consecuencia, nuestros hallazgos probablemente se deben a un efecto de los IAChEs sobre la actividad colinérgica. La reducción del riesgo de fractura en los pacientes tratados con IAChEs dependió de la dosis suministrada (Tabla 10). Esto refuerza la posibilidad de que se deba a un efecto farmacológico. Por otra parte, la asociación que observamos entre el

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

uso de IAChEs y el aumento del IMC también puede confirmar el efecto de estos fármacos sobre la actividad metabólica del sistema colinérgico. Con todo, el diseño retrospectivo de nuestro estudio no nos permite determinar una relación de causa efecto entre el uso de IAChEs, la actividad del sistema colinérgico y el metabolismo óseo, por lo que tendrían que llevarse a cabo ensayos clínicos controlados para confirmar estos resultados potencialmente importantes.

Otro resultado significativo de este estudio es la diferencia en el riesgo de fractura de cadera entre los pacientes con EA en función del tipo de IAChE utilizado. El riesgo de fractura de cadera en pacientes tratados con rivastigmina y donepezilo fue significativamente menor que en los pacientes no tratados con IAChEs. Sin embargo, el tratamiento con galantamina no tuvo ningún efecto beneficioso sobre el riesgo de fractura de cadera. La galantamina tiene un efecto sobre el estado cognitivo de los pacientes con EA similar al donepezilo y la rivastigmina. Luego es improbable que las diferencias entre los distintos tratamientos se deban a un efecto sobre el estado cognitivo de los pacientes. Los IAChEs utilizados en este estudio tienen diferentes mecanismos de acción sobre los distintos tipos de IAChEs, esto podría explicar las diferencias en el riesgo de fractura de cadera apreciadas entre los distintos grupos de pacientes. Por ejemplo, la galantamina es capaz de unirse a todos los nRACh independientemente de su tejido o célula de origen; pero no se puede unir a los mRAChs. Por consiguiente, la acción de la galantamina se limita a un aumento selectivo de la estimulación a nivel nicotínico. Esto contrasta con la estimulación nicotinica y muscarinica inducida por el donepezilo y la rivastigmina. La sobreactividad nicotínica se asocia a una reducción de la capacidad de remodelación ósea, así pues, la acción selectiva de la galantamina sobre los nRACh puede explicar las diferencias significativas entre los pacientes tratados con donepezilo o rivastigmina y los tratados

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

con galantamina. A pesar de esto, la estimulación de los mRACh parece favorecer el remodelado óseo; por esta razón creemos que el efecto de la rivastigmina y el donepezilo sobre los receptores mRACh podría explicar la reducción significativa del riesgo de fractura de cadera en los pacientes tratados con estos fármacos.

Los fármacos más utilizados actualmente en el tratamiento de la osteoporosis son la hormona paratiroidea y los bisfosfonatos. Estos últimos han demostrado su eficacia en la protección contra las fracturas de cadera en comparación con el placebo con un riesgo relativo (RR) del 0,66 (IC , 0,51-0,85 95 %) [ácido zoledrónico 0,58 (IC , 0,41 a 0,82 95 %) , alendronato 0,95 (IC , 0,54-1,68 95 %) , y risedronato 0,73 (IC , 0,37-1,44 95 %)]. Además, el uso de la hormona paratiroidea ha demostrado reducir la incidencia de fracturas de cadera de forma muy significativa con un RR en comparación con el placebo de 0,46 (IC del 95 % 0,25 - 0,86).

En nuestro estudio el OR de la rivastigmina y el donepezilo en comparación con los pacientes no tratados con IAChE fue de 0,22 (IC del 95%: 0,10 a 0,45) y 0,39 (IC, 0,19 hasta 0,76 95 %), respectivamente. Aunque estos resultados no se pueden comparar directamente con el RR observado en los ensayos clínicos mencionados anteriormente, sí parecen indicar que los IAChEs estimuladores de mRAChs podrían utilizarse en la prevención de fracturas de cadera en pacientes ancianos, especialmente en aquellos diagnosticados de EA. Sin embargo, harán falta nuevos ensayos clínicos y estudios experimentales para confirmar esta hipótesis.

Los resultados obtenidos en nuestro estudio indican que el uso de los IAChEs podría ser beneficiosos en la prevención de fracturas de cadera en pacientes osteoporóticos. Aunque estos medicamentos tienen una serie de efectos secundarios que limitan su uso

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

en un porcentaje significativo de la población (ej. Nauseas, vómitos, y vértigo), esto será un factor a tener en cuenta a la hora de recetar esto fármacos.

En este estudio se presenta la primera evidencia clínica que sugiere que la estimulación de los receptores mRACh puede reducir el riesgo de fractura de cadera en pacientes de edad avanzada. Estos receptores están presentes en el sistema nervioso central, así como en células del tejido óseo; por lo tanto, no está claro si el efecto de IAChEs sobre el hueso es sistémico o local. Varios estudios recientes han descrito la presencia de fibras de nerviosas cholinérgicas en el hueso que podrían afectar directamente a los ARChs en el hueso. Por otra parte, los IAChEs podrían ejercer un efecto sistémico sobre el hueso a través de los sistemas autonómico o humoral. Debido a esto, será necesario desarrollar nuevos estudios que determinen el mecanismo exacto por el cual estos fármacos actúan en el hueso.

Limitaciones

Este es un estudio observacional retrospectivo y será necesario el desarrollo de futuros estudios prospectivos para confirmar nuestros resultados. Por otra parte, la alta mortalidad de los pacientes con EA, y el número relativamente limitado de fracturas de cadera registradas en este estudio son factores limitantes que hay que tener en consideración. Otro posible factor de confusión es la menor probabilidad de recibir tratamiento con IAChEs en las fases más avanzadas de la enfermedad, probablemente debido al estado terminal de estos pacientes. Sin embargo, el "Odd ratio" ajustado al grado de EA sigue confirmando el efecto protector de los IAChEs en estos pacientes. Por otra parte, este estudio analiza el efecto de los IAChRs sobre el hueso de forma indirecta mediante la evaluación de la incidencia de fracturas de cadera.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Por estas razones, será necesario realizar futuros estudios prospectivos multicéntricos que confirmen nuestros hallazgos. Estos estudios deberán cuantificar la densidad ósea de los pacientes tratados con IAChEs.

Conclusiones

• Este estudio representa la primera evidencia clínica que demuestra que la administración de los inhibidores de acetilcolinesterasa donepezilo y rivastigmina se asocia a una reducción del riesgo de padecer fractura de cadera en pacientes con enfermedad de Alzheimer.

• La administración del inhibidor de acetilcolinesterasa galantamina, que además estimula alostéricamente receptores nicotínicos, no disminuye el riesgo de padecer fractura de cadera.

• La disminución en el riesgo de padecer fractura de cadera secundario a la administración de rivastigmina y donepezilo podría explicarse por la estimulación de receptores M3 muscarínicos en osteoblastos.

• Estos hallazgos pueden ser potencialmente importantes ya que muchos pacientes ancianos con Alzheimer o con otras demencias podrían beneficiarse de los efectos positivos que los inhibidores de acetilcolinesterasa parecen tener sobre el riesgo de fractura.

• Es necesario realizar estudios observacionales y ensayos clínicos más extensos que confirmen definitivamente estos hallazgos.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Publicaciones y
Divulgación Científica
UNIVERSIDAD
DE MÁLAGA

Addendum

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

INFORME DE VALORACIÓN DE TESIS
THESIS EXAMINER'S REPORT

Título de la tesis / Thesis title: … Cholinergic regulation of bone turnover: Hip fracture risk in Alzheimer patients treated with Acetyl-cholinesterase inhibitors.

Doctorando / PhD candidate: …Iskandar Tamimi Mariño……..

Informe redactado por / Examiner's name: …Dr Mahmoud Ababneh……..

RESUMEN VALORACIÓN / ASSESSMENT SUMMARY

	Excelente Excellent	Buena Good	Suficiente Fair	Deficiente Poor
Originalidad / Originality	X			
Metodología / Methodology		X		
Relevancia de los resultados/ Relevance of results	X			
Discusión y Conclusiones Discussion and conclusions	X			
Bibliografía / References	X			
Presentación / Presentation	X			
Publicaciones / Publications	X			

RECOMENDACIÓN / RECOMMENDATION

[X] Acceptar /**Accept**
[X] Cambios Menores / **Minor Corrections**
[_] Cambios Mayores / **Major Corrections**
[_] Rechazar / **Revise and Resubmit**

Explicación de las recomendaciones. La calificación otorgada dependerá del tipo de comentarios realizados por el evaluador y del tiempo que considera el experto que el doctorando necesita para llevar a cabo los cambios sugeridos: menos de 1 mes para **cambios menores**, menos de 3 meses para **cambios mayores**. En el caso en que el evaluador considere que el doctorando necesita más de 3 meses para incorporar los cambios sugeridos, la evaluación debe ser negativa (**Rechazar**). Una recomendación de **aceptar** indica que el revisor considera que la tesis presentada no necesita cambios.

Explanation of Recommendations. The examiner should recommend **Accept** if no changes are required to the manuscript. Other recommendations suggest that changes are required, and they depend on the kind of changes and the estimated time required to implement them. Minor corrections must be completed within one month, and major modifications may require up to three months to implement. If the examiner considers that the suggested changes are substantial enough to require more than 3 months, the recommendation shall be **Revise and re-submit**.

Por favor, indique en la siguiente página una justificación de la valoración otorgada siguiendo el esquema propuesto, e incluya una lista detallada con los cambios que se sugiere incorporar en la tesis.

Please use the following pages to justify your recommendation and to list your suggested changes.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

INFORME JUSTIFICATIVO DE LA VALORACION OTORGADA / DETAILED Report:
Use tantas páginas como necesite / use as many pages as needed

1. PUNTOS FUERTES DE LA TESIS / STRENGTHS

-I believe this work is very original and may have significant clinical implications on the mid-long term.

-It is supported by two strong publications.

-The results are statistically significant.

-The thesis is well structured and the text is clear and easy to read.

- These studies were developed in the context of multicentre cooperation.

-The review of the literature was meticulous and extensive.

2. PUNTOS DÉBILES / WEAKNESSES

- The introduction is too extensive. The less relevant topics could be omitted.
- One limitation of this study was the reduced number of subjects included, although samples size calculation revealed that sufficient statistical power was achieved.
- In this study AD patients in an advanced stage of the disease were less likely to receive AChEIs, probably due to the terminal status of their condition. This issue could be a limitation because it could drive an artificial risk reduction for hip fracture with AChEIs. However, risk estimates adjusted to the grade of AD and comorbidity were still confirmatory of the protective effect of AChEIs on hip fracture.
- Another limitation of this study was that this retrospective design does not allow determining a cause-effect relationship of AChEIs and hip fracture.
- An additional limitation was that the effect of AChEIs on bone was only examined in an indirect way by assessing risks of hip fractures, which doesn't provide information on the physiological conditions of bone.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Use tantas páginas como necesite / use as many pages as needed

3. CAMBIOS SUGERIDOS / SUGGESTED CHANGES (1/2)

3.1 Cambios obligatorios / Mandatory changes

-The less relevant topics could be omitted from the introduction.

-Add percentages in (results line 3)

-Transfer "Hip fractures appeared to be correlated with the age of the patients (Table 9) and were more common in females than in males (3:1 in the general population and 4:1 in the AD group, Table 9)." To 2. Hip fracture incidence in the general population and Alzheimer patients

- Add " in 2008 (n=381)" to table 10.

- Add "n=80" to table 11.

- Change : "Assessment of risk factor" to "Assessment of risk factors in control and study groups"

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Use tantas páginas como necesite / use as many pages as needed

3. CAMBIOS SUGERIDOS / SUGGESTED CHANGES (2/2)

3.2 Otros cambios propuestos / Optional changes and suggestions for improvement

- Future large-scale prospective cohort studies will have to be performed in order to confirm these findings by increasing the sample size, determining cause-effect relationships, and eliminating the possibility of bias by competing risk of death.
- We recommend Mr Tamimi Mariño to carry on with this line of research

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

INFORME DE VALORACIÓN DE TESIS
THESIS EXAMINER'S REPORT

Título de la tesis / Thesis title: Cholinergic regulation of bone turnover: Hip fracture risk in Alzheimer patients treated with Acetyl-cholinesterase inhibitors

Doctorando / PhD candidate: Iskandar Ahmad Tamimi Mariño .....

Informe redactado por / Examiner's name: Belinda Nicolau.....

RESUMEN VALORACIÓN / ASSESSMENT SUMMARY

	Excelente Excellent	Buena Good	Suficiente Fair	Deficiente Poor
Originalidad / Originality	x			
Metodología / Methodology			x	
Relevancia de los resultados/ Relevance of results	x			
Discusión y Conclusiones Discussion and conclusions		x		
Bibliografía / References		x		
Presentación / Presentation			x	
Publicaciones / Publications				

RECOMENDACIÓN / RECOMMENDATION

[_] Acceptar /Accept
[X] Cambios Menores / Minor Corrections
[_] Cambios Mayores / Major Corrections
[_] Rechazar / Revise and Resubmit

Explicación de las recomendaciones. La calificación otorgada dependerá del tipo de comentarios realizados por el evaluador y del tiempo que considera el experto que el doctorando necesita para llevar a cabo los cambios sugeridos: menos de 1 mes para **cambios menores**, menos de 3 meses para **cambios mayores**. En el caso en que el evaluador considere que el doctorando necesita más de 3 meses para incorporar los cambios sugeridos, la evaluación debe ser negativa (**Rechazar**). Una recomendación de **aceptar** indica que el revisor considera que la tesis presentada no necesita cambios.

Explanation of Recommendations. The examiner should recommend **Accept** if no changes are required to the manuscript. Other recommendations suggest that changes are required, and they depend on the kind of changes and the estimated time required to implement them. Minor corrections must be completed within one month, and major modifications may require up to three months to implement. If the examiner considers that the suggested changes are substantial enough to require more than 3 months, the recommendation shall be **Revise and re-submit**.

Por favor, indique en la siguiente página una justificación de la valoración otorgada siguiendo el esquema propuesto, e incluya una lista detallada con los cambios que se sugiere incorporar en la tesis.

Please use the following pages to justify your recommendation and to list your suggested changes.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

INFORME JUSTIFICATIVO DE LA VALORACION OTORGADA / DETAILED Report:

Use tantas páginas como necesite / use as many pages as needed

1. PUNTOS FUERTES DE LA TESIS / STRENGTHS

This thesis reports the results of a project aiming at investigating whether Acetylcholinesterase inhibitors, which are drugs widely used in the treatment of Alzheimer disease, affect the bone turnover which in turn would decrease the risk of bone fracture. Dr. Iskandar Tamimi is to be congratulated on carrying out such an important and original study. This project has several strengths:

1. Evidence of originality and creativity: the research question of this PhD thesis is original and has major implications for prevention of conditions that affect millions of people around the word.

2. Literature review is comprehensive demonstrating that the author is very familiar with previous studies published on the topic.

3. Usefulness of the results to other workers in the field; value as a contribution to knowledge: This is a new and important area of research and Dr. Tamimi's work adds new information on the effect of use of Acetylcholinesterase inhibitors drugs and risk of fracture. The results from this thesis are definitely relevant and could have a major effect on people's life. This is especially true given the aging of the populations and its associated increase in risk of bone fractures. The identification of a drug that can contribute to bone turnover is a major finding.

4. Grasp of subject and power of criticism and awareness of previous work: Dr. Tamimi demonstrated an excellent understanding of the subject. His arguments are clear and he integrated ideas from different sources making it easier for those outside the field to understand the subject and follow his argumentation.

2. PUNTOS DÉBILES / WEAKNESSES

I provided below a few comments which I would encourage Dr. Tamimi to take into account if he has the opportunity to revisit his work.

1. Quality of presentation: the thesis has several typos and inconsistencies which need to be revised. I provide a list of some of them in the attached document.

2. Topics in the discussion: there are several aspects with regards to the methodology of the study which should be discussed such as:

- Quality of data source: patient files in hospitals are notoriously known to be incomplete and may contain errors. How has this issue been addressed and what could be the effect of it on the results?
- Compliance with medication: it appears that this study assumes that the patients totally complied with the prescription of the Acetylcholinesterase inhibitors. However, it appears that compliance has not been evaluated in the present study. Is there any information on the validity of the assumption that patients took the prescribed medication in the prescribed dose and frequency? How would non-compliancy affect the results?
- Treatment of missing information: there should be a rationale for the inclusion of the missing values in the regression analyses.

3. Publications: you could mention in more details the studies that you have already carried out and published to underline the coherence of your latest results.

Following is a series of questions for the sections "Materials and methods", "Results" and "Discussion"

Materials and methods

1. Demographic information on the study population
   - Were all medical records of hip fractures treated between January 2004 and December 2008 recorded in the computerized medical records? If not, what was the approximate proportion?
   - Why did potential cases need to have been recorded in the database for at least 2 years?
   - The inclusion criterion for age was between 75 and 95 years old. This, however, is not reflected in tables 6-10 but in table 12. There is a need for clarification of the inclusion criteria for age.
2. Exposure assessment
   - It is not clear whether the computerized medical records contained information from patient's files and standardized questionnaires (p. 3 in Materials and methods) or whether additional information ("patient's files, computerized records and standardized questionnaires") was used for the patient's characteristics.
   - How was a possible change in medication prescription handled?
   - Could additional medication be potential confounders? Was information available about other medication?
3. Statistical analysis
   - The author stated that conditional logistic regression was used for the data analysis. I believe he meant unconditional logistic regression since there is no indication of matching of cases and controls. Conditional regression is used for match data. This should be clarified in the statistical method of the thesis. Also, the thesis would benefit from a detailed description of the statistical analysis. For example, how the variables were selected for the final model. Similarly, there seems to be a mistake in the list of confounders: AChEI treatment is the exposure not a confounder. Is that correct? Finally, sample size power calculation should be included in the methodology section of the thesis

Results

1. Demographic information
   - Table 8, title: were these fractures treated anywhere in the Carlos Haya area or specifically at the Carlos Haya hospital?
2. Assessment of risk factors in the control and study groups
   - Which parameters were used for adjustment in table 12? All of them? Please list them individually.
   - I cannot see that there was not difference between BMI groups: compared to the baseline group (<25 BMI) the other groups show significantly decreased probability of hip fracture.
   - Please check the p-values in table 13 as they contradict what you state in the text regarding significant differences between users and non-users of AChEI for specific characteristics.
   - It is not clear how missing values in confounders were treated in the regression analyses.

Discussion

Although Dr. Tamimi states some of the study's limitations, the text lacks depth. More explicit connections of the potential effects of these limitations on the study's findings should be made. For example:

- Small case numbers
- Reliability of the information provided in the patients' files
- Validity of the information related to medication: compliance, change in medication
- Interaction of AChEI and BMI: users of AChEI had a higher BMI which could be a consequence of the medication. If higher BMI is protective for fracture risk, it could either be because it lies on the causal pathway or because there is an interaction between BMI and AChEI. A short discussion of this possibility would be interesting.
- Dr. Tamimi's previous publications highlight different aspects of the relationship between AChEI and hip fracture (healing of hip fracture, for example). This could be discussed too and add to the validity of the results.
- Some epidemiological aspects of the study could be included in the discussion:
  - how do the study results relate to the general population?

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

- How could the results be used and applied by general physicians?
- What are Dr. Tamimi's recommendations for the treatment of the aging population based on this study results? (implications for public health)
- Are there any suggestions for knowledge translation of his study?

- Dr. Tamimi mentions the need for large scale prospective cohort studies and clinical studies of biomarkers. What does he plan to study next?

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Use tantas páginas como necesite / use as many pages as needed

3. CAMBIOS SUGERIDOS / SUGGESTED CHANGES

3.2 Otros cambios propuestos / Optional changes and suggestions for improvement

Following are a series of questions for the sections "Materials and methods" and "Results". The thesis would gain in relevance and validity if these could be addressed.

Materials and methods

Statistical analysis

- There seems to be a mistake in the list of confounders: AChEI treatment is the exposure, isn't it, not a confounder.
- The general rule for the number of parameters in a regression analysis (variables included while counting the number of categories of each one minus 1 for each variable) is to have at least 10 more cases and 10 more controls than parameters. You may have too many variables in the regression analysis based on this rule. This reduces your statistical power and makes the results less reliable. You might want to reconsider the inclusion of certain confounders such as CDR, BDS and the MMSE.

Results

1. Demographic information
   - A flowchart containing the numbers from total population to selected controls and from total hip fractures treated to included cases would be helpful to fully capture the study population.
   - It would be more adequate to indicate the number of the general population over 70 years instead that of the general population over 75 years because all subsequent analyses include the 70-74 years group. In table 6 you might want to include only the population aged 70 and above in order to make the table comparable to tables 7 and up.
   - To make the numbers comparable you should only give the number of hip fractures for the population aged 70 and above (not the total of 381 hip fractures).
   - Table 7 and 8: you should either include a line for Alzheimer patients aged under 70 in table 7 or exclude the line with patients under age 70 in table 8 to make them more comparable.
   - Table 8, title: were these fractures treated anywhere in the Carlos Haya area or specifically at the Carlos Haya hospital?
2. Assessment of risk factors in the control and study groups
   - Both, the control and the case are study groups which mean that the title of this section should rather say "in cases and controls".
   - In the first two paragraphs the inclusion criteria for age is 75 and above. Is this correct?
   - This section could be a little shorter and easier to read if it contained less numbers (which can be easily looked up in table 12). No need to put in detail, for example, "(21 between 75-79 years, 28 between 80-84, and 31 above 85 years) and 2178 controls (594 between 75-79 years, 846 between 80-84 and 738 above 85 years)". You could limit the description to situations where the proportions are different (not necessarily significantly)
   - Table 12: please check why there is an OR for BMI missing when no case had missing BMI.
   - Which parameters were used for adjustment in table 12? All of them? Please list them individually.
   - I cannot see that there was not difference between BMI groups: compared to the baseline group (<25 BMI) the other groups show significantly decreased probability of hip fracture.
   - This table would be more easily readable if the rows were not coloured, especially since the pattern doesn't seem to be simply a light and dark blue but there are intermediary shades which

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

are difficult to interpret (to which variable do they belong?)

- It would be easier to follow the text if table 12 followed the text about table 12 before starting the text about table 13.
- Please check the p-values in table 13 as they contradict what you state in the text regarding significant differences between users and non-users of AChEI for specific characteristics.
- Please check the numbers of cases and controls as well as the total in the column titles in table 15 as they don't add up and are sometimes very similar.
- Table 14: please verify the numbers of cases and controls in this table. They don't add up to the column totals nor to the total of AChEI users.
- Tables 14 and 15: Were the controlled variables those from table 12 or 10?
- It is not clear however how missing values in confounders were treated in these regression analyses.
- Table 15: how is it possible that the RR for Rivastigmine with only 6 cases could be calculated but not that of Donepezil with 12 cases?

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

Reply to reviewer I:

The following changes have been made:

- The less relevant topics have been omitted from the introduction.

- Percentages have been added to the tables is the results section.

- The date 2008 and the value of "n" have been added to table 10.

- The value of "n" has been added to table 9.

-"Assessment of risk factor" has been changed to "Assessment of risk factors in control and study groups" in the results section.

Reply to reviewer II:

The following changes have been made:

-Regarding the quality of data source: All the hip fractures that were treated between January 2004 and December 2008 were reviewed; all these cases were registered in the computerized database of the orthopedic surgery department. All the patients included in the study group were subject to an additional standardized questionnaire in order to complement any missing information in the medical records.

- Data regarding the socioeconomic status of the patients was not registered because the Spanish public healthcare system provides universal free healthcare, and all of our patients have equal access to the drugs assessed in this study.

- Compliance, which could act as a possible confounder, was not measured during the collection of data; however, we believe that it could be high, as most of the patients relied on relatives and caregivers for the administration of the medications which were generally very involved in the care of the patients.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

-The Charlson comorbidity score, uptake of selective serotonin reuptake inhibitors, and smoking were also included as possible confounders.

- Potential cases had to be recorded in the neurology department database for at least 2 years, in order to ensure an adequate record of the treatment received for Alzheimer disease.

-It has been clarified in tables (9, 10 and 11) that the age group between 70-74 years is also illustrated but it is not part of the study group (it was used to calculate the hip fracture incidence in AD patients and in the general population).

- Other medications such as proton pump inhibitors that alter bone metabolism and act as potential confounders were not analyzed in this study, these could influence to a certain point our results; however, there is no known relation between the use of AChEIs and the intake of proton pump inhibitors. Therefore the use of proton pump inhibitors should be similar in AD patients treated with AChEIs and those not treated. Other medications such as ISRS and bisphosphonates were taken in consideration in the material en methods section.

-The statistical analysis performed was an unconditional logistic regression. The use of AChEIs is not a confounder in table 12

-In table 8: All the fractures were treated in the Carlos Haya Hospital in 2008.

-In table 12: all the parameters were used for adjustment: Fracture Risk Assessment Tool, Body Mass Index, Alzheimer's disease, Blessed Dementia Scale, Mini-Mental State Exam, Clinical Dementia Rating Scale, Charlson comorbidity score, selective serotonin reuptake inhibitor, age, gender, smoking and institutionalization.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

-The BMI was higher in patients treated with AChEIs, and this could be explained by an increase in the parasympathetic signal in these patients as a result of these drugs.

-The values of "p" have been checked in table 13.

-One of the limitations of this study was the reduced number of subjects that could be included, which lead to non significant statistical results regarding the effects of galantamine on the hip fracture risk and the relation between galantamine and donepezil dosage and the hip fracture risk; however, the main result observed in our study was that our working hypothesis was confirmed; AD users of AChEIs had significantly lower risk of developing hip fractures than nonusers, despite the small number of cases.

-Despite these generally bearable side effects, we believe that AD patients would benefit from the reduction in the hip fracture risk related to the use of AChEIs, and we encourage their prescription in this population group. However, their indication in healthy elderly groups or in osteoporotic patients will certainly need further evidence, but could become a real alternative option in the prevention of osteoporotic fractures in the future.

-Our research team is currently designing a large scale prospective cohort study that aims to analyze the hip fracture risk in AD patients in the United Kingdom. All the clinical information needed to complete this work will be acquired from the British health system computer database. A second line of research in collaboration with McGill University, University of Malaga and Carlos Haya Hospital is also in progress, it aims to study a possible syndrome of hypocholinergic activity which could include clinical conditions such as osteoporosis, AD, and several autonomic system

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA

dysfunctions. We believe that these studies will provide valuable information that could add further evidence to the results stated in this thesis

-The study population was included in the flowchart containing the numbers of the cases and controls.

-Regarding the assessment of risk factors in the results section we have changed: "in the control and study group" into "in cases and controls".

-The inclusion criteria is age >75 years.

-We have darkened the characters in table 12 and chose a single color for the rows in order to make it easier to read.

-The p-values have been corrected in the text in order to match the figures in table 13.

-The number of cases and controls in table 14 have been corrected.

-The controlled variables for tables 14 and 15 are those in table 12.

Publicaciones y Divulgación Científica
UNIVERSIDAD DE MÁLAGA