Cholinergic Regulation of Bone

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Dedication

This thesis is dedicated to my parents for their endless love and support throughout the course of this thesis. It is also dedicated to my wife, Hala, and kids, Nadia and Zaild, for all the wonderful things they bring to my life.

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Abstract

Recent research indicated that certain neurons (brain cells) can regulate bone metabolism and that their damage results in weaker bones. But little was known about the potential that increased activity by these neurons might have on bone. A family of drugs frequently used to treat Alzheimer disease, Acetylcholinesterase inhibitors, is known to stimulate a group of neurons that play a major role in maintaining memory. While these drugs have been widely used in the treatment of Alzheimer disease and other forms of dementia since the mid-1990s, their potential effects on bone biology had not been explored. This thesis presents a group of *in vivo* and clinical studies that were conducted to test the effects of these drugs on bone.

We *first* have determined that the use of centrally acting acetylcholinesterase inhibitors, such as donepezil, is associated with a beneficial effect on bone strength in mice. Donepezil promoted bone mass accrual by altering the activity of the autonomous nervous system two-arms: the cholinergic and the adrenergic systems.

We *next* have illustrated that the use of peripherally acting acetylcholinesterase inhibitors, such as neostigmine, is associated with an increase in bone quantity and quality. Neostigmine positive-effects on bone were not related to the activity of the central nervous system. However, it was induced by a local effect on the immune system, causing an alteration in the serum level of different immunocytokines (such as IL-17 and IL-23) that are known to regulate bone mass.

We *then* have demonstrated through two cohort retrospective studies that the use of acetylcholinesterase inhibitors in clinics might have beneficial effects on bone. We observed that Alzheimer disease patients who were treated with the acetylcholinesterase inhibitors had lower risk of osteoporotic fracture and faster healing, if the fracture occurred, compared to non-users. Taken together, we believe that the results presented in this thesis might be useful for Alzheimer Disease patients who suffer from bone diseases such as osteoporosis.

Résumé

Des études récentes ont démontré que les neurones peuvent réguler le métabolisme osseux et que leur déséquilibre peut résulter en une faiblesse osseuse. Peu d'information est disponible sur l'activité osseuse lorsque ces neurones sont stimulées. Certains médicaments connue pour contrôler la maladie Alzheimer, les inhibiteurs de l'acétylcholinestérase, stimulent ces neurones et ont un rôle dans le maintien de la mémoire. Ces médicaments ont été largement utilisés dans le traitement de l'Alzheimer et de d'autres types de démence depuis environ l'an 1995. Cependant, leurs effets potentiels sur la biologie de l'os n'ont pas été encore élucidés. Cette thèse comprend des études in vivo ainsi que des études cliniques qui ont permis de tester l'effet de cette classe de médicaments sur le métabolisme osseux.

Tout d'abord, nous avons prouvé que l'utilisation centrale des inhibiteurs de l'acétylcholinestérase tel le donépézil est associée à un effet bénéfique sur la force osseuse chez les rats. Le donépézil augmente la masse osseuse et diminue sa fragilité en changeant l'activité des systèmes nerveux cholinergique et adrénergique.

Ensuite, nous avons démontré que l'utilisation des inhibiteurs l'acétylcholinestérase périphérique telle la néostigmine est associée a une augmentation de la qualité osseuse ainsi que de sa densité. Notons que les effets positifs de la néostigmine sur les os n'ont pas été reliés à l'activité du système nerveux central, mais plutôt par un effet local sur le système immunitaire causant

une alteration des taux sériques des immunocytokines (tels les IL-17 et IL-23), connues pour réguler la densité osseuse.

Finalement, nous avons démontré, via deux études rétrospectives à double insu, que l'utilisation des inhibiteurs de l'acetylcholinesterase en clinique peut resulter en un effet bénéfique sur la densité osseuse. Nous avons observé que les patients souffrant de la maladie d'Alzheimer et qui ont été traité par des inhibiteurs de l'acetylcholinesterase ont un risque moins élevé de fracture ostéoporotique et avaient une guérison plus rapide si une fracture se présentait, en comparaison avec les non utilisateurs. Nous croyons que les résultats présents dans cette thèse donnent espoir dans le traitement des maladies osseuses tel l'ostéoporose.

Originality & Authors Contributions

This thesis is composed of five manuscripts. Materials presented in this thesis represent original contributions to knowledge. Authors' contributions to the work are described below for each of these manuscripts:

Chapter 2:

HE performed the literature search and drafted the manuscript. IT and MM revised and edited the manuscript content. FT supervised and designed the review manuscript.

This work is now published as part of the manuscript: **Eimar H**, Tamimi I, Murshed M, Tamimi F. Cholinergic regulation of bone. J Musculoskelet Neuronal Interact. 2013 Jun;13(2):124-32.

Chapter 4:

HE conducted the *in vivo* experiments (animal handling, injection, urine and blood collection, bioassays, locomotive activity and radiographic, mechanical and physical assessment of bone), and drafted the manuscript. SA performed the histomorphometric analyses. GM performed part of the *in vivo* studies, histomorphometry. FT and MM assisted HE, SA and GM in experimental procedures, supervised and designed the project.

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Chapter 5:

HE conducted the *in vivo* experiments (animal handling, injection, urine and blood collection, bioassays, locomotive activity and radiographic, mechanical and physical assessment of bone), and drafted the manuscript. SA performed the histomorphometric analyses and part of the cell culture experiments. GM performed part of the *in vivo* studies, histomorphometry and cell culture

experiments. FT and MM assisted HE, SA and GM in experimental procedures, supervised and designed the project. (*Authors contributed equally to the study).

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Chapter 6:

HE collected the GPRD codes, designed the study and drafted the manuscript. IT involved in the developing the study design and drafting the manuscript. AK conducted the statistical analyses. BN and IK designed the study and revised the manuscript. FT supervised and designed the manuscript.

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Chapter 7:

HE organized, analyzed the data and drafted the manuscript. APL collected the data and analyzed patients' radiographs. IT collected the data, revised and edited the manuscript content. PMS, IGT, FRT, TGO collected the data. FT supervised and designed the project.

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CHAPTER 1: Introduction and Aim of the Work

Bone is a hard tissue that protects internal organs, helps locomotion and serves as a reservoir for essential minerals required for proper body function. Bone encloses two distinctly different cell types: bone forming cells-osteoblasts and bone resorbing cells-osteoclasts[1]. Osteoblasts are derived from multi-potential mesenchymal stem cells, and their function is to lay down the extracellular matrix and regulates its mineralization. Eventually, osteoblasts get trapped in the bone calcified matrix, and developed into osteocytes, the most abundant cells in bone. Osteoclasts, derived from hematopoietic stem cells, and their function is to resorb the mineralized bone[1].

Bone is a living tissue that undergoes a constant process of formation and resorption, called remodeling[2]. Under physiological conditions, bone formation and resorption are carefully balanced[2]. Disruption of this balance may lead to osteoporosis and risk of fracture[2]. Recent studies have shown that the central nervous system plays an important role in controlling bone remodeling through the adrenergic and cholinergic pathways[3-8]. Adrenergic pathway has been associated with bone resorption and drugs that inhibit this activity have been found to increase bone accrual and reduce the risk of hip fractures[3-6,9]. By contrary, the available literature indicates that the inhibition of cholinergic pathway results in bone loss[7,8]. However, little is known whether stimulating this pathway may have a positive effect on bone mass accrual. Cholinergic pathway can be stimulated by acetylcholinesterase inhibitors (AChEIs).

1.1. Hypothesis:

AChEI could have an anabolic effect on bone accrual.

AChEIs are a group of drugs that prevent acetylcholine degradation resulting in enhanced cholinergic signaling[10]. AChEIs can stimulate central- or peripheral- cholinergic synapses depending on their capability to cross the blood brain barrier (BBB)[10]. However, little is known on the potential clinical effects that these drugs might have on bone.

1.2. Specific Aims:

a. *Determine the effect of central acting ACEIs on bone mass accrual- in vivo study*

b. *Determine the effect of peripheral acting ACEIs on bone mass accrual- in vivo study*

c. *Determine the effects of AChEIs on risk of fracture- Clinical study*

d. *Determine the effect of AChEIs on fracture healing- Clinical study*

CHAPTER 2: Literature Review

Cholinergic Regulation of Bone

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2.1. Abstract:

Bone remodeling is regulated by the two branches of the autonomic nervous system: adrenergic and cholinergic. 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 cholinergic inhibition 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.

2.2. Introduction:

Bone remodeling involves bone resorption by osteoclasts and subsequent formation of new bone by osteoblasts[1,2,11]. This process regulates the biomechanical features of bone and maintains the homeostasis of essential mineral ions in the body[2]. Alteration in the bone remodeling process results in diseases such as osteoporosis that can often cause life-threatening complications[1]. 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[1].

The process of bone formation and resorption is well-regulated, at least, at two different levels: locally and centrally[2]. 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[2]. 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[3,5-8,12-16]. The last two axes are mediated by adrenergic and cholinergic activities, the functional arms of autonomic nervous system[3,5-8,12-14]

The adrenergic activity has been shown to be a negative regulator of bone mass; SNS signaling inhibits osteoblasts proliferation and promotes osteoclasts proliferation and differentiation[3,5,6,17]. Moreover, osteoclasts are also up-regulated indirectly through osteoblast secretion of the receptor activator of NF-κB ligand (RANKL) as a response to SNS signaling[3]. SNS signaling are well controlled by the action of two key proteins: osteocalcin and leptin. Osteocalcin, produced by mature osteoblasts, regulates the production of leptin, an adipocyte-derived hormone. Leptin, in-turn, activates its receptors in the hypothalamus and promotes SNS activity[3,5,6].

Cholinergic activity has been shown to favor bone mass through up-regulating osteoblasts and down-regulating osteoclasts proliferations[7,8]. 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.

2.3. Cholinergic system:

Cholinergic system regulates the body metabolic activity through the use of acetylcholine as a signal transmitter[18]. 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[19]. The secreted acetylcholine targets either nicotinic or muscarinic receptors[20,21]. Nicotinic receptors are compromised of different

subunits α, β, γ, δ and ε that assemble to form ionic channels[22]. Muscarinic receptors are guanine nucleotide protein coupled receptors, and are classified into five subtypes: M1, M2, M3, M4 and M5[21,23]. Acetylcholine signal is terminated by degradation through the enzyme acetylcholinesterase (AChE)[24].

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)[7,8,25-27]. 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 [18,28,29].

2.3.1. Expression of cholinergic components in bone

Bone cells have been shown to express several components of the cholinergic system (transmitter, enzymes and receptors)[7,8,20,21,30-35]. For instance, acetylcholine has been suggested to be produced by osteosblasts due to the presence of the vesicular acetylcholine transporter enzyme in these cells[35]. Cholinergic receptors, both nicotinic and muscarinic, have been identified on the membranes of human primary bone cells, mesenchymal stem cells, osteoblasts and osteoclasts[7,8,30,31,36-39]; and mRNA of several nicotinic receptors subtypes (α_1, α_4, b_1 and g) are also present in osteocytes[40]. AChE is expressed in bone cells such as bone marrow-derived monocytes, osteoclasts and osteoblasts[7,34,41].

2.3.2. 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[28]. Previous studies have shown that acetylcholine might regulate the migration of bone marrow-mesenchymal stem cell[42], a multiprogintor cell capable to differentiate 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[7], whereas muscarinic stimulation *in vitro* increases osteoblasts proliferation[30,35]. Among the cholinergic receptors, the nicotinic subtype-α_2 receptor and muscarinic-3 receptor appear important in bone physiology[7,8,43-45]. Indeed, mice with knockout nicotinic subtype-α_2 receptors are osteoporotic due to the up-regulation of osteoclasts[7], whereas mice with knockout muscarinic-3 receptors are osteoporotic due to an increase in osteoclasts number and a decrease in osteoblasts number[8].

Beside its capability to break down acetylcholine, AChE is thought to play significant roles during remodeling of bone. For instance, AChE has been identified at the sites of new bone formation[41], suggesting a role for AChE as a bone matrix protein[34,41]. Moreover, it has been reported that AChE can regulate bone cells proliferations, differentiations, cell-cell contact as well as mesenchymal stem cells migration[32,34,41,46,47].

2.2.3. Cholinergic innervation of bone

It has been shown that bone tissues are innervated by cholinergic fibers of both the PSNS and the SNS[7,48]. Cholinergic fibers transmit neuronal signaling

from the PSNS nucleus within the CNS to cholinergic receptors located in bone[7]. 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[49]. 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[50]. 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[51-54].

2.2.4. Central nervous system nuclei

2.2.4.1. Hypothalamus

Previous studies have shown that the hypothalamus, a brain structure that encloses cholinergic (muscarinic and nicotinic) and adrenergic components, can affect bone remodeling[55,56]. It has been shown that Alzheimer's disease (AD) patients suffering from cholinergic degradation of the hypothalamus[57,58], are more prone to develop osteoporosis and suffer from a high incidence of fractures[59-62]. In fact, the association between bone mineral density and cholinergic degradation of the hypothalamus is so strong[63,64] that decrease in bone mineral density has been suggested as a predictor of AD[65]. These observations highlight the

importance of cholinergic nucleus of the hypothalamus in regulating bone mass accrual.

One mechanism by which the hypothalamus might use to regulate bone remodeling is by controlling body weight[6], a known positive regulator of bone[66,67]. The hypothalamus encloses two structures that regulate body weight: the arcuate nuclei, ventromedial and the lateral hypothalamic nuclei. The lateral hypothalamic nucleus is concerned in hunger, and any damage to this area can reduce body weight[68]. Lateral hypothalamic nucleus is known to enclose cholinergic neurons and receptors, such as muscarinic receptors[8,69]. 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[70]. 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 pituitary-thyroid-bone axis[5,13,71,72]. It has been reported that the expression of muscarinic receptors centrally might be regulated by the level of calcitonin[73], 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.

2.2.4.2. 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[8]. 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[8].

2.2.4.3. Central IL-1

Central IL-1 is a proinflammatory cytokine produced by brain neurons that are known to regulate learning, memory and sleep patterns[74,75]. Central IL-1 can also affect bone metabolism through nicotinic nerve fibers of the PSNS[7]. Indeed, mice with knockout central IL-1 express very low levels of skeletal acetylcholine[7], and these mice are osteoporotic due to the down-regulation of osteoclasts apoptosis[7,76].

2.2.5. 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 2.8.1**, we suggest possible mechanisms for cholinergic regulation of bone. The presence of cholinergic components in non-neuronal bone cells might indicate that bone could be regulated by the cholinergic activity locally through autocrine/paracrine pathway. On the other hand, recent studies have shown that cholinergic activity might regulate bone mass systemically through neuronal pathways mediated by locus coeruleus and central IL-1 activities[7,8].

2.3. Clinical observations:

2.3.1. 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. 2.3.1.)**[29,77-84]. Decrease cholinergic activity in salivary glands function results in a dry mouth (xerostomia)[85]. Similarly, suppression of cholinergic activity in lacrimal glands results in dry eyes[86]. Damage to muscarinic-3 receptors in langerhans cells result in diabetes type 1[87]. 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[88-91]. 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[92-95]. Interestingly, all these conditions have as a common skeletal feature, a reduction in bone mineral density[92,96,97]. 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[98], a cholinergic-regulated organ[83]. Patients suffering from vertigo are associated with higher risk of osteoporosis[99].

2.3.2. 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[100,101].

2.3.3. Menopause

Another observation that links cholinergic activity to bone is the estrogen replacement therapy, a hormonal treatment for osteoporosis. Estrogen replacement therapy is known to minimize bone loss and risk of fractures[102]. Surprisingly, these patients express an increase in cholinergic activity[103].

2.3.4. Smokers

Clinical studies have shown that heavy smoking is associated with a decreased bone mass and diminished fracture healing capacity[31,104,105,106]. 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[107]. Even though low concentration of nicotine up-regulates osteoblasts through activation of the nicotinic receptors[37,44], excesive levels down-regulate osteoblasts through desentistizating their nicotinic receptors[43]. Also excesive nicotinic levels up-regulate osteoclasts through activation of the niotinic receptors[7].

2.3.5. Poliomyelitis, Botox injection and Myasthenia gravis

Cholinergic neurons innervate muscle fibers[108], and their activity can affect bone mass through mechanical stress[109]. 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[110]. Patients diagnosed with poliomyelitis are known to suffer from impaired bone growth affected by nerve depletion[111]. 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[111].

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[127]. Botulinum neurotoxin causes bone loss that does not improve following the recovery of muscle function[112,113]. Moreover, bone loss due to botulinum neurotoxin has been associated with an increase in bone resorption due to osteoclasts up-regulation[112,114], a phenomena that is recently linked to the inhibited activity of cholinergic fibers of the PSNS[7].

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[116,117]. Surprisingly, despite the low muscular activity, patients diagnosed with Myasthenia graves are not associated with the risk of developing bone disases[115-117]. In contrast, Myasthenia gravis patients are more resistant to develop osteoporosis compared to general population[116]. 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.

2.4. 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[7,8,45]. 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.[7] 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[118]. 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[118]. 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.

2.5. 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. Future studies are also required to determine the interaction between the cholinergic system with endocrine system, and the effects of this interaction on bone mass. Moreover, future research should investigate the possible involvement of paracrine/autocrine pathways in mediating the interaction between the cholinergic components and bone cells.

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 sclerostin secretion, and promote osteoclast proliferation through RANKL secretion[119,120]. Accordingly, nicotinic receptors in osteocytes could be mediating the interaction between these cells with osteoblasts and osteoclasts, although future studies will have to be performed to address this hypothesis.

2.6. 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.

2.7. Acknowledgment:

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2.8. Figures:

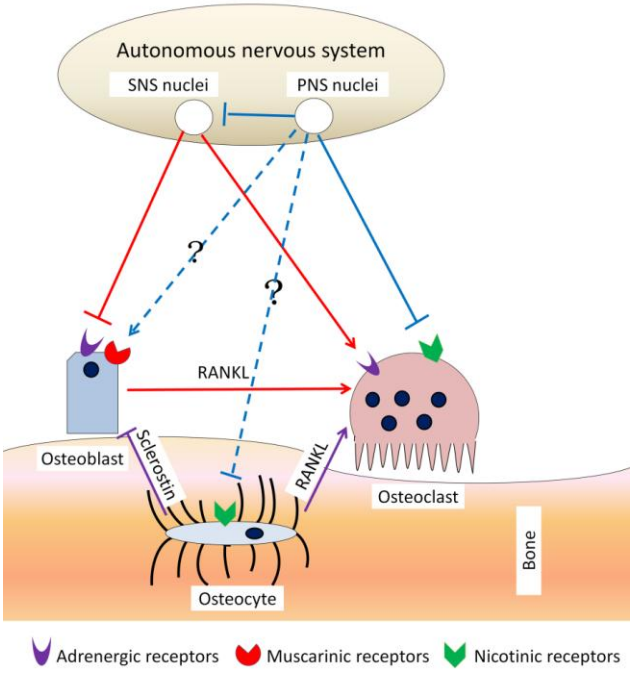

Figure 2.8.1. Model of the autonomic nervous regulation of bone mass accrual. The SNS favors bone loss by down-regulating osteoblasts proliferation, directly through activating the adrenergic receptors. Also, the SNS favors bone loss by up-regulating osteoclasts proliferation, directly through activating the adrenergic receptors and indirectly through stimulating RANKL secretion from osteoblasts. The PNS favors bone mass indirectly by suppressing the SNS signaling and directly by down-regulating osteoclasts proliferation through activating the nicotinic receptors. The two blue-dashed arrows refer to possible effects of the PNS on osteoblasts and osteocytes. Black-dashed arrow refers to a possible interaction between osteoblast and osteoclast by an unknown paracrine pathway using cholinergic components.

2.9. Tables:

Table 2.9.1. The relationship between cholinergic receptors in body tissues with bone turnover in various clinical conditions.

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[77]	Saliva secretion[85]	Dry mouth [85]	Patients with dry mouth are associated with low BMD[88]
Lacrimal glands[79]	Lacrimal secretion[121]	Dry eyes[86]	Patients with dry eyes are associated with low BMD[89]
pancreas[80]	Insulin secretion[122]	Diabetes type 1[87,123]	Patients with diabetes type 1 are associated with low BMD[90,91,124,125]
Respiratory system[126]	Smooth muscle contraction and epithelial regulation[126,127]	Obstructive pulmonary disease [95]	Patients with lung diseases are associated with low BMD [92]
Gastrointestinal system[81]	Smooth muscle contraction[81]	Decrease muscle tone [128]	Patients with gastrointestinal diseases are associated with low BMD[96]
Vestibular organ within ear[83]	Regulate balance and orientation[129]	Unbalance and disorientation (Vertigo)	Patients with vertigo are associated with low BMD[99]

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CHAPTER 3: Materials and Methods

3.1. Methods for the *in vivo* studies:

3.1.1. Mice

Studies presented in chapters 4 and 5 were conducted following an animal use protocol approved by the Animal Care Committee of McGill University. Our experiments were conducted on 5-week-old female wild type C57BL/6J mice which were purchased form Jackson Laboratory (Bar Harbor, MA). The C57BL/6J mice were chosen since they have a relatively low bone density compared with other strains of mice[130]. We used female mice since they have a relatively low bone density and they are more prone to develop bone diseases such as osteoporosis compared to male mice[131]. The mice were kept in a 12 h light/dark cycle. Rodent breeding diet and water were provided *ad libitum*. Mice were treated daily by intraperitoneal injections with specific drugs for specific period of time (please see the materials and methods sections of chapters 4 and 5).

3.1.2. Body metabolites and bioassays

Mice body weights were recorded before the start of the injections (1^{st} reading) and before the sacrifice (2^{nd} reading). Percentile changes in mice body weight were calculated. Visceral fat was dissected from each animal and weighted to assess differences in fat deposition between the treated mice groups.

Before the sacrifice, urine samples were collected from each mouse on the last week before the sacrifice. Samples from 2 collections obtained over three

days were pooled for each mouse. The collected urine samples were stored at -80˚C to prevent deterioration of its protein content. Blood samples were collected from each mouse using cardiac puncture technique. All blood samples were placed on ice at collection. The collected blood samples were centrifuged at 6000 rpm for 5 minutes to produces platelet poor EDTA plasma samples. Samples were stored frozen at – 80˚C for further analysis.

In our studies, we measured serum levels of insulin, leptin, receptor activator of nuclear factor κB ligand (RANKl), osteoprotegerin (OPG), interferon-γ, tumor necrotic factor-α, IL-17, IL-6 and IL-23 using commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kits. This technique relies on the presence of an antibody or an antigen to identify and quantify a substance.

The ELISA kit consists of plate of 96 wells. Initially, plate-wells were coated with specific capture antibodies (primary antibodies) (**Figure 3.1.1.A**). Then, serum/urine and standard control samples were added to different wells and incubated for a specific period of time. Antigens for the specific substance present in serum/urine would have been bound to the primary antibodies (**Figure 3.1.1.B**). The plate was washed to remove the weakly adherent antibodies from the serum/urine samples. Then, secondary antibodies (detection antibodies) attached to an enzyme, which can convert colorless materials into colored products, were added to each well to bind the antigen-antibody complex (**Figure 3.1.1.C**). After specific incubation period, an enzyme-substrate was added to each well to detect the colored product in the wells (**Figure 3.1.1.D**). The amount of colored product at each well was measured by a plate reader at specific wave

lengths. The density of the colored material is proportional to the amount of antigen bound to the primary antibody.

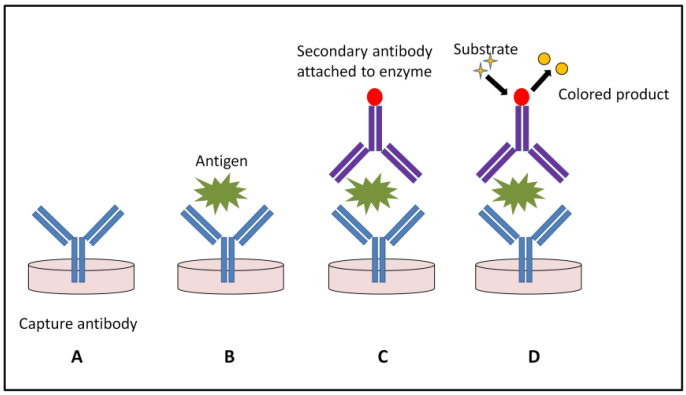

Figure 3.1.1. Schematic diagram illustrating the steps of ELISA test.

3.1.3. Open Field test

Open Field Test is an experiment used to measure levels of general locomotor activity and anxiety in rodents[132]. This test is commonly used to assess the pharmacological effects of drugs on animal movement, mood and anxiety, which are controlled by the central nervous system[132]. Open Field Test consists of a rectangular enclosure with walls that prevent animals from escape and a field that is marked by square crossings **(Figure 3.1.2)**. Rodents' spontaneous behavior would be video-recorded for a specific time period by a camera located above the chamber. From the recorded video, the locomotor activity of the animal can be analyzed based on the number of squares crossed by the animal during the specific time period.

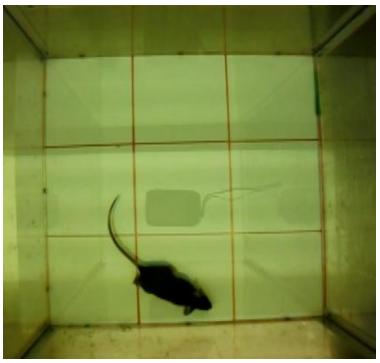

Figure 3.1.2. A photograph captured during the assessment of the locomotive activity for a mouse.

In our *in vivo* studies, we used the Open Field Test to assess whether the study drugs had an effect on the locomotor behavior of the mice. Change in the status of the locomotor activity of the mice after treatment means that the signaling of the central nervous system has been altered, indicating that the tested drug crossed the blood brain barrier and induced a central effect. In our studies, we used an open field box with the following dimension: 30 X 30 X 60 [height] cm. Floor of the box was divided into 9 squares. The number of squares crossed by each mouse was counted for 5 minutes[133].

3.1.4. 3D-micro computed tomography analysis

Axial and long bones, collected from the treated-mice, were submitted for 3D-micro computed tomography (μCT) analysis. μCT is a non-destructive imaging technique that uses the x-rays attenuated data at multiple viewing angles to build a virtual 3D model for the object[134]. The 3D model is created from cross-section images of the object at the micrometer range (voxel size is at micrometer range), which is sufficient to measure trabecular bone in rodents such as mice[135].

The use of μCT to assess bone morphology and microarchitecture has several advantages compared to the 2D measurements provided by histology: 1) it measures the 3D morphology of the trabeculae (trabecular thickness and separation), 2) it analyses a larger volumes of interest of bone specimens, and 3) the time needed for the analysis by μCT is reduced. For all these reasons, μCT is considered as the gold standard technique to evaluate bone morphology and microarchitecture in rodents.

In our *in vivo* studies, long (represented by left tibiae) and axial (represented by lumber vertebra number 5 (L5)) bones of each mouse was scanned by SkyScan 1072 (Bruker-Microct, Kontich, Belgium) machine. The machine was adjusted according to the following parameters: 50kV x-ray energy, 200μA x-ray current, 0.5mm Aluminum filter, 5μm image pixel size and 1000 pixel field width resolution. The analysis of tibia included a region of interest of 2.3 mm distal to growth plates, whereas the analysis of vertebra included the whole body. Bone-analysis software (Version 2.2f, Skyscan, Kontich, Belgium) was used to calculate the following 3D morphological parameters: bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N) and cortical thickness (Ct.Th)[134].

3.1.5. Dual X-ray Absorptiometry (DXA)

In our *in vivo* studies, we assessed bone mineral density (BMD) of mice left tibiae. BMD was measured by Dual-energy X-ray absorptiometry (DXA). DXA is a noninvasive diagnostic tool that estimates the quality and quantity of

bone. DXA uses two x-rays sources of different energy levels: low and high energy. The low energy x-rays is absorbed mainly by soft tissues and the high energy x-rays is absorbed mainly by bone. The amount of x-rays absorbed by the soft tissues is subtracted from the total absorbed x-rays by the body to estimate the patient's bone mineral density. Currently, DXA is considered as the gold standard for diagnosis of bone related disease in human such as osteoporosis[136].

In our *in vivo* studies, the applied DXA machine (PIXIMUS, GE Medical Systems, Schenectady, NY, USA) uses two x-rays sources of different energy levels (80/35 KV at 500 μA) to achieve contrast in extremely low-density bone. The difference in x-rays absorption of each beam from mice tibiae was captured by a detector uses ultra-high resolution pixels (0.18 × 0.18 mm). The obtained DXA images were analyzed to determine the BMD.

3.1.6. Mechanical testing

In our *in vivo* studies, we measured the mechanical properties of long and axial bones using a three-point breaking (bending) test. Three-point breaking test is the most commonly used method to characterize the strength of the appendicular bones[137]. The bone sample is placed on two supporting holders separated by specific distance. A third loading pin is lowered from the top at a consistent rate to induce deformation then fracture of the bone sample.

In our *in vivo* experiments, a three-point breaking test was performed on the midshaft of the right tibiae obtained from all mice (**Figure 3.1.3**). A commercial bench-mounted vertical tensile/compression tester, the Instron 5569

(Instron Corp., Canton, MA, USA), was used. The span of two support points was 10 mm, and the deformation rate was 1.0 mm/min. The extrinsic parameters: young modulus, stiffness, ultimate force and work to failure, were calculated from the resulting load-displacement curves.

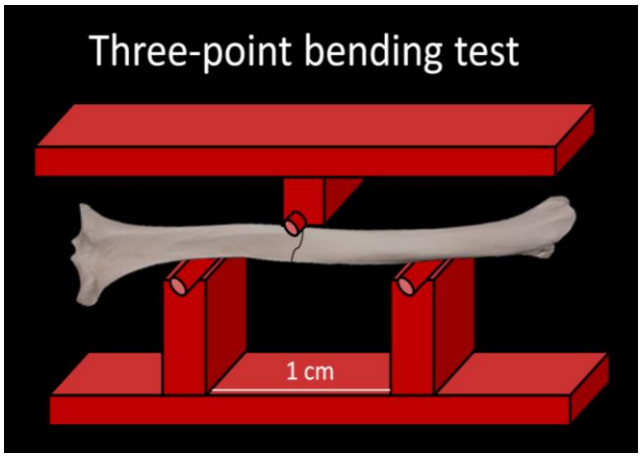

Figure 3.1.3. Schematic diagram illustrating the three-point bending/breaking test

3.1.7. Raman analysis

Raman spectroscopy is a non-invasive analytical technique that helps define the crystallinity and chemical composition of materials (e.g. organic and mineral contents). This technique relies on the scattering of monochromatic light, such as laser, from the sample. The interaction between the Raman laser and the sample induces changes in energy of the laser photons that is translated into information about the vibrational, rotational, and other low-frequency modes in a system. The use of Raman spectroscopy to assess crystallinity and chemical composition of bone is well documented in the literature[138-140].

Crystallinity index analysis gives relative information about the crystallographic ultrastructure (e.g. crystal size and lattice defects) of the material. Such information is important since bone is a polycrystalline material[141], and it is well known that in this kind of materials the average size of the crystals regulates materials resistance to fracture[142]. Chemical composition analysis gives relative information about the organic and mineral contents of the materials. Information about the organic and mineral contents of bone is important since both of them are known to regulate bone mechanical properties.

In our *in vivo* studies, crystallinity index and chemical composition analyses were conducted on the mid-shaft of the left tibia, the area where the fracture occured. A Raman spectrophotometer (Senterra, Bruker, Karlsruhe, Germany) equipped with 785nm diode laser (of 50mW power) coupled with an optical microscope (Olympus Optical Co., Hamburg, Germany). The Raman spectrophotometer was set at a resolution of 3-5 cm^{-1}. The collected Raman spectra were normalized using the Raman spectrophotmer's software (OPUS 7.0.0, Bruker, Karlsruhe, Germany) based on the absorbance of the ν_1PO4 at 960 cm^{-1}. The bone organic content was estimated from the ratio of Amide I (1635 cm^{-1})-to-ν_3PO_4 (960 cm^{-1}) peaks. Crystallinity index was calculated based on the bandwidth at the half peak intensity of the $\nu_1PO_4^{-3}$ band at 960 cm^{-1}.

3.1.8. X-ray diffraction

The crystallographic ultrastructure of mice bones was also analyzed by means of X-ray Diffraction (XRD) spectroscopy. The XRD spectroscopy is a non-

destructive technique used to define the mean size and physical properties of the crystal particles. This technique is based on measuring the intensities and angles of the diffracted x-ray beams from the crystalline atoms of materials. Since bone is a polycrystalline material, the use of XRD to analyze its crystallographic ultrastructure is well document in the literature[143].

In our *in vivo* studies, the left tibia of each mouse was manually crushed into powder, de-fated with Acetone (Sigma–Aldrich, Oakville, Ontario) and left to dry at ambient temperature for 48 hours. The bone powder specimens were submitted to XRD (D8-Discover/GADDS, Bruker, Karlsruhe, Germany). The XRD parameters were adjusted as following: 40 kV and 40mA Cu Kα radiation, 10–60° scanning angle, 0.02 step size and 1800 s scan time. DIFFRACplus EVA software (Bruker AXS, Karlsruhe, Germany) was used to analyze the XRD spectra.

Bone crystals have a hexagonal morphology with two crystal planes: a- and c-axes. Crystal size along a- and c- axes were calculated from the peak broadening of the XRD peaks (002) and (310), respectively, according to Scherer formula[33]. The (002) and (310) Bragg peaks were chosen because they do not overlap with other peaks [144-146].

$$D = \frac{K\lambda}{\beta \cos\theta}$$

Where D is the average diameter, K is the shape factor, λ is the x-ray wavelength, β is the line broadening at half the maximum intensity (FWHM) and θ is the Bragg angle.

3.1.9. Histomorphometric analyses

In our *in vivo* studies, we used histology and histomorphometric analysis to measure bone volume, bone formation, osteoblast number and osteoclast number in mice. We used lumbar vertebrae of mice which were fixed for 24 hr in 4% PFA/PBS, dehydrated in graded ethanol series, embedded in methyl methacrylate resin and sectioned (7-μm thickness)[147]. The undecalcified sections of the lumber vertebra were stained by Von Kossa/Von Gieson, toluidine blue and tartrate-resistant acid phosphatase (TRAP). Von Kossa staining illustrates the calcium salts (e.g., carbonate and oxalate) and phosphate components of the bone mineral, whereas Von Gieson staining shows the collagen. Toluidine blue staining demonstrates the osteoblasts whereas TRAP staining is used to define the osteoclasts duet to their highly expression of TRAP enzyme.

Stained bone sections were analyzed for bone volume-to-tissue volume ratio (BV/TV), osteoblast count and osteoclast count using the Osteomeasure software (Osteometrics Inc., Atlanta, GA). Images were taken using light microscope (DM200; Leica, Heerbrugg, Switzerland) adjusted at 2.5 × 20 × or 40 × objective. All histological images were captured using a camera (DP72; Olympus, Center Valley, PA), acquired with DP2-BSW software (XV3.0; Olympus, Center Valley, PA), and processed using Photoshop (Adobe, Adobe Systems, San Jose, CA)

3.2. Methods for the clinical studies:

Below is a full description for types of the study designs used in health sciences. In chapter 6 and 7 we used nested case-control and cohort studies.

3.2.1. Randomized controlled trial

A randomized controlled trail (RCT) is the gold standard design to conduct clinical studies. Such studies are often used to define the efficacy of a new medical intervention (such as drugs) in a patient population[148]. The strength of the RCT is illustrated by the random allocation in receiving the treatments by the participants. In addition to the random allocation, the RCT contains control groups either receiving no treatment (a placebo-controlled study) or a previously approved treatment (a positive-control study). RCTs have level of evidence of either 1 (high quality RCT) or 2 (lesser quality RCT)[149].

Strengths of RCT design:

1) It provides the strongest evidence for the efficacy and side effects of the medical intervention.
2) Incidence or prevalence can be assessed since the measurement is conducted in patients who were randomly selected from the population.
3) It has the least bias due to the randomization and controlling the confounders.

Weaknesses of RCT design:

1) It is relatively expensive and time consuming to perform.

2) It could have ethical constrains issues such as some individuals are required to accept risk to assess certain conditions that may not benefit them.

3) It is inefficient in assessing delayed outcomes.

4) Its findings might not apply to the population (risk of generalizability) since the selected patients, although it was through a random process, might not be good representatives for the total population.

3.2.2. Cohort study

A cohort study is a longitudinal observational study used commonly in health sciences. It relies on the analysis of risk factors for developing a disease in a group of people followed for a specific time period[150]. The word "Cohort" refers to a group of people who have similar characteristics or shared a particular event together within a defined period (e.g., age, exposed to a specific medical intervention). The comparison group can be the general population or people from the cohort, who have no exposure to the substance (medical intervention) under investigation[150].

A cohort study is conducted either prospectively or retrospectively from archived records. A prospective cohort study defines the data related to exposure before the occurrence of the event to be studied. A retrospective cohort study defines the data related to exposure after the occurrence of the event. Prospective cohort studies provide higher level of evidence in comparison with

the retrospective cohort studies since they assess wider range of exposure-disease associations which minimizes the bias[150].

Strengths of cohort design:

1) Multiple outcomes can be evaluated from a single exposure.
2) Incidence or prevalence can be assessed since the measurements are made in a population based sample.

Weaknesses of cohort design:

1) It is relatively expensive since it required a large sample size of the population and huge amount of data to perform the analyses.
2) Large sample size is required to obtain results with a high enough power.

3.2.3. Case-control study

A case control study is an observational study that aims to identify factor/s associated to a medical condition by comparing participants who developed the condition (defined as cases) with other participants who have similar characteristics to the "cases", however; they never developed the condition (defined as controls)[151]. The case-control studies have level of evidence of 3. The case-control studies are often used as preliminary studies to assess the association between the risk factor and condition of interest[151].

Strengths of case-control design:

1) It is often used for rare disease since it guarantees (with a high enough power) a sufficient number of cases with the condition of interest.
2) It is relatively inexpensive and requires shorter duration in comparison to RCTs and cohort studies.

Weaknesses of case-control design:

1) It is difficult to obtain reliable information about the participants' exposure status over time due to the retrospective design (risk of recall bias).
2) It does not provide information about the incidence or prevalence because no measurements are made in a population based sample.
3) There is a risk of bias in using the case-control design since the selection of the controls might not be based on a fully representative sample of the population.

3.2.4. Nested case-control study

A nested case-control study is a case-control study conducted in the population of an ongoing cohort study. In a nested case-control study, certain number of controls, who did not develop the condition of interest, are time-matched based on the risk set (age, date of entry to cohort, length of time in cohort) to each case, who developed the condition of interest[152].

Strength of nested case-control design:

1) It is relatively less expensive than RCTs and full cohort studies.
2) It is used when the exposure of interest is difficult to obtain and/or the outcome is relatively rare.
3) Controls and cases are selected from the same population which decreases the risk of bias.
4) It requires relatively a smaller sample size than full cohort studies.
5) Data on exposure and confounders are collected before the occurrence of the condition of interest which provides a relative cause-effect interpretation and limits the potential for bias.

Weaknesses of nested case-control design:

1) It is relatively more expensive than case-control study.
2) Risk of bias in selecting the controls, whom might not be fully representative of the original cohort. However, the risk of selection of bias in nested case-control studies is lower than in case-control studies.
3) Risk of recall bias since the data of exposure and confounders were collected retrospectively.

3.2.5. Case-series studies

A case-series study is a detailed descriptive study of a group of people who have the condition of interest[153]. Such studies describe the outcomes in a specific group of people with no comparison with people who do not have the condition of interest (controls). The case-series studies have a level of evidence of 4[153].

Strengths of case-series design:

1) It provides information for a very rare disease with few risk factors.
2) It is relatively inexpensive and less time consuming to conduct compared to RCTs, cohort, nested case-control and case-control studies.

Weaknesses of case-series design:

1) It does not provide information about the cause-effect association since no controls (who did not have the condition of interest) were assessed.
2) It does not provide information about disease frequency.
3) It might suffer from the risk of selection bias since the measurements may be conducted in a non representative sample of the population.

3.2.6. Case-report study

A case-report study is a detailed presentation of unique findings for a single case[154]. A case-report study usually reports information about the clinical course, manifestation, prognosis, follow-up and outcomes for the patient of interest (who developed the unique findings). This type of clinical study is considered as a type of anecdotal evidence[154].

Strengths of case-report design:

1) Facilitate the recognition of new diseases or drugs side effects.

2) Provides clinical information on rare diseases so further hypotheses and studies can be conducted.

Weaknesses of case-report design:

1) It may suffer from risk of recall bias since the data were collected retrospectively.

2) It provides a low level evidence as the observations may be subject to bias.

CHAPTER 4: Donepezil Regulates Energy Metabolism and Favors Bone Mass Accrual

4.1. Preface:

In chapter 2, we reviewed the studies that indicate that the cholinergic system plays an important role in regulating bone mass accrual. For instance, it has been shown using mutated mice models that the loss of specific acetylcholine receptors (either nicotinic or muscarinic) within the central nervous system resulted in bone loss[7,8]. However, little is known on the potential effects of boosting the cholinergic signalling on bone mass. One way of stimulating the cholinergic system is by administration of cholinergic agonists such as AChEIs.

Donepezil is an AChEI that is able to cross the blood brain barrier and act on the cholinergic nervous system[10]. Previous clinical studies suggested that donepezil might have positive effects on the bone quality[118,155]. This chapter presents the results of an *in vivo* study conducted in order to investigate the effects of donepezil on bone mass accrual.

Donepezil Regulates Energy Metabolism and Favors Bone Mass Accrual

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4.2. Abstract:

The autonomous nervous system regulates bone mass through the sympathetic and parasympathetic arms. The sympathetic nervous system (SNS) favors bone loss whereas parasympathetic nervous system (PNS) promotes bone mass. Donepezil, a central-acting cholinergic agonist, has been shown to down-regulate SNS and up-regulate PNS signalling tones. Accordingly, we hypothesize that the use of donepezil could have beneficial effects in regulating bone mass. To test our hypothesis, two groups of healthy female mice were treated either with donepezil or saline. Differences in body metabolism and bone mass of the treated groups were compared.

Body and visceral fat weights as well as serum leptin level were increased in donepezil-treated mice compared to control, suggesting that donepezil effects on SNS influenced metabolic activity. Donepezil-treated mice had better bone quality than controls due to a decrease in osteoclasts number. These results

indicate that donepezil is able to affect body energy metabolism and favors bone mass in young female WT mice.

4.3. Introduction:

Bone remodeling is a lifelong process that involves a balance between bone resorption and bone formation. This process is well-regulated, at least, at two different levels: locally and centrally [2]. Locally, bone remodeling is regulated through a direct interaction between osteoblasts and osteoclasts, and by local interactions among these cells and the cells of the immune system [2]. Centrally, bone remodeling is regulated through the hypothalamic-pituitary-thyroid axis and by the common regulators of bone, adipose tissue and energy metabolism that involve two arms of autonomous nervous system: the sympathetic nervous system (SNS) and parasympathetic nervous system (PNS) [3,5-8,12,14,17,156,157].

The activity of the autonomous nervous arms (SNS and PNS) is known to regulate the buildup (anabolism) and breakdown (catabolism) of the body tissues' energy (energy metabolism). For instance, signalling of the SNS reduces body weight and bone mass accrual, whereas signalling of the PNS is associated with an increase in fat deposition and bone mass accrual [3,7,8,158-162]. In addition, PNS signalling within the central nervous system (*i.e.*, *locus coeruleus*) is known to down-regulate the SNS tone [8]. Accordingly, it would be logical to expect that the stimulation of the PNS receptors within the central nervous system would down-regulate the SNS, affect the body energy metabolic activities and enhance bone mass.

One way of stimulating PNS receptors is by the administration of parasympathomimetic agonists such as acetylcholinesterase inhibitors (AChEIs) [163]. AChEIs are group of drugs that increase PNS neurotransmitter acetylcholine levels by inhibiting the action of butyrylcholinesterase in the peripheral tissues and/or acetylcholinesterase in the CNS [10]. Peripheral acting AChEIs are used in the therapy of myasthenia gravis and glaucoma, whereas central acting ACEIs are used for Alzheimer's Disease (AD) treatment.

Donepezil is the most common AChEI used for AD treatment [164,165], and its beneficial effects on slowing down the progress of AD disease is well established [165]. Compared to other types of AChEIs, donepezil has a greater selectivity for acetylcholinesterase than buytrylcholinesterase, due to the presence of N-benzylpiperidine and an indanone moiety in its chemical structure [166,167]. Due to this unique chemical structure, donepezil expresses a more potent effect on the PNS receptors located within the central nervous system with no expected effect on the peripheral nervous system [164,167-171].

Previously, we showed that AD disease patients who are using donepezil had higher body mass index, lower risk of hip fracture and faster healing of the hip fracture (if occurred) compared to AD non-users [118,155]. However, these observational studies on non-healthy patients cannot determine fully the exact mechanism by which donepezil promotes bone mass. Accordingly, this study was designed to investigate, in healthy mice, the effects of donepezil on body metabolites and bone mass accrual. Our results demonstrate that daily injection of donepezil altered the autonomic mediated-body metabolic activities and promoted

bone mass accrual in healthy mice. Based on being an AD drug, donepezil could also be useful for managing energy metabolism problems such as weight loss and osteoporosis.

4.4. Methods:

4.4.1. Mice

This study was conducted following an animal use protocol approved by the Animal Care Committee of McGill University. Twenty four 5-week-old female wild type C57BL/6J mice were purchased form Jackson Laboratory (Bar Harbor, MA). The mice had similar baseline readings: age and weight as well as they had free access to diet and water (provided *ad libitum)* during the study period. The mice were kept in a 12 h light/dark cycle in 6 cages (4 mice in each cage). Cages were assigned numbers from 1 to 6 respectively. Mice of cages 1, 3 and 5 (n=12) were treated daily by intraperitoneal injections with donepezil (0.6 mg/kg)[172,173], whereas the rest of mice (n=12) received daily intraperitoneal injections of 0.9% saline. The treatment time was for 4 weeks from postnatal week 6 to 10.

We compared the whole-body energy metabolism of donepezil-treated mice with a group of mice treated with saline (control). Following indicators of the body energy metabolism were assessed: locomotor activity, weight changes, intra-abdominal visceral fat, leptin and insulin serum levels and urinary epinephrine (SNS neurotransmitter) level. Locomotive activity is known to be regulated by PNS through the activity of muscarinic cholinergic receptors within

the hypothalamus nucleus[174,175]. Weight changes and fat mass are regulated through different mechanisms: neuronal (including the SNS and PNS signalling within the hypothalamus) and hormonal (via various hormones including leptin and insulin)[176]. Leptin, a hormone secreted from the white adipose tissues, regulates fat mass by acting within the central nervous system[177]. Leptin secretion is inhibited by the activity of SNS[178]. Secretion of insulin hormone, which promotes absorption of the carbohydrate from the blood to muscular and fat tissues, is controlled mainly by food intake, and partially, by the activity of muscarinic cholinergic receptors expressed by β-cells of pancreas[179].

4.4.2. Locomotor activity

Open field box (30 X 30 X 60 cm) was used to assess the locomotor activity of the treated mice groups. The floor of the box was divided into 9 squares and number of squares crossed by each mouse was counted for 5 minutes[133].

4.4.3. Body metabolites and bioassays

Body weight and intra-abdominal fat weight were assessed for each mouse. Intra-abdominal fat was collected from epididymal and retroperitoneal region. Serum leptin (Life Technologies, Gaithersburg, MD), serum insulin (B-Bridge International, BioCat, Cupertino, CA) and urinary epinephrine (Elisa kit, BlueGene, Biotech, Shanghai, China) levels were measured using commercially available ELISA kits. Serum levels of receptor activator of nuclear factor κB

ligand (RANKl) and osteoprotegerin were measured using antibody-based detection ELISA kits (abcam, Toronto, ON).

4.4.4. 3D-micro computed tomography analysis

μCT analyses were performed as previously described[180]. Briefly, left tibiae of each mouse was scanned by SkyScan 1072 (Bruker-Microct, Kontich, Belgium) machine. Bone-analysis software (Version 2.2f, Skyscan, Kontich, Belgium) was used to calculate the following 3D morphological parameters: bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N) and cortical thickness (Ct.Th)[134].

4.4.5. Dual X-ray Absorptiometry

PIXIMUS bone densitometer (GE Medical Systems, Schenectady, NY, USA) was used to assess bone mineral density (BMD) of left tibia collected from different treatment groups. The parameters were adjusted according to previous work in the field[180].

4.4.6. Mechanical testing

A three-point breaking test was performed on the midshaft of the right tibiae obtained from all mice as previously described[180]. Briefly, a commercial bench-mounted vertical tensile/compression tester, the Instron 5569 (Instron Corp., Canton, MA, USA), was used. The span of two support points was 10 mm, and the deformation rate was 1.0 mm/min. The extrinsic parameters, stiffness and ultimate force, were calculated from the resulting load-displacement curves.

4.4.7. Histomorphometric analyses

Histomorphometric analyses were performed as previously described [147,181]. Briefly, calcein solution (0.25% calcein and 2% $NaHCO_3$ dissolved in 0.15 M NaCl) was injected twice i.p. in mice (10 µl/g body weight) at an 8-day interval. Mice were euthanized 2 days after the second calcein injection. Lumbar vertebrae, collected from the treated-mice, were fixed for 24 hr in 4% PFA/PBS, dehydrated in graded ethanol series, embedded in methyl methacrylate resin and sectioned (7-µm thickness) [147]. The undecalcified sections of the lumber vertebra were stained by Von Kossa/Von Gieson, toluidine blue and tartrate-resistant acid phosphatase (TRAP). Stained bone sections were analyzed for bone volume-to-tissue volume ratio (BV/TV), osteoblast count, osteoclast count, and bone formation rate (BFR) using the Osteomeasure software (Osteometrics Inc., Atlanta, GA). Images were taken using light microscope (DM200; Leica) adjusted at 2.5 × 20 × or 40 × objective. All histological images were captured using a camera (DP72; Olympus), acquired with DP2-BSW software (XV3.0; Olympus), and processed using Photoshop (Adobe).

4.4.8. Statistical analyses

All results are shown as descriptive outcomes (mean ± standard deviation (SD)). Normality of the data was checked by the Shapiro-Wilks statistical test. Statistical analyses were performed by Student's two-tailed unpaired t-test. In all experiments, a value of $p < 0.05$ was considered significant as indicated by a single asterisk.

4.5. Results:

4.5.1. Donepezil alters energy metabolism

We observed that donepezil-treated mice had lower locomotor activity (a relative measure of gross motor activity[182]) than the controls **(Figure 4.9.1A)**. In comparison to saline-treated mice, donepezil-treated mice had higher body weight and body fat that accompanied with increased serum levels of leptin and insulin **(Figure 4.9.1B-E)**, most likely due to the up-regulation of the PNS and suppression of the SNS. This donepezil-induced suppression of the SNS was further confirmed by the lower levels of urinary epinephrine content in the donepezil-treated mice compared to controls **(Figure 4.9.1F)**. These results may indicate that donepezil, an AChEI acting on the CNS, is able to affect energy and body metabolism in mice.

4.5.2. Donepezil favors bone mass accrual

We compared bone phenotypes of donepezil-treated mice with that of saline-treated mice. Phenotypes of long bones (tibia) collected from the mice were determined using μCT, DXA and 3-point bending mechanical test. As shown in **Figure 4.9.2**, the administration of donepezil in 5-week-old mice had a positive effect on their long bone mass accrual. Mice treated with donepezil had higher bone volume, bone trabecular number and bone mineral density in comparison to the saline-treated mice **(Figure 4.9.2A, B, C and F,)**. With the increase of trabecular number, trabecular spacing decreased significantly **(Figure 4.9.2D)**. No

difference was observed in the trabecular thickness between donepezil- and saline-treated mice **(Figure 4.9.2E)**. Analysis of bone mechanical properties showed higher bone stiffness and bone ultimate load in donepezil-treated mice than in controls, whereas work to failure was similar in both groups **(Figure 4.9.2G)**.

4.5.3. Donepezil reduces osteoclasts number

Histomorphometric analyses of the axial bone (vertebra) confirmed that the increase in bone volume in donepezil-treated mice compared to saline-treated mice **(Figure 4.9.3A)**. The increase in bone volume in donepezil-treated mice was accompanied with a decrease in osteoclast numbers but no changes in osteoblast numbers or bone formation rate **(Figure 4.9.3B and D)**. These results indicated that donepezil treatment affected the resorption arm of bone remodeling. In agreement with these results, serum RANKL level was lower and serum OPG level was higher with over all lower RANKL/OPG ratio in donepezil-treated mice compared to saline-treated mice **(Figure 4.9.3E)**.

4.6. Discussion:

In this study, we provided evidence for the previously unexplored effects of donepezil on bone mass. We showed that donepezil, a central acting AChEI that up-regulates the PNS and down-regulates the SNS signaling induces changes in body metabolism and favors bone mass accrual.

4.6.1. Donepezil alters body energy and metabolites

In our study, donepezil-treated mice had lower locomotor activity compared to control. The locomotor activity is known to be regulated by the PNS arm of the autonomic nervous system. Indeed, it has been shown that central injections of cholinergic agents such as acetylcholine, carbachol and pilocarpine decreased spontaneous locomotor activity *in vivo*[183-185], and mainly through the muscarinic receptors within the ventromedial- and anteromedial-hypothalamus nuclei [174,175]. However, our study is the first to report this effect on the locomotor activity by the use of an AD drug such as donepezil.

Our study demonstrated that the donepezil treatment was associated with a significant increase in mice body weight compared to non-treated mice. Previous clinical studies have shown that AD patients suffer from weight loss [186-190]. However, AD patients treated with donepezil are associated with higher body mass index than those untreated, indicating that donepezil could counteract or minimize the AD-mediated body weight loss [118]. These observational clinical studies can only provide associations between body mass index and donepezil. However, our study is the first to confirm a cause-effect relationship between body mass gain and donepezil treatment.

In the present study, we report that the observed increase in mice body weight following 1-month of donepezil treatment (which is equivalent to ~30 months in human age) was related to the increase in fat mass. This observation was further confirmed by the significant increase in adipocyte-derived hormone leptin and pancreatic-derived hormone insulin levels, key regulators of body energy and food intake[191,192]. Findings of our study are different from those

reported in a previous clinical study, which showed that 6-month donepezil treatment induced a linear decrease of serum leptin levels in AD patients [190]. Different findings between the current and previous studies might be related to the differences between donepezil treatment time and study models (healthy mice vs. AD patients). Additionally, the surroundings or settings (access to food and physical activity) are very controlled in animal studies compared to those for AD patients. However, future studies are required to assess short- and long-term effects of AChEIs, including donepezil, on leptin secretion.

The mechanism by which donepezil increased fat mass, serum leptin and insulin levels might be related to the observed reduction in the locomotive activity of the mice described above. Donepezil treatment might have altered the parasympathetic receptor activity of the hypothalamus within the central nervous system. Hypothalamus encloses the lateral hypothalamic nucleus that is concerned in hunger, and regulates body weight [68,161]. Lateral hypothalamic nucleus is known to enclose parasympathetic neurons and receptors, such as muscarinic receptors [8,69,193]. Accordingly, stimulating the lateral hypothalamus causes a desire to eat, which could explain why donepezil-treated rats had higher weight, fat, serum leptin and insulin levels [70]. However, future research has to be done to test these hypotheses.

The above described changes in metabolic activities could be caused directly by an increase in PNS tone following donepezil treatment [194-196]. Alternatively, the increase in fat mass, serum leptin and insulin levels may also occur as a result of the decrease of the SNS tone [197-199]. Our study indicated that

the level of SNS neurotransmitter epinephrine was significantly lower in donepezil-treated mice compared to control, an observation that is similar to a previous *in vivo* study [200]. The down regulation of the sympathetic nervous system following donepezil treatment might have occurred through the activity of the muscarinic cholinergic receptors that are expressed in adrenergic neurons of the ventromedial hypothalamus nucleus [3,5] and/or nonadrenergic neurons of locus coeruleus nucleus [8].

4.6.2. Donepezil favors bone mass accrual

In our study we show that donepezil-treated mice had more bone mass accrual compared to saline-treated mice. This could explain recent clinical studies on AD patients indicating that the use of donepezil was associated with low risk of hip fracture [118] and better healing of hip fracture, when it occurred, compared to AD-nonusers [155]. In this study, we showed donepezil favors bone quality by reducing the number of the bone-resorbing osteoclasts. The decrease in osteoclast number was accompanied by a decrease in serum RANKL-to-OPG ratio. Similar to specific anti-osteoclasts drugs, we did not observe any significant change in osteoblast number and bone formation rate upon donepezil treatment, probably due to lack of secondary inhibition of osteoblasts [201-203].

In this study, we showed donepezil may have affected the signalling of the ANS arms: the SNS and PNS. The SNS is known to negatively affect the bone mass accrual by inhibiting osteoblast proliferation, directly through the adrenergic receptors [3,5,6]. 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 [3,5,17]. On the other hand, the PNS favors bone mass indirectly by suppressing the SNS signaling through the activity of muscarinic receptors expressed in the locus coeruleus and directly by promoting apoptosis in osteoclasts through activating the nicotinic receptors [7,8]. Accordingly, it is logical to observe an increase in bone mass following donepezil treatment due to the mediated--suppression of SNS and -stimulation of PNS.

Pyridostigmine, an AChEI have been shown to act peripherally on the osteoclasts. It is possible that donepezil may exert a similar direct effect on osteoclasts. However, donepezil acts mainly within the central nervous system with minimal peripheral effects[164,167-171]. However, donepezil has weak inhibiting effects on plasma AChE and a dose of more than 5m/kg (8 folds higher than the treatment dose used in current study) is needed to cause a peripheral effect[204,205]. Additionally, the concentration of donepezil in the brain is estimated to be 6-7 times larger than its concentration in plasma[206]. Therefore, it is unlikely that it would act directly on bone cells. Altogether, future studies are required to confirm this hypothesis. A recurring notion in the field suggest that bone, body mass and energy metabolism are regulated by common regulators[207]. Our data presented in this study strongly suggest this notion.

4.6.3. Significance and future research

The current study showed that donepezil, may have beneficial effects on body tissues favoring bone mass and weight gain. These results may provide therapeutic benefits for patients suffering from multiple energy metabolism problems such as AD patients. For instance, AD patients manifest body weight loss[187,189], and they are more prone to develop osteoporosis and suffer from a high incidence of hip fractures than rest of population[60-62]. Based on being an AD drug, donepezil could also be useful for managing energy metabolism problems in these patients such as weight loss and osteoporosis. However, future studies are required to confirm these hypotheses.

The present study assessed the effect of donepezil in young female healthy (WT) mice. Female mice were chosen since they are more prone to metabolic and bone diseases such as osteoporosis compared to males [208]. We used WT mice rather than AD mice in order to determine the effects of these drugs under healthy condition. Young and old mice usually respond similarly to osteoporotic therapeutics[209]. Therefore, we would expect that our results could be extrapolated to other conditions including osteoporosis. However, future studies on osteoporotic (ovarictomized and senile) animal models are required to determine the potential positive effects of donepezil in preventing bone mass loss. Additionally, future research required to assess the potential effects of donepezil on bone phenotype in dementia (AD) animal models.

The current study showed that donepezil treatment favored bone mass accrual, probably, due to a reduction in the SNS activity. However, future studies are needed to confirm this hypothesis.

Our study assessed the effect of single-daily dose of donepezil (0.6mg/kg), similar to several previous *in vivo* studies[172,173]. Clinically, initial low dose (0.3mg/Kg) is frequently prescribed for AD patients for 4 to 6 weeks followed by higher doses (0.6mg/Kg or 1.0mg/Kg). Previous study showed no significant differences between low or high donepezil doses in cognitive state of AD mice [173]. However, future studies will have to be performed to determine the effect of different doses of donepezil on the body metabolites and bone health.

4.7. Conclusion:

The results of this study demonstrated that administration of donepezil, a central acting AChEI, affects body energy metabolism and favors bone mass accrual in young healthy WT mice.

4.8. Acknowledgment

This work was supported by operating grants from Canadian Institutes of Health Research (CIHR) Fund # 102678 to MM, the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Fondation de l'Ordre des Dentistes du Québec (FODQ) to FT. HE and SA receive studentships from the FRQS Réseau de Recherche en Santé Buccodentaire et Osseuse (RSBO) and the Ministry of Higher Education of Saudi Arabia, respectively. MM is an FRQS Chercheur-boursier. The authors thank Dr. Laura Stone for help with the locomotor activity test. The authors have no conflict of interest.

4.9. Figures:

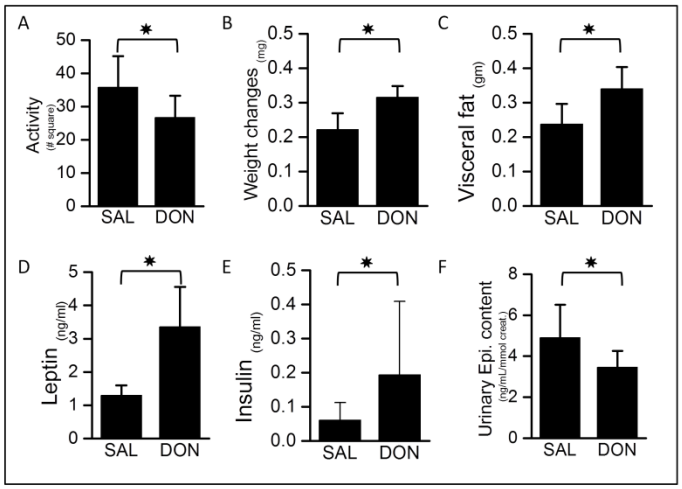

Figure 4.9.1. Donepezil affects body energy and metabolic activities. A-F. Graphs showing the locomotor activity, average weight and the average visceral fat weight as well as serum leptin and insulin levels, and urinary epinephrine content in the donepezil- (n=12) and saline-treated (n-12) mice. **A.** Donepezil-treated mice had lower locomotor activity than saline-treated mice. **B and C.** In comparison to control, donepezil-treated rats had higher weight mass, visceral fat. **D and E.** Serum leptin and insulin levels were higher in donepezil treated mice than in control mice. **F.** The urinary epinephrine content was lower in donepezil-treated mice compared to saline-treated mice. Data are presented as mean±SD. *$p<0.05$.

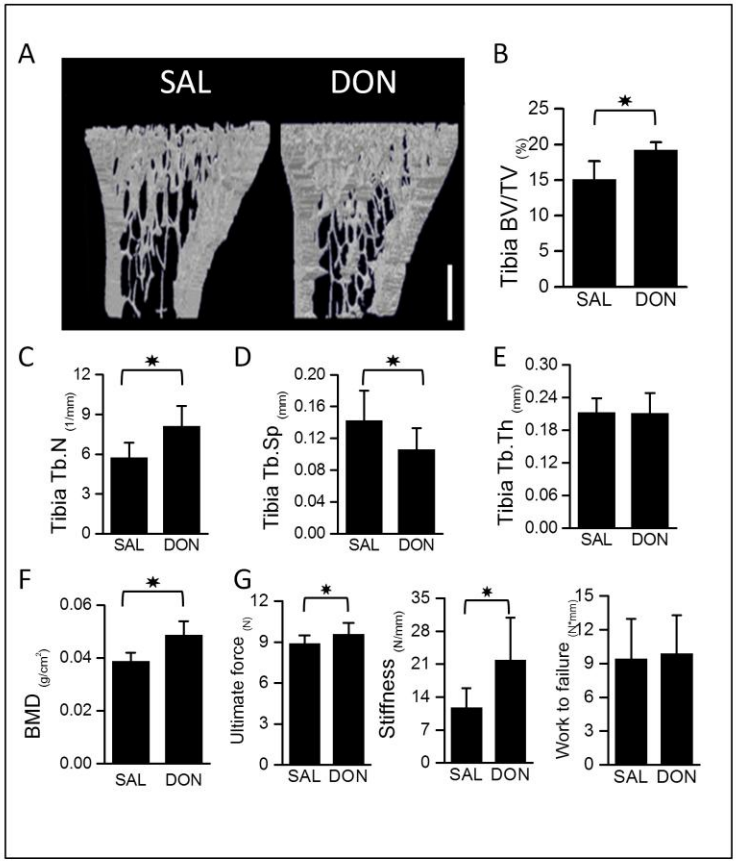

Figure 4.9.2. Donepezil favors bone mass accrual and enhances its mechanical properties. **A.** μCT images of tibiae retrieved from the saline (SAL)- and donepezil (DON)-treated mice. (Scale bar = 500μm). **B-E**. Donepezil-treated mice (n=12) had higher bone volume fraction (BV/TV), and trabecular number (Tb.N) and lower spacing between the trabecula (Tb.Sp) compared to saline-treated mice(n=12). No significant differences were observed in cortical thickness (Ct.Th) and trabecular thickness (Tb.Th) between the control and experimental groups. **F.** Bone mineral density (BMD) of tibiae, measured by DXA, was higher in mice treated with donepezil compared to those treated with saline (control). **G**. Illustrates the biomechanical results obtained from the three point bending test of the collected tibiae. Mice treated with donepezil (DON) had higher stiffness and

ultimate force values compared to those treated with saline (SAL). Work to failure was similar in both groups. Data are presented as mean±SD. *p<0.05.

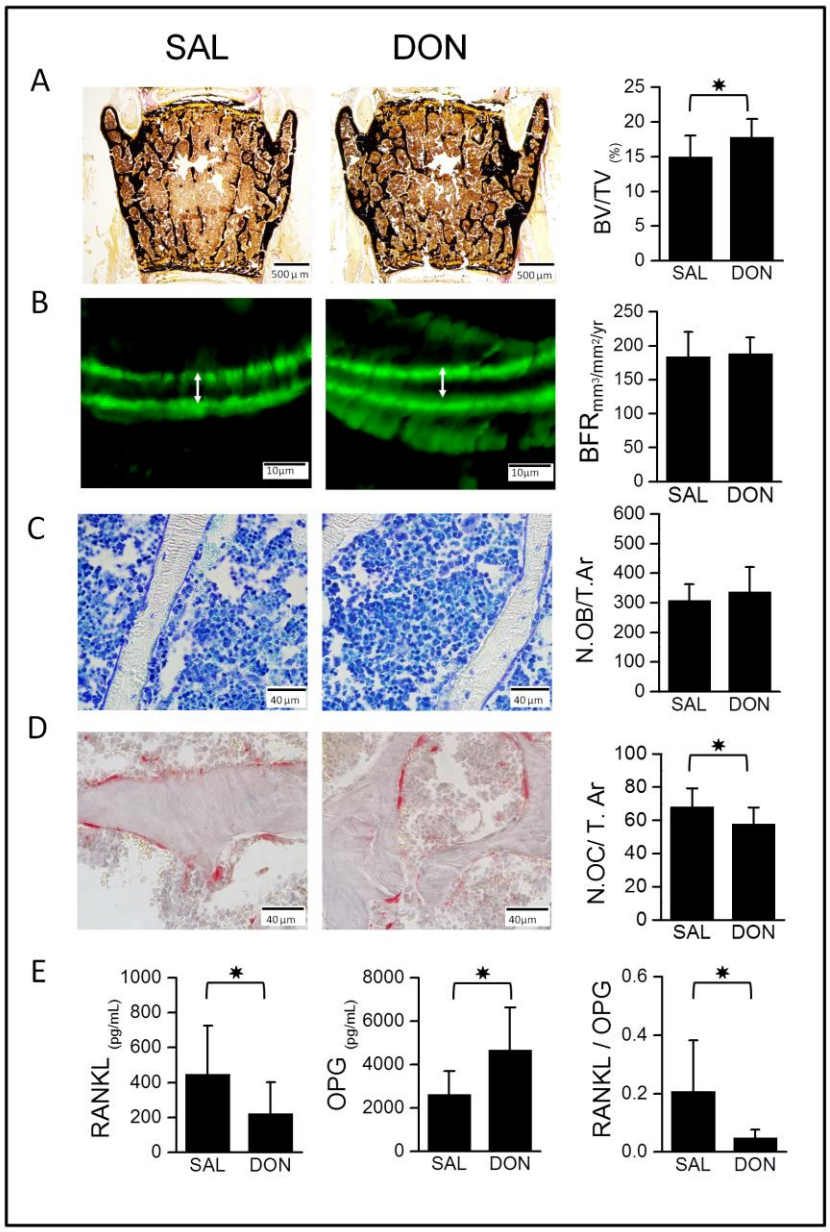

Figure 4.9.3. Donepezil affects osteoclast numbers. A. Von Kossa and van Gieson-stained lumbar vertebra sections showing that mice treated with donepezil (DON, n=12) had higher BV/TV compared to saline-treated mice (SAL, n=12). **B.**

Calcein double labeling shows no significant change in BFR between the control and experimental groups. The white arrows show the distance between calcein double labels **C and D.** Toluidine blue- and Tartrate-resistant acid phosphatase (TRAP)-staining of lumbar vertebra sections demonstrates that there was no change in osteoblast numbers, however, the osteoclast numbers were significantly lower in donepezil-treated mice than in control mice. **E.** Compared to saline-treated mice, donepezil-treated mice had low RANKL serum level, high OPG serum level and low RANKL/OPG ratio. Data are presented as mean±SD. *$p<0.05$.

CHAPTER 5: Non-Neuronal Cholinergic Stimulation Favors Bone Mass Accrual

5.1. Preface:

In chapter 4, we showed that the administration of donepezil in mice enhances their bone quality. In addition to its effect on bone, donepezil regulated the body metabolites, indicating that the donepezil crossed the blood brain barrier and stimulated the cholinergic receptors located within the central nervous system. Interestingly, both cholinergic receptors (muscarinic and nicotinic) are expressed in the peripheral tissues including bone. In addition, cholinergic receptors are very abundant in immune cells[210], and their stimulation affects the cytokine secretion pattern[211,212].

Since the immune system is also a major regulator of bone metabolism[2], stimulating the cholinergic receptors located peripherally might affect bone mass accrual directly by acting on bone cells or indirectly through the immune system (cholinergic-immune pathway). This chapter presents the results of an *in vivo* study conducted in order to investigate the effects of neostigmine, a peripheral acting AChEI[10], on bone mass accrual.

Non-Neuronal Cholinergic Stimulation Favors Bone Mass Accrual

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5.2. Abstract:

Non-neuronal cholinergic receptors are expressed in immune cells and their stimulation has been shown to regulate the secretion of several cytokines. Some of these cytokines, such as interleukin (IL)-17, IL-23, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), are known to regulate bone mass. Accordingly, we hypothesize that stimulating cholinergic receptors in non-neuronal cells, such as immune cells, promotes bone mass accrual. To test this hypothesis, we used neostigmine, a drug that increases acetylcholine levels by inhibiting acetylcholinesterases in the peripheral tissues. Here we show that neostigmine treatment promotes bone mass accrual in endochondral bones of both the axial and appendicular skeleton. The body mass index, body weight, visceral fat pad weight and epinephrine levels in the neostigmine-treated mice were similar to those of saline-treated mice, indicating that neostigmine favored bone

mass accrual by acting peripherally rather than centrally. The increased bone mass in the neostigmine-treated mice was caused by an increase of osteoblast proliferation and bone formation rate. We also observed an increase of circulating immunocytokine IL-17 levels in the neostigmine-treated mice. Statistical analysis showed that the increase of serum IL-17 level was associated with the increase of osteoblast number. In agreement with our findings from the *in vivo* experiments, IL-17 treatment increased the proliferation of MC3T3.E1 preosteoblasts *in vitro*, while acetylcholine or neostigmine did not have any significant effect. Taken together, these findings demonstrate that the peripheral cholinergic stimulation of non-neuronal tissues promotes bone mass accrual indirectly through the immune system.

5.3. Introduction:

Bone remodeling is a lifelong process which is critical to maintain a healthy bone mass. This process involves a balance between bone resorption and bone formation and can be regulated both locally and centrally [2]. The direct interactions between bone cells (i.e. osteoblasts and osteoclasts) and their interaction with immune cells (i.e. T-cells) regulate the net outcome of bone remodeling locally[2,213]. On the other hand, centrally, bone remodeling is regulated through the hypothalamic-pituitary-thyroid axis, which coregulates bone, adipose tissue and energy metabolism via the adrenergic sympathetic arm (SNS) of the autonomic nervous system [3,5,6,12,14,17,156,157]. Recent studies have provided

evidence that the other arm of the autonomic nervous system, the cholinergic parasympathetic nervous system (PNS), also affects bone remodeling [7,8,118].

The main neurotransmitter of the PNS is acetylcholine [18]. In cholinergic neurons, choline acetyl transferase, a cytoplasmic enzyme, synthesizes acetylcholine from acetyl-CoA and choline[19]. During neurotransmission, acetylcholine released from the nerve-ends exerts its signalling effects by targetting either nicotinic (α, β, γ, δ and ε) or muscarinic receptors (ChRM 1-5) present in the post-synaptic neurons or non-neuronal cells [20,21]. The signal is then terminated when acetylcholine is hydrolyzed by acetylcholinesterase, present mainly in the cholinergic nerve synapses and junctions[24].

The first proof of cholinergic regulation of bone mass came from an animal study in which it was shown that mice lacking muscarinic receptor 3 (ChRM3) globally present a low bone mass phenotype caused by decreased bone formation and increased bone resorption [8]. These mice showed an increased sympathetic tone, suggesting a neuronal role for ChRM3 in the regulation of bone mass [8]. However, the role of peripheral (non neuronal) ChRM3 on bone mass was not clarified.

The cholinergic pathway is also known to regulate the immune system through a newly discovered cholinergic-immune pathway [214]. This pathway plays a critical role in controlling systemic and local inflammatory processes via the peripheral cholinergic receptors in immune cells [214]. Cholinergic receptors are expressed in the immune cells, such as T-lymphocytes and macrophages, and their activation has been shown to regulate the secretion of several cytokines

[211,212,215-217]. Some of these cytokines, such as IFN-γ and TNF-α, are known to regulate bone mass accrual by affecting osteoclast activity; whereas one cytokine in particular, IL-17, has the ability to stimulate osteoblast proliferation [2,211,212,215-218].

Based on the above findings, we hypothesize that cholinergic activity regulates bone metabolism through the immune system. Accordingly, we investigated the effects of neostigmine, a cholinergic agonist that acts peripherally, on bone mass accrual. Neostigmine is an acetylcholinesterase inhibitor (AChEI) that prevents acetylcholine degradation resulting in enhanced cholinergic signaling both in the synaptic spaces and in the peripheral tissues[10].

5.3. Methods:

5.4.1. Mice

All *in vivo* experimental procedures of this study were performed following an animal use protocol approved by the Animal Care Committee of McGill University. Five-week-old female wild type C57BL/6J mice were procured form Jackson Laboratory (Bar Harbor, MA). All mice were kept in a pathogen-free standard animal facility, maintained under a 12 hours light - dark cycle with *ad libtum* access to food and water. The mice were randomly divided into 2 assigned groups of 12 mice each, and treated daily with one specific drug for 6 weeks. Group 1 was injected intraperitoneally (i.p.) with neostigmine (0.08 mg/kg); and group 2 was injected i.p. with normal saline (0.2 ml) as control group. The mice were sacrificed 6 weeks after the start of drug injection.

5.4.2. Locomotor activity

Locomotor activity of the different treated mice groups was evaluated using open field box. The box consisted of a transparent plastic box (30 X 30 X 60 cm). The floor of the box was divided into 9 squares. Mice were placed individually in the box for 5 min, and number of squares crossed by each mouse during this period was counted[133].

5.4.3. Body metabolites and bioassays

Body weight and abdominal fat pad weight were assessed for each mouse. Blood and urine samples from each mouse were collected before **sacrifice**. Serum leptin (Life Technologies, Gaithersburg, MD) and insulin (B-Bridge International, BioCat, Cupertino, CA) levels were measured using commercially available ELISA kits. Urinary catecholamine (Elisa kit, BlueGene, Biotech, Shanghai, China) contents were measured in acidified spot urine samples, and creatinine (Elisa kit, Quidel Corporation, San Diego, CA) was used to standardize between urine samples. Serum C terminal telopeptides of type I collagen (CTX) level was measured using RatLaps EIA (IDS) kits. Serum IL-17, TNF-α and IFN-γ levels were assessed using commercially available Elisa kits (eBioscience, San Diego, CA).

5.4.4. 3D-micro computed tomography analysis

μCT analyses were performed as previously described[180]. Briefly, proximal left tibiae of each mouse were scanned using SkyScan 1072 (Bruker-Microct, Kontich, Belgium) machine and analyzed using bone-analysis software

(Version 2.2f, Skyscan, Kontich, Belgium). The following 3D morphological parameters were evaluated: bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N) and cortical thickness (Ct.Th)[134].

5.4.5. Dual X-ray Absorptiometry

Bone mineral density (BMD) of left tibia collected from different treatment groups was measured using a PIXIMUS bone densitometer (GE Medical Systems, Schenectady, NY, USA). The DXA parameters were adjusted according to previous work in the field[180].

5.4.5. X-ray diffraction (XRD)

The left tibia of each mouse was manually crushed into powder, de-fated with Acetone (Sigma–Aldrich, Oakville, Ontario) and left to dry at ambient temperature for 48 hours. The bone powder specimens were submitted to XRD (D8-Discover/GADDS, Bruker, Karlsruhe, Germany). The XRD parameters were adjusted according to previous work in the field[219]. DIFFRACplus EVA software (Bruker AXS, Karlsruhe, Germany) was used to analyze the XRD spectra. Crystal dimensions along a- and c- axes were calculated from the peak broadening of the powder x-ray diffraction peaks (002) and (310), respectively, according to Scherer formula[219].

5.4.7. Raman analysis

Crystallinity index analysis on mid-shaft of the left tibia was conducted by means of Raman spectroscopy. A Raman spectrophotometer (Senterra, Bruker, Karlsruhe, Germany) equipped with 785nm diode laser (of 50mW power) coupled with an optical microscope (Olympus Optical Co., Hamburg, Germany). The Raman spectrophotometer parameters were adjusted according to previous work in the field [220]. Crystallinity index was calculated based on the bandwidth at the half peak intensity of the $\nu_1 PO_4^{-3}$ band at 960 cm^{-1}.

5.4.8. Mechanical testing

A three-point breaking test was performed on the midshaft of the mice right tibiae using Instron 5569 (Instron Corp., Canton, MA, USA) machine [180]. The span of two support points was 10 mm, and the deformation rate was 1.0 mm/min. The extrinsic parameters: stiffness and ultimate force were calculated from the resulting load-displacement curves.

A Vickers microhardness indenter machine (Clark CM100 AT, HT-CM-95605, Shawnee Mission, KS) was employed on cortical bone of the resin-embedded lumber vertebral bodies [221]. The indentation load was adjusted to 10g per 10s. A computer software (Vision PE 3.5, Clemex Technologies Inc., Shawnee Mission, KS) was used to measure the microhardness value at the site of indentation from images captured with a built-in camera. Due to the variations in microhardness within the cortical bone, 10 readings were performed for each cortical bone sample with a minimum distance apart of 50 μm [219]. The

microhardness profile of each cortical bone was obtained by calculating the average of the 10 readings.

5.4.9. Histomorphometric analysis

Histomorphometric analyses were performed as previously described[147,181]. Briefly, calcein solution (0.25% calcein and 2% $NaHCO_3$ dissolved in 0.15 M NaCl) was injected twice i.p. in mice (10 μl/g body weight) at an 8-day interval. Mice were euthanized 2 days after the second calcein injection. Lumbar vertebrae were fixed for 24 hr in 4% PFA/PBS, dehydrated in graded ethanol series, embedded in methyl methacrylate resin according to standard protocols and sectioned (7-μm thickness). The undecalcified sections of the lumber vertebra were stained by von Kossa/van Gieson, toluidine blue and tartrate-resistant acid phosphatase (TRAP). Stained bone sections were analyzed for bone volume-to-tissue volume ratio (BV/TV), osteoblast count, osteoclast count and bone formation rate-to-bone surface (BFR/BS) using the Osteomeasure software (Osteometrics, Inc.). All histological images were captured using a camera (DP72; Olympus), acquired with DP2-BSW software (XV3.0; Olympus), and processed using Photoshop (Adobe).

5.4.10. BrdU assay

MC3T3.E1 preosteoblasts cell line was used for the cell culture studies. Preosteoblasts cultures were treated either with acetylcholine (100 μM), muscarine (10 μM), nicotine (10 μM), neostigmine (10 μM) or saline (control).

Proliferation rate in cell culture studies were assessed with BrdU labeling using a commercially available kit (abcam, Toronto, ON).

5.4.11. Statistical analyses

All results are shown as descriptive outcomes (mean ± standard deviation (SD)). Normal distribution of data was checked by the Shapiro-Wilks statistical test. Statistical analyses were performed by Student's two-tailed unpaired t-test. In all experiments, a value of $p < 0.05$ was considered significant as indicated by a single asterisk.

5.5. Results and Discussion:

5.5.1. Neostigmine favors bone mass accrual without affecting the central nervous system

We compared the bone phenotypes of 5-week-old C57BL/6 mice that were treated for 6 weeks with either neostigmine or saline (control). The axial (vertebra) and long (tibia) bones collected from these mice were analyzed using microcomputed tomography (μCT), histomorphometry, Raman spectroscopy, X-ray diffraction (XRD) and mechanical tests. Mice treated with neostigmine had higher bone volume, trabecular number and mineral density in comparison to the saline-treated mice **(Figure 5.8.1A)**. With the increase of trabecular number, trabecular spacing decreased significantly in the experimental group **(Figure 5.8.1A)**. Analyses of bone mechanical and physical properties showed higher bone stiffness, ultimate force, microhardness and increased crystal dimensions in the neostigmine-treated bones compared to the control bones **(Figure 5.8.1.B-D)**.

We observed a higher crystallinity index, however, the mineral-to-organic ratio remained unaltered between the two groups **(Figure 5.8.1.E)**.

No significant differences were observed in locomotor activity, body weight, body fat and serum leptin levels or urinary epinephrine levels between the neostigmine- and saline-treated mice **(Figure 5.8.1F)**. These findings indicate that neostigmine did not affect the sympathetic tone, thus ruling out any involvement of the neuronal system.

5.5.2. Neostigmine increases bone mass by stimulating bone formation rate

Histomorphometric analyses of the lumbar vertebrae further confirmed an increase of bone volume in neostigmine-treated mice and showed that it was accompanied by an increase in bone formation rate over bone surface (BFR/BS) **(Figure 5.8.2A and B)**. Both osteoblast and osteoclast numbers were significantly higher in neostigmine-treated mice than in control mice, indicating an increase in bone turnover that was confirmed by the increase in serum collagen C-terminal telopeptide (CTX) levels **(Figure 5.8.2C and D).**

Our data showing a stimulatory effect of neostigmine on the bone mass is in agreement with that of Bajayo *et al* reporting an increase of bone mass in pyridostigmine-treated mice [7]. However, treatment of mice with pyridostigmine, a reversible AChEI, has been shown to result in the apoptosis of osteoclasts, without affecting bone formation by osteoblasts. In contrary, our data show a bone anabolic effect of neostigmine as a result of increased bone formation by osteoblasts[7]. The discrepancy between the effects of these two different classes of

AChEIs on the dynamic parameters of bone remodeling could be explained by their differential mode of action in the target tissues. This explanation is supported by the observation that, pyridostigmine has fewer muscarinic effects compared to neostigmine[222-224].

5.5.3. Neostigmine stimulates secretion of the immunocytokine IL-17

Our *in vivo* experiments showing unaltered sympathetic activity upon neostigmine treatment indicated that the observed increase of osteoblast number in these mice was solely regulated by a peripheral mechanism. In order to investigate a direct effect of cholinergic stimulation on osteoblast proliferation, we performed cell culture experiments using MC3T3.E1 preosteoblasts. As shown in **Figure 5.8.3A**, 4-day-treatments of these cells with neostigmine, acetylcholine, nicotine (nicotinic receptor agonist) or muscarine (muscarinic receptor agonist) did not affect their proliferation. These results rule out the possibility of a direct effect of cholinergic stimulation on osteoblast proliferation and cannot explain the observed increase in osteoblast number following neostigmine treatments *in vivo.*

Considering the known effects of cholinergic stimulation on the paracrine activities of immune cells [211,212,215-217], we measured the serum levels of several pro-osteogenic cytokines, e.g. TNF-α, IFN-γ, IL-17 and IL-23 in neostigmine-treated mice. As shown in **Figure 5.8.3B**, neostigmine-treated mice showed a 3-fold increase in IL-17 and 1-fold increase in IL-23 levels in comparison to control. No significant differences in the levels of TNF-α or INF-γ were observed.

Accordingly, our data suggests that there is a stimulation of immune cells upon neostigmine treatment.

The increase in IL-23 has been previously shown to stimulate osteoclast proliferation[225,226], which might explain the observed increase in osteoclast number in neostigmine-treated mice. However, changes in IL-23 could not explain the observed increase in osteoblast number following neostigmine treatment.

IL-17, which is primarily produced by the cells of the macrophage/monocyte lineage, more specifically by the T-helper 17 cells, has been shown to affect both osteoclast and osteoblast numbers. For instance, IL-17 is able to stimulate osteoclastogenesis by upregulating the expression of RANKL via the osteoblasts [227,228]. On the other hand, recent evidences have linked the IL-17 with osteoblasts proliferation [218,229-232]. It has been shown that IL-17 can stimulate the differentiation of mesenchymal stem cells towards osteoblasts and away from adipocytes [218,229-232]. Moreover, IL-17 has been found to enhance the differentiation of MC3T3-E1 pre-osteoblasts [229] and stimulate the expression of mature bone markers (Collagen-1, Collagen-2, bone sialoprotein and osteocalcin) in cell culture studies [233]. Accordingly, the significant increase in the serum level of IL-17 might explain the increase in osteoblast number in neostigmine-treated mice.

We investigated the association between serum IL-17 levels and the following parameters: bone volume/tissue volume and osteoblast numbers in both saline- and neostigmine-treated mice. Interestingly, we found a significant

positive association between serum levels of IL-17 and both the bone parameters **(Figure 5.8.3C).**

5.5.4. Osteoblast proliferation was stimulated by IL-17

We next investigated whether IL-17 had any direct mitogenic effect MC3T3.E1 preosteoblasts. In agreement with our findings from the *in vivo* experiments, IL-17 treatment for 4 days significantly increased the proliferation of these cells, while acetylcholine or neostigmine did not show any such effects **(Figure 5.8.3D**). Taken together, our results provide convincing evidence that the local stimulation of cholinergic activity regulates bone metabolism through the immune system.

5.6. Conclusion:

The findings of this study add important components to the existing model of the regulation of bone mass by the autonomic nervous system **(Figure 5.8.4)**[157]. The SNS favors bone mass loss by inhibiting osteoblasts proliferation and increasing osteoclasts proliferation through the local adrenergic activity [3,5,6,12,14,17,156,157]. The PNS appears to favor bone mass through both central and local pathways. The central pathway affects bone mass by suppressing the sympathetic tone through the activity of muscarinic receptors (e.g. ChRM3) expressed in the locus coeruleus [8]. On the other hand, it has been recently shown that the local pathway prevents bone resorption by signaling through the nicotinic receptors present in the osteoclasts [7]. Our current study provides further evidence of a local effect of cholinergic signaling on the bone mass accrual by showing that

cholinergic signalling modulates the peripheral non-neuronal secretion of immunocytokines, promoting osteoblast proliferation and bone formation indirectly.

Although it is well known that the parasympathetic nervous system regulates the activity of the immune cells [234], until recently, the influence of the nervous system or the immune system on bone mass regulation has been assessed as two independent mechanisms [2]. Our work demonstrates that bone mass is regulated through a neuro-immune pathway, in which the nervous system modulates bone remodeling indirectly by acting on the immune cells. Further work is needed to identify the mediators regulating the crosstalk among the nervous, immune and skeletal systems and to elucidate the possible mechanisms underlying this novel axis of bone regulation.

5.7. Acknowledgment:

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5.8. Figures:

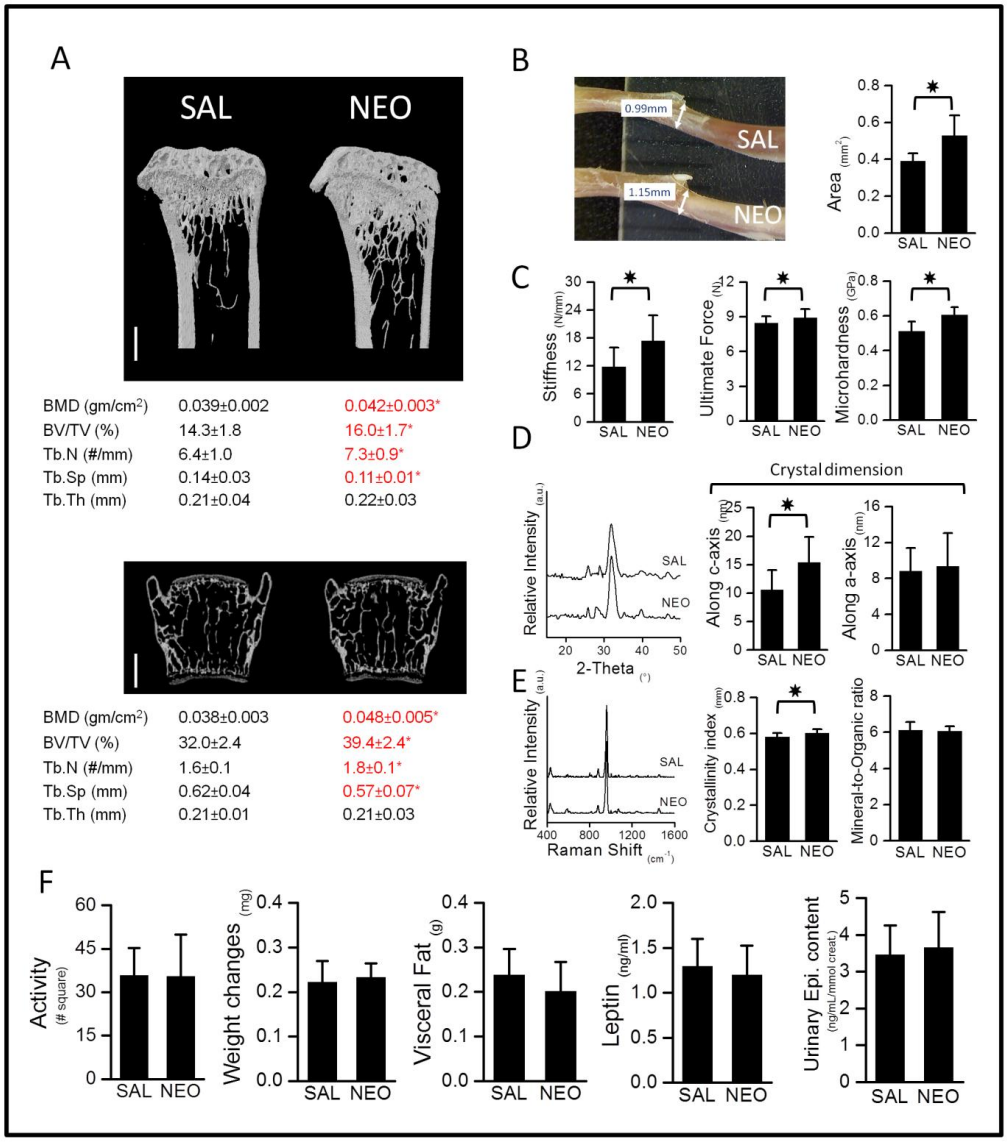

Figure 5.8.1. Neostigmine, a peripherally acting AChEI, favors bone mass and enhances the biomechanical properties of bones. A, μCT images of tibia (top) and vertebra (bottom). Scale bars, 500μm. Neostigmine-treated mice had higher bone mineral density (BMD), bone volume (BV/TV), trabecular number

(Tb.N) and lower spacing between trabeculae (Tb.Sp) compared to the saline-treated mice. No significant change in trabecular thickness (Tb.Th) was observed between the two groups. **B,** A photograph showing the diameter of tibiae obtained from mice treated with neostigmine (bottom) or saline (top). Cross sectional area of mid shaft tibiae in neostigmine-treated mice were larger compared to the saline-treated mice. **C,** Three-point-bending and Vickers' microhardness tests showed higher bone stiffness, ultimate force and microhardness in neostigmine-treated mice compared to control. **D and E**, XRD and Raman spectroscopy analyses revealed that bones of the neostigmine-treated mice had larger crystal dimensions along the c-axis and increased crystallinity with no changes in mineral-to-organic ratio compared to control group. **F,** No significant differences were observed in locomotor activity, body weight, body fat and serum leptin levels or urinary epinephrine levels between the neostigmine- and saline-treated mice, indicating that neostigmine did not alter the SNS tone. Data are mean ± SD. *$p<0.05$.

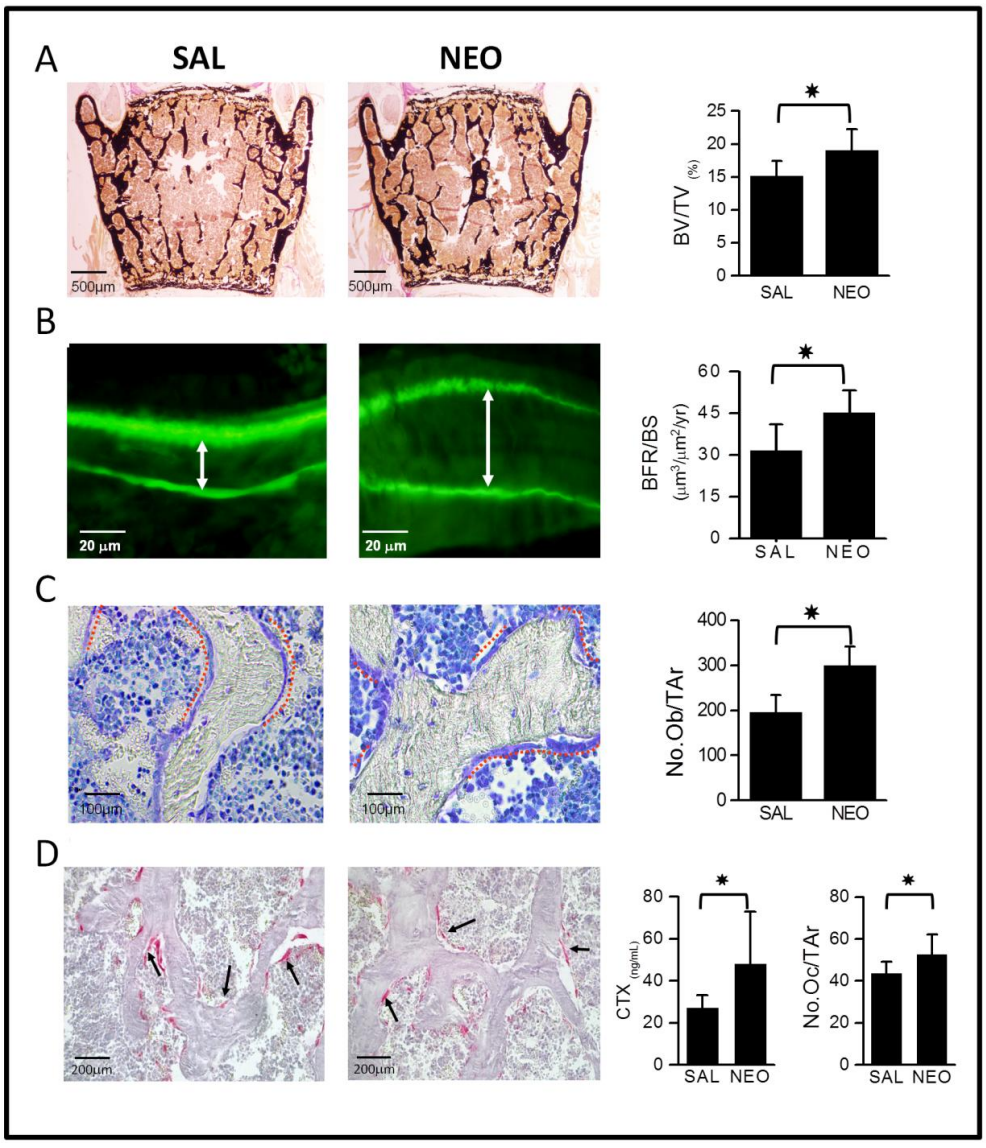

Figure 5.8.2. Neostigmine treatment increases bone mass by stimulating bone formation rate. A, Von Kossa and van Gieson-stained lumbar vertebra sections showing that mice treated with neostigmine (NEO) had higher BV/TV compared to saline (SAL). **B,** Calcein double labeling shows a significantly increased BFR/BS in neostigmine-treated mice compared to controls. The white arrows show the distance between calcein double labels. **C and D,** Toluidine blue- and

tartrate-resistant acid phosphatase (TRAP)- staining of lumbar vertebra sections demonstrates that the osteoblast and osteoclast numbers were significantly higher in neostigmine-treated mice than in control mice. In agreement with the increase in osteoclast number, the serum collagen C-terminal telopeptide (CTX) was increased in the neostigmine-treated group compared to the saline-treated group. Data are mean ± SD. *p<0.05.

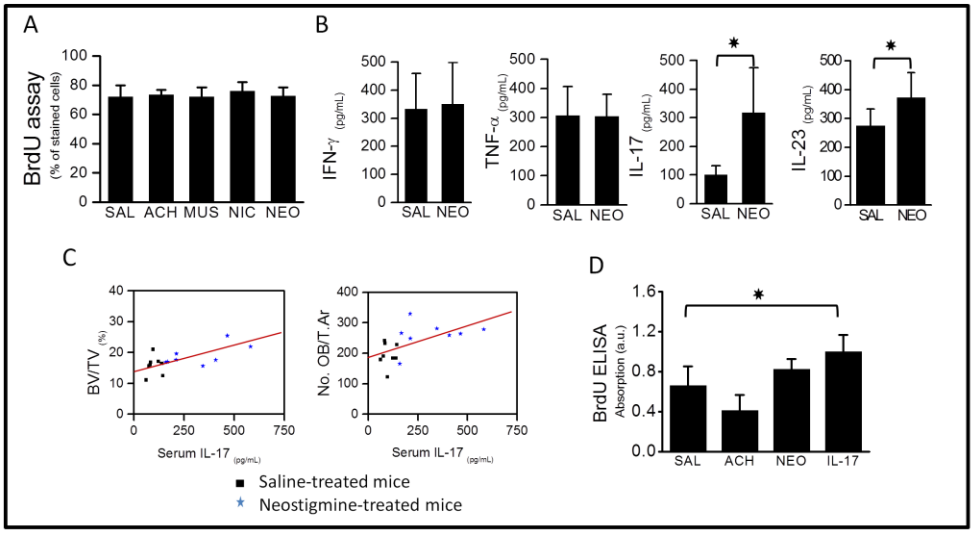

Figure 5.8.3. Osteoblast proliferation was stimulated by the immunocytokine IL-17. A, BrdU assay showing that proliferation, of MC3T3.E1 preosteoblasts, was not stimulated upon treatment with cholinergic ligands, acetylcholine, muscarine, nicotine and an AChEI, neostigmine. **B**, Graphs showing serum levels of TNF-α, IFN-γ, IL-17 and IL-23 in neostigmine- and saline-treated mice. Serum IL-17 level was 3-fold higher in neostigmine-treated group compared to saline-treated group. Serum level of IL-23 was also increased in neostigmine-treated

mice. **C**, Graphs showing the associations between serum IL-17 level, obtained from neostigmine- and saline-treated mice, and bone volume (R=0.624; p=0.009) and osteoblasts number (R=0.579; p=0.029). **D**, Proliferation rates of different MC3T3.E1 preosteoblasts cultures treated for 4 days with either acetylcholine, neostigmine, IL-17 or saline (control) were assessed by BrdU analysis assay. As illustrated, the proliferation of MC3T3.E1 preosteoblasts treated with IL-17 was significantly higher than those treated with saline (control). Data are mean ± SD. *$p<0.05$.

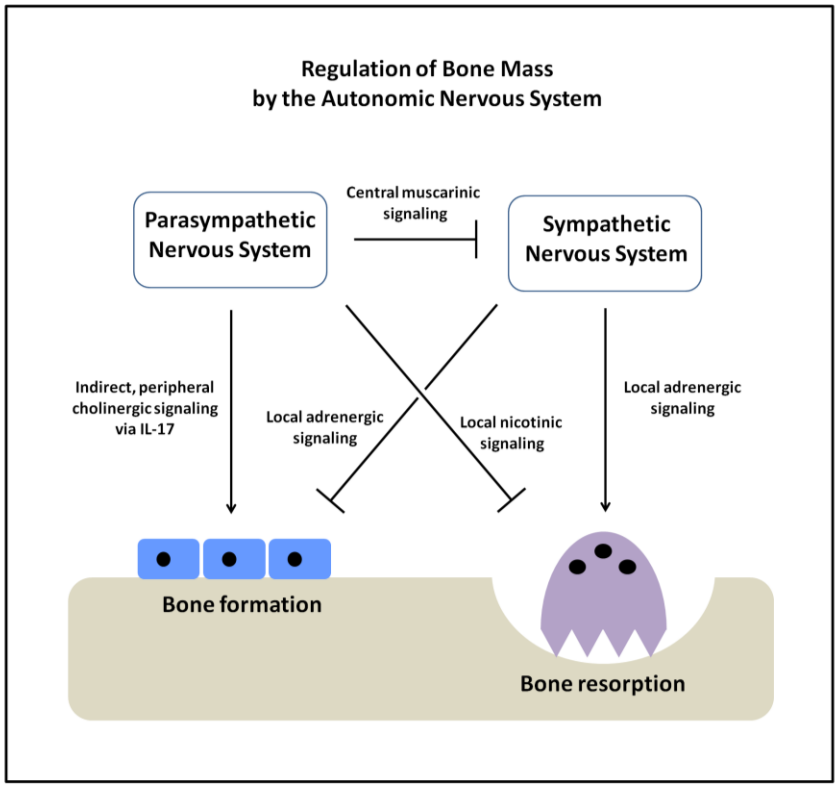

Figure 5.8.4. Schemes showing the regulation of bone mass by the autonomic nervous system (ANS). The autonomic nervous system has two arms, the sympathetic (SNS) and parasympathetic nervous systems (PNS), which function in an opposing yet complementary manner. The SNS inhibits osteoblast proliferation and bone formation. At the same time it promotes osteoclast-mediated bone resorption causing a net loss of bone mass. On the other hand, the PNS favors bone mass accrual through three different axes; *firstly*, by suppressing the SNS tone through the activity of muscarinic receptors within the CNS, *secondly*, by directly stimulating the apoptosis of osteoclasts through the activity of the nicotinic receptors and *thirdly*, by stimulating osteoblast proliferation and bone formation by elevating the secretion of immunocytokine IL-17.

CHAPTER 6: Exposure to Acetylcholinesterase Inhibitors and Risk of Fracture

6.1. Preface:

Data presented in the previous chapters of this thesis suggest that administration of AChEIs increases bone accrual in mice. Building on these important findings, we asked ourselves whether these drugs have clinical effects on bone in humans. AChEIs are already FDA-approved and commercialized, and have been widely used in the treatment of Alzheimer's disease (AD) since the mid-nineties. Accordingly and in order to address our aim, we examined the records of AD patients collected from the United Kingdom Healthcare System Database. We investigated the association between the use of this specific group of drugs by AD patients and their bone quality, represented by the risk of fracture. This chapter presents results of a nested case-control study, conducted in order to assess the effect AChEIs on the risk of bone fracture in an elderly AD population.

Exposure to Acetylcholinesterase Inhibitors and Risk of Fracture

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6.2. Abstract:

Importance: The central nervous system may affect bone remodeling through the cholinergic branch of the autonomous nervous system. Certain drugs used to treat Alzheimer's disease (AD) such as acetylcholinesterase inhibitors (AChEIs), stimulate cholinergic receptors and thus may affect bone mass accrual and quality.

Objective: To determine whether exposure to AChEIs treatments is associated with a reduced in the risk of osteoporotic related fractures.

Design, Setting and Participants: A nested case-control study was conducted using the Clinical Practice Research Database (1998-2012), which contains information on patients in the United Kingdom. The study cohort consisted of AD patients who were older than 65 years. Cases included all patients who had a bone fracture, and were matched to 4 patients who did not suffer from bone fracture.

Main Outcome Measures: Use of AChEIs in case patients *vs.* control patients.

Results: We identified 745 cases and 2980 controls. Compared to nonusers, past use of AChEIs was associated with a reduced osteoporotic bone fracture risk [adjusted odds ratio 0.76 (CI 95%, 0.58-0.99)]. Lower AChEIs compliance (e.i. medication possession ratio 0.0-1.9) was also associated with a reduced fracture risk compared to non-users [adjusted odds ratio 0.74 (CI 95%, 0.56-0.96)]. *Conclusions:* Findings of this study suggest that past exposure and low compliance to AChEIs treatment are associated with a lower risk of osteoporotic bone fractures in elderly AD population.

6.3. Introduction:

Studies have shown that the autonomic nervous system regulates bone remodeling through the activity of its two arms: the adrenergic system and the cholinergic system. Activity of the adrenergic system has been shown to have a catabolic effect on bones[3-6], and drugs (e.g., β-blockers) that inhibit this activity increase bone accrual *in vivo*[5], and increase the bone mineral density[235] and reduce the risk of hip fractures in humans[9]. On the other hand, studies have suggested that the activity of the cholinergic system has an anabolic effect on bones[157]. *In vitro* studies have shown that cholinergic agonists (e.g., nicotine and muscarine) might stimulate the proliferation of osteoblasts, bone forming cells[30,35]. *In vivo* studies on mutant mice models have reported that the loss of specific cholinergic receptors (muscarinic-3 receptor and nicotinic subtype-α_2) is

associated with bone loss[7,8]. Indeed, mice with knockout nicotinic subtype-α_2 receptors are osteoporotic due to the up-regulation of osteoclasts (bone resorbing cells)[7], whereas mice with knockout muscarinic-3 receptors (ChRM3) are osteoporotic due to an increase in osteoclasts number and a decrease in osteoblasts number[8]. These results suggest that the use of cholinergic agonists such as acetylcholinesterase inhibitors (AChEIs) may have positive effects on bone quality in humans.

AChEIs are a group of drugs that prevent acetylcholine degradation resulting in enhanced cholinergic signaling[10]. These drugs have been widely used in the treatment of Alzheimer's disease (AD) and other dementias since the mid-nineties. However, little is known on the potential clinical effects that these drugs might have on bone. We recently published a case-control study on 2178 patients, demonstrated that treatment with AChEIs is associated with a lower risk of hip fracture in elderly AD patients. However, this study was limited by the relatively small sample size.. Moreover, our study was restricted to a population from one healthcare center that covered a limited area raising the question of generalizability of our findings.

Our aim is to re-assess the association between the use of AChEIs with the risk of fracture in a high-quality population-based database that covers a well-defined population. In order to address our aim, we have designed a nested case-control study in which we compared the risk of fracture in a group of patients under AChEI treatment with another group of AD patients receiving no AChEIs in a large cohort representative of general population.

6.4. Methods:

6.4.1. Regulatory approval

The study was approved by the Scientific and Ethical Advisory Group from the Clinical Practice Research Database (CPRD) (previously known as the General Practice Research Database), and the ethics review board of the McGill University Health Centre.

6.4.2. Data source

We conducted a population-based cohort design, with a nested case-control analysis using data set from the Clinical Practice Research Database (CPRD) The CPRD is the world's largest computerized database of longitudinal records from primary care[236-239]. It contains computerized medical records of more than 650 general practices across the UK and complete primary care medical records of approximately 14 million inhabitants of the total registered UK population (corresponding to approximately 22% of the UK population). The age and sex distribution of patients in the CPRD is recorded and it has been shown to be representative of the UK population[240]. The CPRD also includes information on demographic and lifestyle variables, such as height, weight and smoking status[240].

Clinical data collection was done using the Oxford Medical Information System (OXMIS) and read codes for disease, morbidity and mortality that are cross referenced to the International Classification of Diseases (ICD-10). Prescriptions are recorded using a coded drug dictionary based on the UK

Prescription Pricing Authority Dictionary. Only data that pass a quality control is included in the CPRD database and considered "up-to-standard"[241]. As previously noted the CPRD database has a high level of validity and completeness and has been validated as a reliable source for epidemiological data on patients with hip fractures, and on AD patients. Also, the recorded information on drug exposures and diagnoses has been validated and proven to be of high quality[236-240]. For these reasons the CPRD is the most used database for research, with over 700 peer-reviewed publications (information provided by the CPRD).

6.4.3. Cohort definition and follow-up

We identified patients aged 65 years and above with a first-time diagnosis of any type of dementia or AD according to the ICD-10 and OXMIS coding systems, which were recorded in the computerized database between January 1998 and December 2013, or patients who received a first-time prescription of an AChEIs or memantine during the same period. To increase the probability of including only well-defined AD patients in the study population, we used an algorithm previously described by Imfeld et al, which was applied to all potential cases with a first-time diagnosis of AD (7). In order to be considered as eligible AD cases, patients must had either: 1) a diagnosis of AD followed by at least one prescription for an AD specific medication or vice versa; 2) two prescriptions for an AD specific medication; 3) at least two recordings of a AD diagnosis; 4) a AD diagnosis after a specific dementia test (e.g., Clock Drawing Test, Mini Mental State Examination or Abbreviated Mental Test [7-Minute Screen]), a referral to a

specialist (e.g., geriatrician, neurologist or psycho-geriatrician), or an evaluation based on a neuroimaging technique (e.g., magnetic resonance imaging, computed tomography, or single photon emission computed tomography); or 5) an AD diagnosis preceded or followed by any registered dementia symptoms (e.g., memory impairment, aphasia, apraxia, or agnosia) (19) .

Patients diagnosed with conditions known to substantially affect bone metabolism (i.e., osteoporosis, osteomalacia, Paget´s disease, cancer [excluding non-melanoma skin cancer], HIV, rheumatoid arthritis, congestive heart failure, cerebrovascular accident, peripheral vascular disease or alcoholism) as well as patients who used medications known to affect bone metabolism (i.e., corticosteroids, anti-epileptic drugs, anxiolytics, calcium-vitamin D supplements, antihypertensive drugs or bisphosphonates) prior to the diagnosis of AD were excluded. All the participants had at least two consecutive years of up-to-standard follow-up in the CPRD records before the diagnosis of AD.

6.4.4. Case definition and validation

Cases consisted of all the individuals in the study cohort with the diagnosis of an incident fracture (index date) at least 1 year after the diagnosis of AD (to ensure a sufficient period of exposure), potential cases were identified without any exposure information. We included only fractures which are associated with osteoporosis ”osteoportic fractures” such as proximal femur, pubic rami, ribs, distal radius, proximal humerus and vertebral body, using the ICD-10 and OXMIS coding systems. The proportion of patients recorded in the

CPRD with fractures secondary to severe polytrauma is relatively low (0.5%-1%)[242], therefore we decided to include all causes of fracture identified in the computerized database. Patients who suffered more than one fracture during the study period were only included once, and only the first fracture was considered for analysis.

6.4.5. Controls

Controls were AD patients selected from the study cohort who did not suffer from an osteoporotic fracture during the study period. We matched up to 4 controls per case, using incidence density sampling [243]. Controls were matched on age (±2 years), sex, up-to-standard follow-up in the CPRD records (±1 year), calendar time (same index date as for cases), and duration of AD (±6 months). Furthermore, controls had to be alive on the index date. The same exclusion criteria were applied to controls as to case patients.

6.4.6. Exposure assessment

We identified exposure to AChEIs (e.i., donepezil, oral rivastigmine, transdermal rivastigmine and galantamine) for all cases and controls prior to the index date. The use AChEIs is associated with a risk of orthostatic hypotension [244] ***and*** syncope related-falls[245-248], which could depend on the time of exposure. For this reason we decided to classify patients as current users if the last prescription for a study medication was registered 1 to 59 days prior to the index date, as recent users if it was registered 60 to 119 days prior to the index date, and as past

users if it was registered more than 120 days before index date. All others were considered nonusers.

For further analysis we divided the users of AChEIs into 5 different treatment categories: users of oral rivastigmine, transdermal rivastigmine, donepezil, galantamine or combinations of treatment. Patients were included in the "combinations of treatment" subgroup if they had used different AChEIs within 1 year prior to the to the index date [249,250]. In the UK, AChEIs are commonly provided in 1 month prescriptions; however, a significant number of prescriptions are given for periods of less than one month [251]. To overcome these differences and avoid possible bias, prescriptions of 28 or more unit doses (i.e, tablets, patches, solutions, capsules) were assumed as monthly, because all the study drugs are taken at least once a day [251]. On the other hand, when prescriptions were of less than 28 unit doses, the duration of treatment was calculated by dividing the unit dose by the daily regime of the specific drug (intake times per day). Moreover, and in order to assess the drug compliance, we analyzed the number of days of treatment registered in the database during the year prior to the index date, and calculated the medication possession ratio (MPR). Patients were divided into three different categories of compliance according to the MPR (0.0-0.19, 0.20-0.79, and 0.80-1.0) [252]. The dose effect on the fracture risk was analyzed using the defined daily dose (DDD) of each drug according to the World Health Organization (i.e., donepezil 7.5 mg, oral rivastigmine 9 mg, transdermal rivastigmine 9.5 mg, galantamine 16mg) [46]. The

average daily dose in the year prior to index date was calculated for each study drug by dividing the sum of the number of daily doses by the duration of treatment [46,253]. Therefore, patients were divided into two categories according to the average daily dose per year (≤DDD or >DDD) [253].

6.4.7. Statistical analysis

We performed conditional logistic regression analyses using SAS version 9.3 (SAS Institute Inc, Cary NC). Crude and adjusted risk estimates are presented as odds ratios (ORs) with 95% confidence intervals (CIs). *P* values were considered statistically significant if less than 0.05.

Odds ratios were adjusted at the index date for the following confounders, age, sex, body mass index (BMI; <20, 20-24, 25-29, >30 kg/$m^2$and unknown), smoking status (none, current, ex-smokers, unknown), MPR (0.0-0.19, 0.20-0.79, and 0.80-1.0), dose (≤DDD or >DDD), fall risk assessment tool (FRAT) score, long stay at hospital, poor mobility (e.g, home visits by the GP, or use of walking aid), institutionalization (e.g, patient receiving residential care, living in a care home, or nursing care), medical conditions associated with an increased fracture risk (i.e. chronic pulmonary disease, diabetes, hemiplegia, chronic liver diseases, peptic ulcer, renal diseases) and exposure to drugs such as memantine or medications that could increase the fracture risk (i.e, proton pump inhibitors, statins, and selective serotonin reuptake inhibitors)[254,255]. Patients with medication-related variables were defined dichotomously as users (ie, ≥12 months

of cumulative use before index date and last prescription ≤6 months before the index date) and nonusers.

6.5 Results:

After applying the above described algorithm, we concluded with 10459 patients with a well-defined diagnosis of AD. A total of 745 osteoporotic fractures were identified within this cohort; cases were matched with 2980 controls. The most frequent fractures were of the proximal femur (n=398 [53.4%]) and wrist/forearm (n=149 [20.0%]). The age and sex distribution of cases and controls, as well as the distribution per years of CPRD follow-up, smoking, BMI, duration of AD, FRAT score, comorbidity factors, and drugs known to increase fracture risk, are displayed in **Table 6.9.1**. Fractures were distributed according to age categories and were more frequent in women than in men (78.7% vs 21.3%). There was an association between low BMI and increased fracture risk. The use of SSRI was associated with an overall higher risk of fracture.

To analyze the association between the use of AChIEs (i.e, any use, oral rivastigmine, transdermal rivastigmine, donepezil, galantamine and combination of treatments) and fracture risk we divided the cases into three categories (current, recent and past users). Current users of AChEIs had an adjusted OR of 1.15 (95% CI, 0.75-1.79), whereas recent users had an adjusted OR of 1.28 (95% CI, 0.86-1.92). On the other hand, past users of AChEIs who received the last prescription at least 120 days before the index date had an adjusted OR of 0.76 (95% CI, 0.58-0.99). Past users of galantamine had an adjusted OR of 0.54 (95% CI, 0.31-0.94).

The overall fracture risk for any users of AChEIs as well as past users of oral rivastigmine, transdermal rivastigmine and donepezil was lower when compared with non-users: however these results were not statistically significant (**Table 6.9.2).** Interestingly, current and recent AChEIs users also had a higher number of falls compared to non-users; nevertheless, these differences were not significant. On the other hand, past users had similar number of falls compared to non-users (**Table 6.9.3)**. Lower AChEIs compliance (e.i., MPR 0.0-0.19) was associated with reduced fracture risk when compared to non-users, OR 0.74 (CI 95%, 0.56-0.96); higher MPR values (e.i., 0.19-0.79 and 0.8-1.0) were not associated with significant changes in the fracture risk when compared to non-users (**Table 6.9.4**). Finally, we did not observe a dose effect on the fracture risk within the different treatment categories.

6.6. Discussion:

In this study, we found that the use of AChEIs can significantly affect bone health in elderly AD patients. Indeed, our analyses indicate that past usage of AChEIs was associated with a lower risk of osteoporotic fractures. These findings provide further evidence on the association between the cholinergic system and bone tissue.

Current or recent exposure to AChEIs did not significantly alter the fracture risk compared to non-users despite being at a higher risk of fall. The use of AChEIs is known to affect the cardiovascular system causing bradycardia, orthostatic hypotension, and thus increasing the risk of syncope related falls [245-

[248]. In our study, we observed that current AChEIs users tend to have higher falling rates compared to non-users. Although these finding were not statistically significant due to the relatively small number of cases, these results are in concordance with previous reports in the literature[256,257]. There is a well-known casuistic association between the rate of falls and the risk of osteoporotic fractures[258]; therefore, the potential positive effects of AChEIs on bone tissue could be counteracted by an increase in the number of falls among current users. In our study, we did not find differences in the fracture risk between current AChEIs users and non-users despite having higher falling rates.

We found a significant reduction in the risk of osteoporotic fractures among the AChEIs past users compared to non-users. Interestingly, AChEIs past users had similar falling rates to nonusers, but yet lower risk of fracture. This may suggest that past users, who are no longer exposed to the cardiovascular side effects of AChEIs[244], could benefit from a residual positive effect of these medications on bone remodelling.

This study suggests that exposure to galantamine can influence bone tissue in AD patients. A previous case-control study, conducted by our group in Spain, failed to find an association between galantamine and the risk of hip fracture due to the limited sample size[118]. In the current study, we found that past users of galantamine had a significantly lower fracture risk compared to nonusers. Galantamine stimulates both nicotinic and cholinergic receptors; it acts as an allosteric potentiation ligand to nicotinic acetylcholine receptors, and as a weak

competitive reversible cholinesterase inhibitor. Nicotinic receptors, specifically α_2 receptors, are expressed in osteoclasts, and it is known that their stimulation with nicotinic agonists induces osteoclast apoptosis and a marked reduction in the number of TRAP-positive osteoclasts in wild type mice[7]. Moreover, a low stimulation of the nicotinic receptors expressed by osteoblasts is associated with an increase in their proliferation whereas higher stimulation decreases their proliferation, possibly due to receptor desensitization[7]. On the other hand, muscarinic receptors, such as M3 receptors, have been found to influence bone remodelling favouring bone formation and decreasing bone resorption. M3 receptor knockout mice are osteoporotic due to an increase in the number of osteoclasts and a decrease in the number of osteoblasts[259]. Accordingly, it seems that galantamine could have several positive effects on bone remodelling such as increasing osteoblastic proliferation and inducing osteoclastic apoptosis, mediated by both muscarinic and nicotinic receptors[7,259].

Our previous case-control study suggested that AChEIs had a strong protective effect against hip fractures which was observed regardless of the time of exposure OR 0.42 (CI 0.24-0.72)[118]. However, the present study only observed a significant protective effect on past users and patients with galantamine. Differences between the two studies could be explained by disparity in the exclusion-inclusion criteria, sample size and prescription regimes. In contrary to our previous work, in this study we excluded all patients previously diagnosed with high blood pressure as well as individuals on antihypertensive drugs.

It has been reported that there is a higher prevalence of osteoporosis in hypertensive patients, which could be explained by low calcium intake , vitamin D and vitamin K deficiency, high consumption of sodium or high nitric oxide levels [239,260]. However, we believe that the activity of the sympathetic system could be an additional etiological factor, as hypertensive individuals are known to have a higher sympathetic tone [261], and there is solid evidence suggesting that an increase in the adrenergic sympathetic signal can reduce the bone mass [5,262,263].

On the other hand, the use of antihypertensive drugs such as beta-blockers, thiazide or a combination of these two drugs is known to decrease the fracture risk in patients with high blood pressure[9]. For all these reason we believe that the cholinergic stimulation of bone caused by AChEIs could be particularly beneficial in hypertensive patients [157].

Less compliance among AChEIs users (i.e., MPR 0.0-0.19) was associated with a lower fracture risk compared to non-users. On the other hand, individuals who were more compliant (e.i., MPR 0.2-0.79 and 0.8-1.0) did not experience a reduction in the fracture risk.

Patients taking AChEIs irregularly could be less exposed to the undesirable cardiovascular side effects mentioned earlier but could still benefit from the potentially positive effects AChEIs may exert on bone remodeling. On the other hand, more compliant individuals, could be more exposed to bradychardia and syncope and consequently be at a higher risk of fall. In this study, we did not find differences in the fracture risk between more compliant

patients (e.i., MPR 0.2-0.79 and 0.8-1.0) and non-users, probably because in these patients the two potential mechanisms by which AChEs may influence the fracture risk (e.i., stimulation of bone remodelling, and cardiovascular side effects) are neutralizing each other. Our results suggest that the scattered administration of AChEIs could have a protective effect against osteoporotic fractures in AD patients and avoid the unwanted cardiovascular side effects associated with these medications. This is not a new concept as other medication known to influence bone remodelling are such risedronic acid, denosumab are administered monthly or every 6 months respectively[264,265].

6.6.1. Strength and limitations

This study was performed on a high quality population-based database that covers a well-defined population with high levels of ascertainment of cases with a low likelihood of selection bias. Previous studies have validated the accuracy of the CPRD in the ascertainment of patients with AD and patients with fractures[9,266]. In addition, this study included only well-defined AD patients from the study population, following an algorithm that has been previously validated [267]. Moreover, our analyses of odds ratios were adjusted to several confounders that may affect the results of our study including: age, sex, years in the CPRD, body mass index, smoking status, duration of AD, history of falls, medical conditions associated with an increased fracture risk and exposure to medications related to an increase in the fracture risk.

However, our study may be subject to several problems that routinely accompany non-experimental pharmaco-epidemiological research. This occurred mainly due to the small sample size of the cohort population due to our extremely restricted inclusion criteria that aimed to minimize the selection bias in our study. Moreover, our study assessed the association of AChEIs with non-site specific osteoporotic fractures (hip/femur/pelvis, ribs, forearm/wrist, proximal part of the humerus and vertebra bone fractures) due to the insufficient data for each type of fracture.

6.7. Conclusion:

Our results indicate that AD patients who were past users or less compliant to AChEIs treatment were associated with a lower risk of osteoporotic fractures. Although future research is required, results of this study provide useful information on the novel use of an existing class of approved and commercialized drugs for the treatment of bone diseases such as osteoporosis.

6.8. Acknowledgment:

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6.9. Tables:

Table 9.6.1. Characteristics of Case Patients With Fracture and Matched Controls*.

Characteristics	No. %	
	Cases (n= 745)	**Controls (n= 2,980)**
Age, y‡		
65-69	1.34	1.21
70-74	7.92	7.52
75-79	14.77	15.17
80-84	27.11	27.01
≥85	48.86	49.09
Sex ‡		
Men	21.34	21.34
Women	78.66	78.66
Years in the CPRD‡		
Mean ±SD	7.46 ± 3.54	7.39 ± 3.65
Median (IQR)	7.35 (5.48)	7.13 (5.93)
Smoking status		
None	59.06	62.82
Current	10.60	10.00
Ex-smoker	25.23	22.35
Unknown	5.10	4.83
Body mass index §		
<20	16.51	12.65
20-24	35.70	34.19
25-29	21.34	23.19
≥30	5.50	9.19
Unknown	20.94	20.77
Duration of Alzheimer disease ‡		
<2	32.89	32.89
2-6	59.60	59.60
≥6	7.52	7.52
FRAT		
Low risk	90.07	95.50
Moderate risk	8.86	4.23
High risk	1.07	0.27
Comorbidity factors		
Chronic pulmonary disease	18.79	17.65
Diabetes	7.38	7.68
Hemiplegia	0.40	0.44
Chronic liver disease	0.67	0.47
Peptic ulcer	11.41	10.84
Renal diseases	2.68	2.68
Long stay in hospital	6.98	7.05
Poor mobility	5.91	6.07
Institutionalization	20.81	18.62
Drugs known to increase the fracture risk		
PPI	22.15	19.56
Statins	21.07	20.94
SSRI	24.16	17.48
Other drugs		
Memantine	2.82	2.62

Abbreviations: CI, confidence interval; FRAT, Fall Risk Assessment Tool; PPI, proton pump inhibitors; SSRI, selective serotonin reuptake.
*Percentages may not sum 100% due to rounding.
‡Matching variables.
§Measured as weight in kilograms divided by the square of height in meters.

Table 9.6.2. Use of Acetylcholine Inhibitors and Fracture Risk (vs. Nonuse)*.

Exposure	No. % Cases (n= 745)	No. % Controls (n= 2,980)	Crude Odd Ratio (95% CI)	Adjusted Odd Ratio (95% CI)†
Non user of acetylcholinesterase inhibitors	24.03	24.56	1.0	Ref
Any users of acetylcholinesterase inhibitors	75.97	75.44	1.03 (0.85,1.25)	0.84 (0.65,1.07)
Current, No. days of treatment‡	45.23	42.45	1.10 (0.89,1.35)	1.15 (0.75,1.79)
Recent δ	13.15	10.74	1.25 (0.94,1.66)	1.28 (0.86,1.92)
Past γ	17.58	22.25	0.80 (0.62,1.03)	**0.76 (0.58,0.99)**
Oral rivastigmine tablets				
Current, No. days of treatment‡	2.95	3.12	0.97 (0.59,1.58)	0.99 (0.53,1.85)
Recent δ	0.94	0.67	1.43 (0.60,3.41)	1.60 (0.62,4.11)
Past γ	1.21	1.64	0.75 (0.36,1.55)	0.69 (0.33,1.45)
Transdermal rivastigmine				
Current, No. days of treatment‡	0.81	0.40	2.08 (0.77,5.62)	2.16 (0.73.,6.40)
Recent δ	0.00	0.03	0.00	0.00
Past γ	0.13	0.17	0.84 (0.10,7.20)	0.94 (0.11,8.22)
Donepezil				
Current, No. months of treatment‡	31.68	29.50	1.11 (0.89,1.38)	1.18 (0.75,1.843)
Recent δ	9.80	7.89	1.27 (0.93,1.73)	1.32 (0.86,2.01)
Past γ	12.89	14.87	0.87 (0.66,1.15)	0.83 (0.63,1.11)
Galantamine				
Current, No. months of treatment‡	7.38	7.28	1.05 (0.75,1.48)	1.14 (0.68,1.92)
Recent δ	2.01	1.44	1.44 (0.78,2.68)	1.51 (0.76,3.03)
Past γ	2.28	3.93	0.59 (0.34,1.02)	**0.54 (0.31,0.94)**
Combination of treatment				
Current, No. months of treatment‡	2.42	2.15	1.14 (0.66,1.98)	1.12 (0.56,2.21)
Recent δ	0.40	0.70	0.59 (0.17,1.98)	0.50 (0.14,1.78)
Past γ	1.07	1.64	0.66 (0.305,1.42)	0.61 (0.28,1.34)

Abbreviations: CI, confidence interval

*Percentages may not sum 100% due to rounding

† Adjusted to age, sex, smoking status (none, current, ex-smokers, unknown), medication possession ratio (0.0-0.19, 0.2-0.79, 0.8-1.0) ,body mass index, (<20, 20-24, 25-29, >30 and unknown), Fall Risk Assessment Tool score, comorbidity factors (chronic pulmonary disease, diabetes, hemiplegia, chronic liver diseases, ischemic heart disease, peptic ulcer, renal diseases, long stay in hospital, poor mobility, institutionalization), and prior history of use of proton pump inhibitors, statins, selective serotonin reuptake inhibitors and memantine.

‡ Current users: last prescription within 0-59 days.

δ Recent users 60-119 days.

γ Past users >120 days.

Table 6.9.3. Number of Falls in Users of Acetylcholine Inhibitors (vs. Nonuse) According to Time of Intake [ε].

Characteristic‡	Nº falls/patient (Mean ± SD)
Non user of acetylcholinesterase inhibitors	1.06 ± 0.24
Any users of acetylcholinesterase inhibitors	1.19 ± 0.47
Current ‡	1.29 ± 0.58
Recent δ	1.08 ± 0.29
Past γ	1.06 ± 0.25

Abbreviations: CI, confidence interval, SD, standard deviation.
[ε] 120 days before index date,
*Percentages may not sum 100% due to rounding
‡ Current users: last prescription within 0-59 days.
δ Recent users 60-119 days.
γ Past users >120 days.

Table 6.9.4. Drug compliance and Fracture Risk in Users of Acetylcholinestersase Inhibitors (vs. Nonuse)*.

	No. %			
Overall MPR±	**Cases (n= 745)**	**Controls (n= 2,980)**	**Adjusted Odd Ratio (95% CI)†**	***P* Value**
Non user of acetylcholinesterase inhibitors	24.03	24.56	1.0	
Any users Acetylcholinestersase Inhibitors ±				
0.0-0.19	15.57	20.17	**0.75 (0.57,0.98)**	0.0329
0.19-0.79	21.07	18.66	1.14 (0.89,1.46)	0.3117
0.8-1.0	39.33	36.61	1.05 (0.85,1.31)	0.6471

Abbreviations: CI, confidence interval; MPR, medication possession ratio.
*Percentages may not sum 100% due to rounding.
± MPR in the year prior to index date.
† Adjusted to age, sex, smoking status (none, current, ex-smokers, unknown), body mass index, (<20, 20-24, 25-29, >30 and unknown), recency , duration of Alzheimer disease, Fall Risk Assessment Tool score, comorbidity factors (chronic pulmonary disease, diabetes, hemiplegia, chronic liver diseases, ischemic heart disease, peptic ulcer, renal diseases, long stay in hospital, poor mobility, institutionalization), and prior history of use of proton pump inhibitors, statins, selective serotonin reuptake inhibitors and memantine. .

CHAPTER 7: Acetylcholinesterase inhibitors and healing of hip fracture in Alzheimer's disease patients: a retrospective cohort study

7.1. Preface:

In previous chapters, we showed that the use of AChEIs is associated with a better bone quality. Indeed, in chapter 6, the data suggests that use of cholinergic agonists is associated with a reduced risk of osteoporotic fractures in elderly AD patients, and patients who are at risk of developing osteoporosis may potentially benefit from these drugs. These results raise another question; do these drugs have a beneficial effect on bone healing? This chapter presents results of a retrospective cohort study conducted in order to assess the effect AChEIs on bone healing in an elderly population.

Acetylcholinesterase inhibitors and healing of hip fracture in Alzheimer's disease patients: a retrospective cohort study

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7.2. Abstract:

Objectives: This study was designed to assess effects of cholinergic stimulation using acetylcholinesterase inhibitors (AChEIs), a group of drugs that stimulate cholinergic receptors and are used to treat Alzheimer's disease (AD), on healing of hip fractures.

Methods: A retrospective cohort study was performed using 46-female AD patients, aged above 75 years, who sustained hip fractures. Study analyses included the first 6-months after hip fracture fixation procedure. Presence of AChEIs was used as predictor variable. Other variables that could affect study outcomes: age, body mass index (BMI), mental state or type of hip fracture, were also included. Radiographic union at fracture site (Hammer index), bone quality (Singh index) and fracture healing complications were recorded as study

outcomes. The collected data was analyzed by student's-t, Mann-Whitney-U and chi-square tests.

Results: No significant differences in age, BMI, mental state or type of hip fracture were observed between AChEIs-users and nonusers. However, AChEIs-users had better radiographic union at the fracture site (relative risk (RR),2.7; 95%confidence interval (CI),0.9-7.8), better bone quality (RR,2.0; 95%CI,1.2-3.3) and fewer healing complications (RR,0.8; 95%CI,0.7-1.0) than nonusers.

Conclusion: In elderly female patients with AD, the use of AChEIs might be associated with an enhanced fracture healing and minimized complications.

7.3. Introduction:

The central nervous system affects bone remodeling through the adrenergic and cholinergic branches of the autonomous nervous system[8,31,37,268]. Adrenergic activity has been associated with bone resorption and drugs that inhibit this activity have been found to increase bone accrual, reduce the risk of hip fractures and accelerate fracture healing[9,269]. In contrast, cholinergic activity has been recently found to have a positive effect on bone accrual[7,8,45,118,157], however, this physiological mechanism has never been explored as potential therapy for bone diseases such as osteoporosis and fracture healing.

One way of stimulating cholinergic activity is by administration of cholinergic agonists such as acetylcholinesterase inhibitors (AChEIs)[10]. AChEIs are a group of drugs that cause stimulation of cholinergic receptors by inhibiting the action of acetylcholinesterase; thus, increasing the levels of acetylcholine in

the synaptic space. These drugs are widely used to treat Alzheimer's disease (AD) and other forms of dementia since the mid-1990s[270].

Alzheimer's disease (AD) is characterized by a degradation of the hypothalamus, a brain structure that encloses cholinergic components[271]. Surprisingly, AD patients suffer from a low bone mineral density that is highly correlated with the cholinergic degradation of the hypothalamus[65,254]. [63,272]Due to the reduced bone density, it has been shown that AD patients are more prone to bone fractures, particularly hip fractures, compared with the rest of population[59,256,273]. Healing of hip fractures in AD patients is usually slow and often results in delayed-union, non-union, need for re-intervention, or even death[257]. Previously, we have demonstrated that AD patients who are receiving AChEIs, are associated with lower risk of hip fracture[118]. However, the potential effect of these drugs on fracture healing has not been explored yet.

We hypothesize that the administration of AChEIs may have a beneficial effect on bone regeneration that could accelerate fracture healing. In order to test this hypothesis, we have designed a *retrospective cohort* study in which we compared the healing of hip fractures in a group of AD patients under AChEI treatment with another group of AD patients receiving no AChEIs. In this paper, we provide evidence for a potential approach to enhance fracture healing and reduce clinical complications, through stimulation of cholinergic activity by AChEIs.

7.4. Methods:

7.4.1. Study design

Approval from the ethical committee at Carlos Haya Hospital, in Malaga, Spain, was obtained to carry out a retrospective cohort study on AD patients who sustained hip fracture injuries at the same hospital. Patients records were identified in the computerized database of the Department of Traumatology, and the original hardcopy files were retrieved for manual examination. The overall study period was 8 years, between January 1, 2004 and December 31, 2012. All the hip fractures in AD patients that occurred within the study period were reviewed.

Patients, who were female, aged between 75 and 95 years at the date of the fracture, were included in this study. Our study did not include male patients since the number of male patients who satisfied our inclusion and exclusion criteria was too low. Accordingly, our study excluded male patients to control the number of parameters (variables) that could affect the study outcomes. Patients who were smokers or previously diagnosed with any of the following diseases, which are known to substantially affect bone metabolism and fracture healing, were excluded: osteomalacia, paget disease, Vitamin D deficiency, hyperthyroidism, Cancer [excluding non-melanoma skin cancer], Alcoholism, patients on corticosteroids, patients on anti-epileptic drugs, patients on bisphosphonates. Patients were also excluded if their five-week postsurgical radiographs were missing.

Hip fractures were defined according to the International Classification of Diseases (Tenth Revision, Codes 72.0–72.2) as a fracture of the proximal femur ranging from the femoral neck (intracapsular) to the subtrochanteric (extracapsular) region. The analyses included the first six months after operation.

A total of 135 AD patients who suffered hip fractures were identified in our health care area during the study period; among these patients 80 fulfilled our medical inclusion criteria **(Figure 7.9.1)**. Due to missing radiographs, 34 patients were excluded; and therefore, 46 patients were included in our final assessment model **(Figure 7.9.1).**

7.4.2. Study variables

The following parameters which may affect the study outcomes were retrieved from the patients' files, computerized records and standardized questionnaires: patient age, body mass index (BMI), Charlson comorbidity score (CCS) and the grade of AD according to the clinical dementia rating scale (CDR)[274-277]. In addition, information about type of hip fracture: intracapsular or extracapsular fractures were retrieved. The presence of AChEIs was considered as the predictor variable.

7.4.3. Study outcomes

7.4.3.1. Degree of calcification and bone quality

Five weeks-postsurgical plain pelvic radiographs (antero-posterior), taken as part of the routine follow-up, showing the fractured hip and non-fractured hip of the AD patients were retrieved. All of the retrieved radiographs were

performed at our department using a digital conventional hip x-rays machine (General Electric Corporation, Milwaukee, WI) with an adjusted X-ray exposure to match the anatomy of patient being examined. The retrieved radiographs were examined by two experienced musculoskeletal radiologists blinded to the patient treatment group. Inter-observer agreement among the two radiologists was significantly high (κ=0.824; 95%CI=0.691-0.957; p<0.001).

The following parameters were retrieved from the radiographs: the degree of calcification (radiographic union) at the fracture site and the bone quality at the non-fracture site. Degree of radiographic union at the fracture site was assessed following the criteria proposed by Hammer *et al*[278]. Hammer *et al* classified the degree of radiographic union of bone fracture into 5 grades; from grade 1 (corresponding to a complete calcification) to grade 5 (corresponding to a fracture without any evidence of calcification), based on the quality of the bridging callus and the presence or absence of a fracture line (Hammer union index)[278].

Bone quality at the non-fracture site was assessed following the criteria proposed by Singh *et al*[279]. Singh *et al* classified bone quality into 6 grades; from grade 1 (corresponding to a poor bone quality) to grade 6 (corresponding to a normal bone quality), based on the trabeculae morphology and distribution at the proximal femur (Singh index)[279].

7.4.3.1. Healing complication

Fracture healing complications that occurred within 6 months from the date of hip fracture were retrieved from the patients' files. The investigated

complications included the presence of infection, delayed-union, new hip fractures and surgical re-intervention.

7.4.4. Statistical analyses

Inter-observer agreements for the evaluations of the degree of bone calcification (radiographic union) of the fracture site and bone quality using radiographs were done by Kappa Test (κ). The value ranged from +1, with perfect agreement, to -1, which corresponds to absolute disagreement.

Clinical outcomes (degree of fracture calcification, bone quality and fracture healing complications) as a function of patients' characteristics (age, BMI, CCS, CDS and type of hip fracture) were tested using unpaired Student T-test and Mann-Whitney U test. AD Patients' characteristics and clinical outcomes as a function of the presence of AChEIs treatment were tested using unpaired Student T-test and chi-square test. Ratios of the probabilities (relative risk) of the study outcomes were presented with 95% confidence intervals (CIs), and accompanied p values. Values of p were two-sided and considered statistically significant if less than 0.05.

Sample sizes calculation to reject the null hypothesis, that states the study outcomes in AChEIs users and non-users are equal, were conducted using the chi-square test based on the following inputs: (i) expected fair radiographic union at the fracture site (hammer index ≤ 3) at the 5^{th}-week in 100% of AChEIs users versus 60% in nonusers (controls)[280]; (ii) type I error probability (α) equals to 0.05. It was determined that at least fifteen patients in each group would be needed to reach a

power of 80%. In addition, Post-hoc power calculations (following Fleiss test), to reject our null hypothesis, were conducted based on the following inputs: (i) number of included AChEIs users; (ii) ratio between AChEIs nonusers to AChEIs users; and (iii) probabilities of each outcome in AChEIs users and non-users[281].

7.5. Results:

7.5.1. Study outcomes distribution in AD patients

7.5.1.1. Degree of radiographic union (calcification)

Analyses of the retrieved radiographs demonstrated that 3 hip fractures had grade 2, 11 hip fractures had grade 3, 10 hip fractures had grade 4 and 11 hip fractures had grade 5 on fracture healing scale (Hammer union index). Degree of calcification of the fracture site could not be evaluated in 11 radiographs since the hip fracture was treated with a hip hemiarthroplasty.

Due to the small number of hip fractures in each grade, we categorized them into 2 groups: hip fractures that showed grade 3 or less (fair healing) and hip fractures that showed higher than grade 3 (poor healing) on fracture healing scale. The distribution of these two groups as function of AD patients' characteristics is shown in **Table 7.10.1**. AD patients who showed calcification of the fracture site of grade 3 or lower did not have significant differences in age, BMI, mental state or type of hip fracture compared to those who had degree of calcification higher than grade 3 (**Table 7.10.1**). Moreover, degree of radiographic union at the fracture site was not significantly related to bone quality measured at the non-fracture hip (RR,1.5; 95%CI, 0.9-2.7; p=0.129).

7.5.1.2. Degree of bone quality

Analyses of the retrieved radiographs demonstrated that 10 non-fracture hips had grade 2, 19 non-fracture hips had grade 3, 10 non-fracture hips had grade 4, 3 non-fracture hips had grade 5 based on bone quality scale (Singh index). Bone quality could not be evaluated in 4 radiographs due to the poor quality of radiographs showing the non-fracture hip.

Due to the small number of non-fracture hips in each grade, we categorized them into 2 groups: non-fracture hips that showed grade 3 or less (poor bone quality) and non-fracture hips that showed higher than grade 3 (fair bone quality) on bone quality scale. The distribution of these two groups as function of AD patients' characteristics is shown in **Table 7.10.1**. AD patients who showed bone quality higher than grade 3 at the non-fracture site did not have significant differences in age, BMI, mental state or type of hip fracture compared with those who had bone quality of grade 3 or less (**Table 7.10.1**).

7.5.1.3. Healing complications

The distribution of healing complications observed at the fracture site as function of AD patients' characteristics is shown in **Table 7.10.1**. AD patients with fracture healing complications did not have significant differences in age, BMI or mental state compared with those without complications (**Table 7.10.1**). However, all of the reported complications at the fracture site were observed in patients who had poor bone qualities (bone quality index ≤ 3) (RR,1.5; 95%CI, 1.2-1.9; p=0.159).

Three, out of four complications, were observed in patients who suffered from intracapsular hip fractures and the forth complication was in a patient who suffered from an extracapsular hip fracture (RR=0.8; 95%CI, 0.7-1.0; Power=0.56; p=0.152; **Table 7.10.1**). The reported complications were 2 cases of post-surgical infections, 1 case of new hip fracture at a nearby site and 1 delayed-union case. Both infection cases occurred in patients who were previously treated with a hip hemiarthoplasty. These cases who suffered from infections were retreated with Gridlestone osteotomy, however, one of them died 4 months following the operation, while second patient survived for more than 5 years. Patient with the delayed-union of the hip fracture was retreated with Gridlestone osteotomy; due to high risk of surgical complications and low mobility demand.

7.5.2. AChEIs distribution in AD patients and study outcomes

Among the patients included, 24 were AChEIs users and 22 were non-users. Out of 24 AChEIs users, 10 patients had been treated with rivastigmine 3-12mg/day for 12-48 months, 7 patients had been treated with donepezil 5-10mg/day for 12-36 months and 7 patients had been treated with galantamine 6-24mg/day for 12-48 months prior to enrolment.

The distribution of the presence of AChEIs in the study group as a function of age, BMI, CDR, CCS and type of hip fracture is shown in **Table 7.10.2**. Users of AChEIs did not have significant differences in age, BMI, mental state or type of hip fracture compared with AChEIs non-users (**Table 7.10.2**).

Interestingly enough, users of AChEIs were associated with a better degree of calcification at the fracture site (fracture healing index ≤ 3) and a better bone quality (bone quality index > 3) than nonusers (**Figure 7.9.2** and **Table 7.10.3**). Moreover, patients using AChEIs had no complications following hip fracture fixation than nonusers.

7.6. **Discussion:**

In this study we provided the first clinical evidence for the previously unexplored potential role of cholinergic stimulation on fracture healing. Based on our cohort study, AD patients receiving AChEIs such as rivastigmine, donepezil or galantamine expressed accelerated radiographic union (calcification) at the fracture site, better bone quality and less postoperative comorbidity compared to AChEIs-nonusers.

Healing of hip fractures in elderly, such as AD patients, is slow and usually requires a long-term immobilization of the fracture ends leading to greater morbidity and mortality[282-287]. Accordingly, several studies were conducted to find new therapeutic approaches to accelerate bone regeneration and fracture healing[288-290]. Nowadays, the only approved drugs that are commonly prescribed to improve fracture healing are parathyroid hormone (PTH) analogs[288,291]. However, even though PTH analogs are successful therapies for fracture healing, their use is limited due to the high cost and side effects[292]. In our study, we show that AChEIs might accelerate the radiographic union at the fracture sites, which may enhance the healing process. Accordingly, future *in vivo* and longitudinal

clinical studies should be conducted in order to investigate the positive effect of AChEIs on bone quality and fracture healing.

7.6.1. AChEIs accelerate calcification at the fracture site

In this study, healing of hip fractures was evaluated radiologically, by two radiologists who showed a high level of agreement, following criteria proposed by Hammer *et al*[278]. This method has been previously used to evaluate radiographic union of the fracture in several clinical studies[32,33] with a moderate overall general agreement[293].

In this study, AD patients who showed a high degree of calcification of their hip fractures (grade 3 or less on fracture healing index) had similar demographic characteristics to those patients who showed a poor calcification degree (higher than grade 3 on fracture healing index). Moreover, our study illustrated that types of hip fracture (intracapsular and extracapsular) or quality of bone (assessed by Singh index) did not significantly influence the radiographic union of the hip fracture. However, our results indicated that most hip fractures showing higher degree of calcification or radiographic union were observed in AD patients receiving AChEIs (**Table 7.10.3**), indicating that more bone regeneration might have occurred in these patients. These findings represent the first clinical evidence demonstrating that administration of AChEIs might be associated with an enhanced hip fracture healing. However, future research will have to be performed in order to assess whether the administration of these drugs are also associated with faster stability of the fracture site.

7.6.2. AChEIs favor bone mass

This study investigated the bone quality at non-fracture sites using Singh index as described by Sernbo *et al*[294]. This method has been previously used to evaluate bone mineral density in clinical studies, and it has been shown to have a good correlation with results obtained from Dual-energy X-ray Absorptiometry (DXA), the most popular tool to measure bone mineral density[295-299].

In this study, AD patients with bone quality higher than grade 3 based on bone quality index had similar patients' characteristic to those with bone quality of grade 3 or below. However, our results indicated that AChEIs users had better bone quality (bone quality index >3) than non-users. These results are aligned with our previous clinical study, in which it has been shown that users of AChEIs were associated with a lower risk of hip fracture[118], and it might open a new insight to treat bone diseases such as osteoporosis and minimize bone fractures.

Singh index refers to the architectural quality of trabecular network which is affected mainly by bone resorption rather than bone formation. The higher Singh index score observed in AChEIs users compared to nonusers might be related to the fact that these drugs would suppress bone resorption rate by promoting osteoclasts (bone-resorbing cells) apoptosis[7], independently or in addition to their potential role in promoting the proliferation of osteoblasts (bone-forming cells).[31] However, future research will have to be performed to confirm these hypotheses.

7.6.3. AChEIs minimize healing complications

Healing complications following hip fractures in elderly patients are common. A metaanalysis study reported that healing complications might occur in 49% of patients who suffered hip fractures[300]. Hip fracture typically results in a 1.8-year decrease in life expectancy[285,286]. Most of deaths due to hip fractures occur within the first 3-6 months following the event[285,286]. The remaining mortality beyond the initial 6 months is associated with institutionalization and new deficits that are only indirectly related to the fracture itself[285,286].

In this study, we detected four healing complications that occurred within 6 months after the hip fracture fixation procedure. Healing complications were not associated with patient's age, gender, BMI, or with their mental status. Interestingly, our study illustrated that all complications at the fracture site were observed in patients who were not AChEIs users, indicating a potential positive effect of AChEIs on minimizing the fracture healing complications. However, healing complications were also found in patients who showed poor bone quality as assessed by Singh index. Accordingly, the observed complications at the fracture sites might not be solely dedicated to the un-usage of AChEIs.

7.6.4. The mechanism by which AChEIs accelerate fracture healing

In this study, we investigated the effect cholinergic stimulation through the use AChEIs on bone mass. We included three AChEIs: rivastigmine, donepezil and galantamine, that are capable to stimulate cholinergic receptors: nicotinic and muscarinic, located peripherally and within the central nervous

system[301]. Cholinergic activity is known to favor bone mass either indirectly through suppression of the adrenergic activity and/or directly through suppression of osteoclasts proliferation[7,8]. Also, substantial observations pointed toward a possible direct up-regulation of osteoblasts by cholinergic activity[30,31,35].

7.6.5. Limitations and future directions

One limitation of this study was the small number of patients included. Despite this, we found that there is a strong association with a good power between radiographic union of hip fractures, bone quality and healing complications in one hand and the use of AChEIs on the other hand. Another limitation in our study was the use of X-rays radiography to assess the effect of AChEIs on bone healing and bone quality. Analysis of radiographs is subjective; however, it was conducted by two radiologists who were blinded to the treatment patient group, and yet showed a high inter-observer agreement among their results.

One more limitation in this study is that Hummer index was applied to assess the radiographic union of hip fractures from radiographs recorded in an antero-posterior view. Ideally, in order to assess the degree of radiographic union of bone fracture using Hammer index, two radiographs (antero-posterior and lateral radiographs) taken from perpendicular positions is recommended[278]. Another limitation present in our study is that the radiographic union assessment of the fracture site did not include 4 AChEIs users and 7 AChEIs non-users, since their hip fractures were fixed by prostheses. However, those missing participants

did not significantly affect the results of radiographic union of the fracture site (**Table 7.10.3**). We also could not assess the bone quality for other 4 AChEIs users due to the poor quality of radiographs showing the non-fracture sites (**Table 7.10.3**). However, comparing the characteristics, radiographic union of the fracture site and fracture healing complications of these excluded AChEIs users versus the rest of participants were not significant (**Table 7.10 Supplementary 2**), indicating a small potential effect of these patients on bone quality results reported in this study.

An additional limitation of this study is that the relationship of rivastigmine, donepezil and galantamine dosages and durations with the study outcomes could not be assessed for statistical significance due to the small number of participants who used these drugs. In **Table 7.10 supplementary 1**, we assessed the association of each of the included AChEI with the study outcomes.

One more limitation in this study is the missing information about the recovery process that patients received after the hip fracture fixation procedure which might affect the study outcomes. A further limitation in this study was the missing information about the criteria used by physicians to prescribe AChEIs for AD patients[302]. It has been found that physicians are less likely to prescribe AChEIs for patients with high number of comorbidities[302], which could affect our results. Nevertheless, the comorbidity grades, based on CCS, of the AD patients included in our study were not associated with the study outcomes: fracture healing, bone quality and healing complication.

For all of these reasons, future large-scale prospective randomized clinical trials, adjusted to all possible variables that might influence study outcomes, will have to be performed in order to confirm the findings of this study. Moreover, *in vivo* and clinical studies assessing biochemical bone markers, bone density measurements, and bone microarchitecture will have to be performed to explore the effect of AChEIs on bone structure and mineral content.

7.7. Conclusion:

To the best of our knowledge this is the first clinical evidence demonstrating that the administration of AChEIs might be associated with a faster calcification (radiographic union) of hip fracture and a better bone quality in female, aged between 75 and 95 years AD patients. These findings might help identify new therapeutic approaches to accelerate bone regeneration and fracture healing, although additional clinical studies are needed to confirm these potentially important findings.

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7.8. Figures:

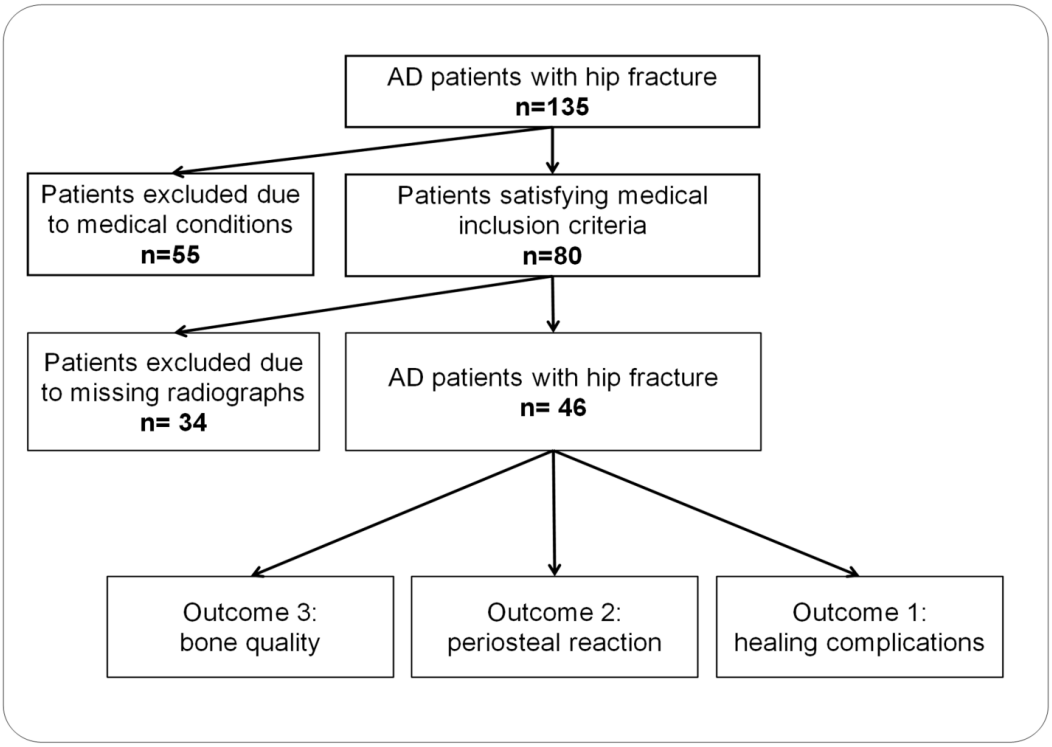

Figure 7.9.1. Flow chart describing the selection process of the participants in our cohort study.

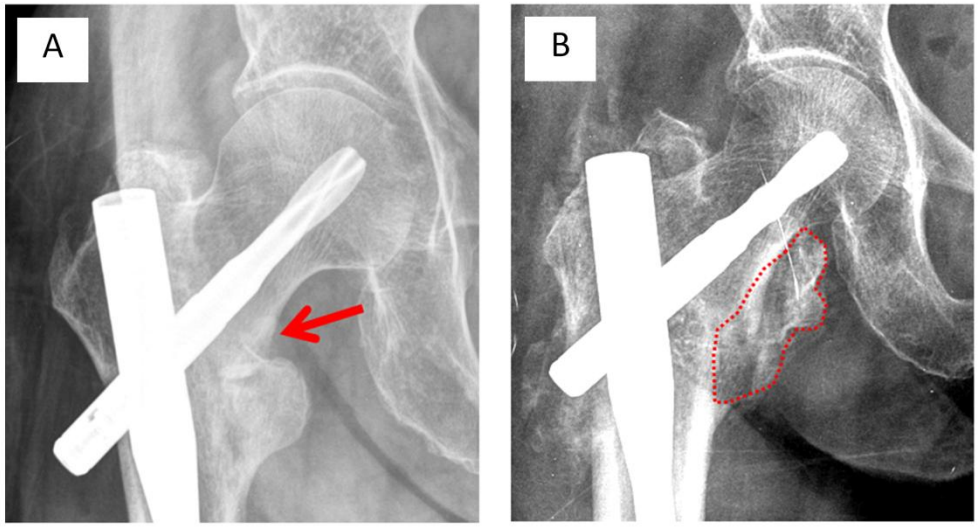

Figure 7.9.2. Frontal radiograph of two trochanteric hip fractures fixed with a proximal femoral nail antirotation (PFN-A, Synthes®, Solothurn, Switzerland) in an AD patient not receiving AChEIs **(A)** and another one receiving AChEIs treatment **(B)**, respectively. Radiographs were taken 5 weeks after the initial intervention. It can be observed that there is a large callus formation in the fracture of the patient treated with AChEIs (area within red-dot-line), but there is no periosteal reaction in the fracture of the patient not treated with AChEIs (red arrow).

7.9. Tables:

Table 7.10.1. AD patients' characteristics as a function of the study outcomes.

Characteristics	Fracture healing index [c]			Bone quality index [f]			Healing complication		
	≤3 (n=14) [d] (fair)	>3 (n=21) [d] (poor)	p-value [e]	>3 (n=13) [g] (fair)	≤3 (n=29) [g] (poor)	p-value [e]	Yes (n=4)	No (n=42)	p-value [h]
Age, years[a]	82.2±4.2	83.4±2.3	0.337	83.0±3.4	82.9±3.5	0.906	81.8±2.2	83.2±3.7	0.460
BMI [a, b]	26.8±4.6	25.4±3.9	0.359	27.4±5.6	24.9±3.5	0.081	22±1.6	25.9±4.3	0.076
CDR[a]	1.8±0.7	2.2±0.8	0.118	1.8±.7	2.0±0.8	0.193	2.2±0.7	2.0±0.7	0.608
CCS[a]	2.3±1.3	2.4±1.6	0.407	1.9±1.0	2.2±1.2	0.418	1.8±0.5	2.3±1.4	0.407
Type of hip fracture									
Extracapsular	12	15	-	-	-	-	1 (25%)	26	-
Intracapsular	2	6	0.424	-	-	-	3 (75%)	16	0.152

Abbreviation: AD: Alzheimer's disease; AChEIs: acetylcholinesterase inhibitors; BMI: body mass index; CDR: clinical dementia rating scale; CCS: Charlson comorbidity score.[a] Values are expressed as the mean and standard deviation.

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

[c] Measured based on criteria proposed by Hammer *et al*.

[d] Missing data: degree of fracture healing could not be evaluated for 11 patients since their hip fractures were treated with a hip hemiarthroplasty.

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

[f] Measured at the non-fracture hip based on criteria proposed by Singh *et al* .

[g] Missing data: Singh index could not be evaluated for 4 patients since the radiographs showing the non-fractured hip were poor

[h] Calculated with Mann-Whitney U test for independent samples.

p-value was calculated by chi-square test.

Table 7.10.2. AD patients' characteristics as a function of AChEIs treatment.

Characteristics	Users of AChEIs (n=24)	Nonusers of AChEIs (n=22)	p-value
Age, years[a]	82.9±3.6	83.2±3.6	0.807[b]
BMI [a, d]	26.1±4.3	24.5±3.9	0.102[b]
CDR[a]	1.8±0.7	2.3±0.7	0.069[b]
CCS[a]	2.5±1.3	2.0±1.1	0.170[b]
Type of hip fracture			
Extracapsular	15 (63%)	12 (55%)	-
Intracapsular	9 (37%)	10 (45%)	0.584[c]

Abbreviation: AD: Alzheimer's disease; AChEIs: acetylcholinesterase inhibitors; BMI: body mass index; CDR: clinical dementia rating scale; CCS: Charlson comorbidity score.
[a] Values are expressed as the mean and standard deviation.
[b] p-value was calculated by Student's t-test for independent samples.
[c] p-value was calculated by chi-square test.
[d] Measured as weight in kilograms divided by the square of height in meters

Table 7.10.3. Study outcomes as a function of AChEIs treatment.

Characteristics	Users of AChEIs (n=24)	Nonusers of AChEIs (n=22)	Crude RR (95%CI)	*Power*	*p-value* [e]
Fracture healing index [a]					
>3 (poor)	9 (38%)	12 (56%)	-		-
≤3 (Fair)	11 (46%)	3 (14%)	2.7 (0.9-7.8)	0.56	0.036
Missing [b]	4 (16%)	7 (32%)	0.9 (0.5-1.6)	0.05	1.000
Bone quality index [c]					
≤ 3 (Poor)	9 (37%)	20 (91%)	-		-
>3 (Fair)	11 (46%)	2 (9%)	2.0 (1.2-3.3)	0.92	0.001
Missing [d]	4 (16%)	0 (0%)	0.31 (0.2-0.5)	0.04	0.018
Healing complication					
No	24 (100%)	18 (82%)	-		-
Yes	0 (0%)	4 (18%)	0.8 (0.7-1.0)	0.56	0.029

Values are mean (95% confidence interval).

Abbreviation: AD: Alzheimer's disease; AChEIs: acetylcholinesterase inhibitors; RR: Relative Risk.

[a] Measured based on criteria proposed by Hammer *et al*.

[b] Missing data: degree of fracture healing could not be evaluated for 4 AChEIs users and 7 AChEIs nonusers since their hip fractures were treated with a hip hemiarthroplasty.

[c] Measured based on criteria proposed by Singh *et al*.

[d] Missing data: Singh index could not be evaluated for 4 AChEIs users since the radiographs showing the non-fractured hip were poor.

[e] p-value was calculated by chi-square test

Table 7.10.Supplementary 1. Study outcomes as a function of AChEIs treatment (no treatment, Rivastigmine, Donepezil and Galantamine).

Characteristics	Fracture healing index [a, b]				Bone quality index [c, d]				Healing complication			
	≤3 (n=14)	>3 (n=21)	RR (95%)	P [e]	>3 (n=13)	≤3 (n=29)	RR (95%)	P [e]	Yes (n=4)	No (n=42)	RR (95%)	P [e]
No Drug	3	12	-	-	2	20	-	-	4	18	-	-
Rivastigmine	4	4	1.6 (0.8-3.3)	0.156	4	4	1.8 (0.9-3.7)	0.013	0	10	0.8 (0.7-1.0)	0.148
Donepezil	4	2	2.4 (0.8-7.7)	0.064	4	2	2.7 (0.9-8.5)	0.002	0	7	0.8 (0.7-1.0)	0.224
Galantamine	3	3	1.6 (0.7-3.7)	0.198	3	3	1.8 (0.8-4.1)	0.020	0	7	0.8 (0.7-1.0)	0.224

Abbreviation: AChEIs: acetylcholinesterase inhibitors; RR: Relative Risk.

[a] Measured based on criteria proposed by Hammer *et al*.

[b] Missing data: degree of calcification at the fracture site could not be evaluated for 7 AChEIs nonusers, 2 Rivastigmine, 1 donepezil and 1 neostigmine users since their hip fractures were treated

[c] Measured based on criteria proposed by Singh *et al*.

[d] Missing data: Singh index could not be evaluated for 2 Rivastigmine, 1 donepezil and 1 neostigmine users since the radiographs showing the non-fractured hip were poor.

[e] p-value was calculated by chi-square test.

Table 7.10.Supplementary 2. Characteristics of patients who had their bone quality not evaluated (missing data) vs. Characteristics of rest of patients who had their bone quality assessed.

	Patients with missing information	Rest of the patients	P
Age, years [a]	84.5±5.4	82.9±3.5	0.405 [d]
BMI [a, b]	27.8±4.4	25.3±4.2	0.266 [d]
CDR [a]	2.5±0.6	2.0±0.7	0.224 [d]
CCS [a]	3.6±2.2	2.2±1.6	0.018 [d]
Fracture healing [c]			
Poor	3	11	-
Fair	1	20	0.193 [e]
complication			
No	4	38	-
yes	0	4	0.686 [e]

Abbreviation: BMI: body mass index; CDR: clinical dementia rating scale; CCS: Charlson comorbidity score.

[a] Values are expressed as the mean and standard deviation.

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

[c] Measured based on criteria proposed by Hammer *et al*.

[d] p-value was Calculated with Mann-Whitney U test for independent samples.

[e] p-value was calculated by chi-square test.

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CHAPTER 8: General Conclusions

From the present set of studies, we concluded that AChEIs have an anabolic effect on bones. This conclusion was based on the following findings:

- Our *in vivo* studies demonstrated that daily treatment with donepezil, a central acting AChEIs, was associated with an increase in bone mass accrual and better biomechanical properties in mice. The increase in bone mass accrual was due to a reduction in osteoclast proliferation as a result of the altered activity of the autonomous nervous system arms: the cholinergic and the adrenergic systems.
- Our *in vivo* studies indicated that daily treatment with neostigmine, a peripherally acting cholinergic agonist, was associated with an increase in bone quantity and quality. The increase in bone mass was caused by the increase in bone formation and osteoblast proliferation. Neostigmine treatment did not alter the activity of the central nervous system, thus it could not explain the increase in osteoblast proliferation. Moreover, the increase in bone mass following neostigmine treatment was not due to a direct local effect on osteoblasts, confirmed by cell culture experiments. However, neostigmine treatment altered the serum levels of immune-cytokines such as IL-17, which is known to promote osteoblast proliferation. The role of IL-17 as a stimulator of osteoblasts proliferation was confirmed through series of *in vitro* studies.

- Our clinical studies showed that the AChEIs could have a positive effect on bones in elderly AD population, which was presented by the risk of osteoporotic bone fractures and healing of bone fractures. AChEIs users had lower risk of osteoporotic fractures and faster healing, if the fracture occurred, compared to non-users.

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APPENDIX II: Additional Co-authored Articles Published by the Candidate during Ph.D. Studies

During the PhD studies, the candidate was involved in several research projects that were not included in the main text of the thesis. In each research project the candidate contributed significantly in generating the results and drafting the manuscript. This appendix includes the first pages of the articles published by the candidate as first author or co-author (6 published and 2 accepted articles). The articles were published in the following journals: Journal of Biomechanics, Rejuvenation Research Journal, Clinical Implant Dentistry and Related Research Journal, Journal of Clinical Periodontology, Springerplus Journal, Journal of Dental Research, Journal of Dentistry and Acta Biomaterialia.

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Regulated fracture in tooth enamel: A nanotechnological strategy from nature

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ABSTRACT

Tooth enamel is a very brittle material; however it has the ability to sustain cracks without suffering catastrophic failure throughout the lifetime of mechanical function. We propose that the nanostructure of enamel can play a significant role in defining its unique mechanical properties. Accordingly we analyzed the nanostructure and chemical composition of a group of teeth, and correlated it with the crack resistance of the same teeth. Here we show how the dimensions of apatite nanocrystals in enamel can affect its resistance to crack propagation. We conclude that the aspect ratio of apatite nanocrystals in enamel determines its resistance to crack propagation. According to this finding, we proposed a new model based on the Hall–Petch theory that accurately predicts crack propagation in enamel. Our new biomechanical model of enamel is the first model that can successfully explain the observed variations in the behavior of crack propagation of tooth enamel among different humans.

1. Introduction

Tooth enamel is the most highly mineralized and hardest tissue in mammals (Chai et al., 2009; Imbeni et al., 2005; Xu et al., 1998). Enamel inorganic consists of crystal key-hole shape structures composed of prisms (~6–8 μm) that are made of carbonate apatite (CAP) nanocrystals (Cui and Ge, 2007). Enamel organic is highly birefringent and is mainly composed of proteins and minor amounts of proteoglycans and lipoids that fill the spaces between the crystals (Cerny et al., 1996).

Enamel suffers from continuous mechanical stress that causes the formation of cracks favoring bacteria growth, caries and tooth fracture (Huang et al., 2010; Imbeni et al., 2005). Enamel has certain damage tolerance to sustain cracks (Chai et al., 2009) that could be an important factor in the adaptation of tooth to diet in evolution (Ang et al., 2010; Barani et al., 2011; Janis and Fortelius, 1988). It is also of interest for biomaterials research in the development of biomimetic materials (Ang et al., 2010). It has been suggested that geometrical and microstructural characteristics of enamel have an effect on its damage tolerance (Ang et al., 2010; Bajaj and Arola, 2009; Chai et al., 2009; Imbeni et al., 2005; Lawn and Lee, 2009; O'Brien et al., 2013; Xu et al., 1998).

The fact that tooth enamel is tougher than geologic-hydroxyapatite seems to indicate that the specific characteristics of enamel such as the organic content, enamel prisms and crystals might have an effect on defining its mechanical properties (An et al., 2012; He and Swain, 2007; White et al., 2001; Yahyazadehfar et al., 2013). Removal of enamel organic content reduces its fracture resistance (Baldassarri et al., 2008; Zheng et al., 2013). Prisms degree of decussation and its crystal orientation affect tooth enamel mechanical properties (An et al., 2012; Bajaj and Arola, 2009; Chai et al., 2009; Xu et al., 1998; Yahyazadehfar et al., 2013).

However, the specific contribution of enamel crystal size on crack propagation remains largely unknown. Crack propagation in polycrystalline materials is known to be regulated by their crystallographic dimensions. Increasing the crystal size in polycrystalline materials results in lower resistance to crack propagation (Mercer and Soboyejo, 1996; Wang and Shaw, 2009; Yusheng et al., 2004; Zhou et al., 2011), because cracks can propagate more easily around bigger crystals than around smaller ones (Yusheng et al., 2004).

We hypothesize that crack propagation in enamel might be influenced by its crystallographic dimensions. This study was designed to analyze the associations of enamel crystallographic

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Article number 2.

Resveratrol as anti-aging therapy for age-related bone loss

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"Word count": 2827

Key words: Resveratrol, aging, rats, bone microstructure, micro-computed tomography, biomechanical properties

Abbreviated tittle: Effects of resveratrol on old bones

Article number 3.

Melatonin dietary supplement as an anti-aging therapy for age-related bone loss

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Word count: 1947

Key words: melatonin, aging, rats, bone mass, micro-CT scan, biomechanical properties

Abbreviated tittle: Effects of melatonin on age-related bone loss

Article number 4.

Membranes over the lateral window in sinus augmentation procedures: a two-arm and split-mouth randomized clinical trials

Jesús Torres García-Denche[1,2], Xixi Wu[3], Pedro-Pablo Martinez[2], Hazem Eimar[3], Daher Jalil-Abumalham Ikbal[2], Gonzalo Hernández[1], Enrique López-Cabarcos[4], Isabel Fernandez-Tresguerres[1] and Faleh Tamimi[3]

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Torres García-Denche J, Wu X, Martinez P-P, Eimar H, Ikbal DJ-A, Hernández G, López-Cabarcos E, Fernandez-Tresguerres I, Tamimi F. Membranes over the lateral window in sinus augmentation procedures: a two-arm and split-mouth randomized clinical trials. J Clin Periodontol 2013; 40: 1043–1051. doi: 10.1111/jcpe.12153.

Abstract

Objective: This study evaluates whether or not, among other factors, membrane-coverage of antrostomy defects improves implant survival in sinus augmentation procedures.

Materials and Methods: We performed a two-arm and split-mouth randomized controlled clinical trial on 104 and 5 patients respectively. In the two-arm study, antrostomy defects were membrane-covered in 66 procedures and uncovered in 69, before placing a total of 265 implants that were followed up for 1 year. In the split-mouth study, following bilateral sinus augmentation, antrostomy defects were membrane-covered on one side and left uncovered on the contra-lateral. Bone biopsies from each sinus were histologically analysed 6 months later.

Results: In the two-arm study, implant survival rates were similar ($p = 0.08$) in the membrane-covered (96.1%) and uncovered (94.2%) groups. In the split-mouth study, bone augmentation was similar in both groups ($p = 0.52$). Delayed implant placement ($p = 0.04$), thick Schneider's membrane (≥2 mm) ($p < 0.01$), treatment for hypertension ($p = 0.04$) and non-smoking ($p = 0.01$) seemed to be associated with lower risk of implant failure.

Conclusions: Implant survival in sinus lifting procedures could be influenced significantly by timing of implant placement, Schneider's membrane thickness, antihypertensive treatment and smoking habits, but not by antrostomy membrane coverage.

Key words: barrier membranes; bone regeneration; dental implants; risk factors; sinus floor augmentation

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Conflict of interest and source of funding statement

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Insufficient bone volume is a common obstacle for placement of endosseous implants in the posterior maxilla. One of the most frequently used surgical techniques to address this problem is maxillary sinus grafting (Boyne & James 1980, Tatum 1986, Chanavaz 1990). This procedure involves creating an antrostomy defect to access the sinus, detaching Schneider's membrane from the sinus floor, and placing a graft material into the sinus cavity to promote vertical bone augmentation (Summers 1994, 1995, Hirsch & Ericsson 2002). Different grafting materials have been successfully used in sinus augmentation procedures including allografts (Groeneveld & Burger 2000), xenografts (Valentini & Abensur 2003), autogenous bone (Keller et al. 1987, Jensen et al. 1990) or combinations of these materials (Zinner & Small 1996, Galindo-Moreno et al. 2009).

Many clinicians use membranes to cover antrostomy defects in sinus

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RESEARCH Open Access

Trace elements can influence the physical properties of tooth enamel

Elnaz Ghadimi[1], Hazem Eimar[1], Benedetto Marelli[2], Showan N Nazhat[2], Masoud Asgharian[3], Hojatollah Vali[1] and Faleh Tamimi[1*]

Abstract

In previous studies, we showed that the size of apatite nanocrystals in tooth enamel can influence its physical properties. This important discovery raised a new question; which factors are regulating the size of these nanocrystals? Trace elements can affect crystallographic properties of synthetic apatite, therefore this study was designed to investigate how trace elements influence enamel's crystallographic properties and ultimately its physical properties.

The concentration of trace elements in tooth enamel was determined for 38 extracted human teeth using inductively coupled plasma-optical emission spectroscopy (ICP-OES). The following trace elements were detected: Al, K, Mg, S, Na, Zn, Si, B, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Sb, Se and Ti. Simple and stepwise multiple regression was used to identify the correlations between trace elements concentration in enamel and its crystallographic structure, hardness, resistance to crack propagation, shade lightness and carbonate content. The presence of some trace elements in enamel was correlated with the size (Pb, Ti, Mn) and lattice parameters (Se, Cr, Ni) of apatite nanocrystals. Some trace elements such as Ti was significantly correlated with tooth crystallographic structure and consequently with hardness and shade lightness. We conclude that the presence of trace elements in enamel could influence its physical properties.

Keywords: Crystal domain size; Trace elements; Tooth enamel; Physical properties

Background

Tooth enamel is composed of both an organic and an inorganic phase. The organic phase is composed of proteins such as amelogenin, ameloblastin and tuftelin, as well as minor concentrations of proteoglycans and lipoids (Belcourt and Gillmeth 1979; Eggert et al. 1973; Glimcher et al. 1964). The enamel inorganic phase is composed of well-packed nanocrystals made of calcium phosphate apatite (HA) with small amounts of incorporated trace elements (Sprawson and Bury 1928). The organization and size of apatite crystals in tooth enamel affects its hardness (Jiang et al. 2005) and optical properties (Eimar et al. 2011, 2012). These findings raise the following question: what determines the size of apatite crystals in tooth enamel? One possibility is that the tooth protein content could affect its crystal domain size, however we had found that the concentration of protein in enamel is not associated with the crystallographic structure of mature teeth (Eimar et al. 2012). Therefore this study was designed to investigate other factors, namely the presence of trace elements that can influence the size of apatite crystals in enamel.

The crystallographic properties of synthetic hydroxyapatite (HA) have been found to be influenced by the incorporation of trace elements (Table 1). Some of the trace elements expand the crystal cell lattice parameters of synthetic HA along the a-axis (Fe^{2+}, Fe^{3+}, Sr^{2+} and Zn^{2+} (molar fraction > 10%)) while others shrink it (SiO_4^{4-}, CO_3^{2-}, Mg^{2+}, Zn^{2+} (molar fraction < 10%) and Ti^{4+}). The crystal domain size along c-axis can be increased by some trace elements (SiO_4^{4-}, CO_3^{2-}, Zn^{2+}, Fe^{2+}, $Fe^{3+}$and Sr^{2+}) and decreased by others (Mg^{2+}, Ni^{2+}, Cr^{3+}, Co^{2+} and Ti^{4+}). Some trace elements can increase the crystallinity (degree of structural order of atoms) and crystal domain size (average length of individual crystals) of synthetic HA (Cr^{3+}, Co^{2+} and Ni^{2+}), while others have the opposite effect (SiO_4^{4-}, Zn^{2+}, CO_3^{2-}, Fe^{2+}, Ti^{4+}, Sr^{2+}, Ce^{3+} and Mg^{2+}) (Christoffersen et al. 1997;

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Article number 6.

RESEARCH REPORTS
Biological

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Local Regulation of Tooth Mineralization by Sphingomyelin Phosphodiesterase 3

ABSTRACT

Sphingomyelin phosphodiesterase 3 (*Smpd3*) encodes a membrane-bound enzyme that cleaves sphingomyelin to generate several bioactive metabolites. A recessive mutation called *fragilitas ossium* (*fro*) in the *Smpd3* gene leads to impaired mineralization of bone and tooth extracellular matrix (ECM) in *fro/fro* mice. In teeth from *fro/fro* mice at various neonatal ages, radiography and light and electron microscopy showed delayed mantle dentin mineralization and a consequent delay in enamel formation as compared with that in control *+/fro* mice. These tooth abnormalities progressively improved with time. Immunohistochemistry showed expression of SMPD3 by dentin-forming odontoblasts. SMPD3 deficiency, however, did not affect the differentiation of these cells, as shown by osterix and dentin sialophosphoprotein expression. Using a transgenic mouse rescue model (*fro/fro; Col1a1-Smpd3*) in which *Smpd3* expression is driven by a murine *Col1a1* promoter fragment active in osteoblasts and odontoblasts, we demonstrate a complete correction of the tooth mineralization delays. In conclusion, analysis of these data demonstrates that *Smpd3* expression in odontoblasts is required for tooth mineralization.

KEY WORDS: neutral sphingomyelinase 2, dentin, enamel, extracellular matrix, matrix vesicle, *fro* mutation.

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INTRODUCTION

While extracellular matrix (ECM) mineralization in bones and teeth is driven by developmentally distinct cell types, the mineralization process appears to be regulated by several common determinants. Indeed, the mechanisms underlying bone ECM mineralization and those of tooth dentin and cementum are so similar that genetic diseases affecting bone mineralization often also manifest as tooth mineralization defects (Waymire *et al.*, 1995; Boukpessi *et al.*, 2006; Majorana *et al.*, 2010; Opsahl Vital *et al.*, 2012).

In an attempt to identify the novel regulators of hard-tissue mineralization and to understand their modes of action, we are investigating mouse models with bone and tooth mineralization defects in which the known determinants of ECM mineralization are unaffected. These critical determinants of ECM mineralization include 2 mineral ions, inorganic phosphate (P_i) and calcium, the mineralization inhibitor inorganic pyrophosphate (PP_i), and alkaline phosphatase (ALPL), an ectoenzyme that regulates tissue PP_i levels (Fleisch *et al.*, 1965; Terkeltaub, 2001; Hessle *et al.*, 2002; Murshed *et al.*, 2005; Murshed and McKee, 2010). We recently reported the cell-autonomous requirement of sphingomyelin phosphodiesterase 3 (SMPD3) in osteoblasts for bone ECM mineralization (Khavandgar *et al.*, 2011). SMPD3 cleaves sphingomyelin in the cell membrane and generates phosphocholine and ceramide (Merrill *et al.*, 1997), 2 bioactive metabolites that, in turn, affect a variety of cellular activities. A recessive mutation in *Smpd3* called *fragilitas ossium* (*fro*) leads to poor bone and tooth mineralization, impaired apoptosis of hypertrophic chondrocytes, and severe skeletal dysplasia in *fro/fro* mice (Guenet *et al.*, 1981; Muriel *et al.*, 1991; Aubin *et al.*, 2005; Goldberg *et al.*, 2008; Khavandgar *et al.*, 2011). Interestingly, the known factors important in influencing ECM mineralization appear to be unaffected in this model (Khavandgar *et al.*, 2011), thus making it useful for deciphering the direct effects of SMPD3 on mineralization.

The objectives of the present study were to characterize the tooth mineralization defects in *fro/fro* mice and to determine, in transgenic mice, whether SMPD3 regulates tooth mineralization locally. Herein we report on the temporal appearance (and recovery) of tooth mineralization delays in young *fro/fro* mice. Analysis of the radiographic and histological data suggests that the tooth mineralization delays caused by the *fro* mutation are attributable not to abnormal cell differentiation and patterning, but rather to altered mineralization caused by a local effect of the absence of SMPD3 activity (from odontoblasts) in the dentin ECM. Finally, we show that transgenic expression of *Smpd3* in odontoblasts in *fro/fro* mice completely corrects the neonatal tooth abnormalities.

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Hydrogen peroxide whitens teeth by oxidizing the organic structure

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ABSTRACT

Objectives: The mechanism of tooth bleaching using peroxide oxidizers is not fully understood. It is unknown whether peroxide radicals make teeth whiter by deproteinizing, demineralizing, or oxidizing tooth tissues. This study was designed to define the mechanism of tooth bleaching and determine which of tooth enamel chemical components is/are affected by bleaching.

Methods: Sixty sound teeth were collected from adult patients. The teeth were divided into 6 equal groups ($n = 10$). Groups 1, 2, 3 and 4 were treated for 4 days with one of the following solutions: deproteinizing (NaOH) that removes organic content, demineralizing (EDTA) that decalcifies the mineral content, oxidizing (H_2O_2) and distilled water (control). Group 5 and 6 were pre-treated with either deproteinizing or demineralizing solutions before treating them with oxidizing solutions for 4 days. Changes in enamel elemental ratios, crystallinity index and tooth shade parameters of the treated teeth were examined by means of EDS, Raman spectroscopy and shade-spectrophotometry. The data obtained was analysed with Wilcoxon Signed-Ranks Test, and the statistical signicance was set at $p < 0.05$.

Results: Tooth deproteinization increased the lightness by $4.8 \pm 2.7°$, tooth demineralization resulted in $8.5 \pm 5.6°$ decrease in the lightness and tooth oxidization induced $19.9 \pm 6.5°$ increase in the lightness. Oxidization of the deproteinized teeth did not influence shade parameters, but oxidation of the demineralized teeth resulted in $10.7 \pm 5.8°$ increase in the lightness.

Conclusion: Hydrogen peroxide does not induce significant changes in tooth enamel organic and inorganic relative contents, and it whitens teeth just by oxidizing their organic matrix. These findings are of great clinical significance since they explain the mechanism of tooth bleaching, and help understanding its limitations and disadvantages.

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The effect of autoclaving on the physical and biological properties of dicalcium phosphate dihydrate bioceramics: Brushite vs. monetite

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ABSTRACT

Dicalcium phosphate dihydrate (brushite) is an osteoconductive biomaterial with great potential as a bioresorbable cement for bone regeneration. Preset brushite cement can be dehydrated into dicalcium phosphate anhydrous (monetite) bioceramics by autoclaving. This heat treatment results in changes in the physical characteristics of the material, improving in vivo bioresorption. This property is a great advantage in bone regeneration; however, it is not known how autoclaving brushite preset cement might improve its capacity to regenerate bone. This study was designed to compare brushite bioceramics with monetite bioceramics in terms of physical characteristics in vitro, and in vivo performance upon bone implantation. In this study we observed that monetite bioceramics prepared by autoclaving preset brushite cements had higher porosity, interconnected porosity and specific surface area than their brushite precursors. In vitro cell culture experiments revealed that bone marrow cells expressed higher levels of osteogenic genes *Runx2*, *Opn*, and *Alp* when the cells were cultured on monetite ceramics rather than on brushite ones. In vivo experiments revealed that monetite bioceramics resorbed faster than brushite ones and were more infiltrated with newly formed bone. In summary, autoclaving preset brushite cements results in a material with improved properties for bone regeneration procedures.

1. Introduction

Calcium phosphate biomaterials are of special interest in bone regeneration due to their similar composition to bone. Dicalcium phosphate dihydrate, mineral name brushite, is a calcium phosphate biomaterial that can be prepared in the form of hydraulic cements with a wide range of applications [1]. Besides their ability to regenerate bone, brushite biomaterials can resorb in vivo faster than most calcium phosphates, enabling the replacement of the bioceramic by newly regenerated tissues. However, in vivo studies have shown that even though brushite is initially resorbable after implantation, the bioceramic tends to react with the surrounding medium, forming insoluble hydroxyapatite [1,2]. This reaction results in a severe reduction in the resorption rate of the biomaterial, limiting its clinical applications.

Monetite is the anhydrous form of brushite, and it is also a useful biomaterial for bone regeneration. Monetite bioceramics can be prepared by modifying the precipitation conditions of brushite cements [2]. For instance, setting brushite cements in excessively low pH conditions, in water-deficient environments, or in the presence of metallic ions would disrupt brushite crystals favouring monetite formation [2–5]. Another method of preparing monetite bioceramics is by thermal dehydration of already set brushite cements [6]:

$$CaHPO_4 \cdot 2H_2O \rightarrow CaHPO_4 + 2H_2O$$

Thermal dehydration of brushite bioceramics can cause shrinkage of the material and damage its mechanical properties. However, by maintaining high pressure and humidity during the dehydration process, overall shrinkage can be prevented [7]. Sterilizing pre-set brushite cements by autoclaving provides the adequate temperature, pressure and humidity conditions that result in their dehydration into monetite without altering the overall macroscopic geometry of the material. Monetite bioceramics prepared by this method have been shown to stimulate vertical bone augmentation, and regeneration of bone defects in animals as well as in human patients [6,8,9], and can achieve higher volumes of bone regeneration than hydroxyapatite-based biomaterials [6,10].

Monetite bioceramics prepared by autoclaving of brushite preset cements have inferior mechanical strength to that of their brushite precursor, and similar levels of cytotoxicity [11]. However, monetite bioceramics prepared by this method release ions at a slower rate than their brushite precursors, do not form insoluble hydroxyapatite in vivo [6,10], and upon subcutaneous

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